REVIEW ARTICLE

Structure, function and regulation of pyruvate carboxylase

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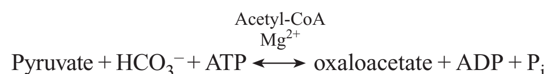
Pyruvate carboxylase (PC; EC 6.4.1.1), a member of the biotin-dependent enzyme family, catalyses the ATP-dependent carboxylation of pyruvate to oxaloacetate. PC has been found in a wide variety of prokaryotes and eukaryotes. In mammals, PC plays a crucial role in gluconeogenesis and lipogenesis, in the biosynthesis of neurotransmitter substances, and in glucose-induced insulin secretion by pancreatic islets. The reaction catalysed by PC and the physical properties of the enzyme have been studied extensively. Although no high-resolution three-dimensional structure has yet been determined by X-ray crystallography, structural studies of PC have been conducted by electron microscopy, by limited proteolysis, and by cloning and sequencing of genes and cDNA encoding the enzyme. Most well characterized forms of active PC consist of four identical subunits arranged in a tetrahedron-like structure. Each subunit contains three functional domains: the biotin carboxylation domain, the transcarboxylation domain and the biotin carboxyl carrier domain. Different physiological conditions, including diabetes, hyperthyroidism, genetic obesity and postnatal development, increase the level of PC expression through transcriptional and translational mechanisms, whereas insulin inhibits PC expression. Glucocorticoids, glucagon and catecholamines cause an increase in PC activity or in the rate of pyruvate carboxylation in the short term. Molecular defects of PC in humans have recently been associated with four point mutations within the structural region of the PC gene, namely $Val^{145} \rightarrow Ala$, $Arg^{451} \rightarrow Cys$, $Ala^{610} \rightarrow Thr$ and $Met^{743} \rightarrow Thr$.

Key words: biotin-dependent carboxylase, gluconeogenesis, insulin secretion, lipogenesis, pyruvate carboxylase deficiency.

METABOLIC ROLES OF PYRUVATE CARBOXYLASE (PC) IN LOWER AND HIGHER ORGANISMS

PC (EC 6.4.1.1) was first described by Utter and Keech [1] in the course of defining the gluconeogenic pathway in chicken liver. The reaction catalysed by PC is:



$$\text{Pyruvate} + HCO_3^- + \text{ATP} \xleftrightarrow[]{\text{Acetyl-CoA, } Mg^{2+}} \text{oxaloacetate} + \text{ADP} + P_i$$

Subsequently, PC has been found to be distributed widely among both vertebrates and invertebrates, as well as in many micro-organisms (reviewed in [2]).

Among prokaryotes, PC has been identified in *Pseudomonas* spp., *Rhodobacter* spp., *Bacillus* spp., *Rhizobium* spp., etc., but not in Enterobacteriaceae [2]. The last group of bacteria can synthesize oxaloacetate directly from phosphoenolpyruvate using phosphoenolpyruvate carboxylase [3]. A genetic study has indicated that expression of *Escherichia coli* phosphoenolpyruvate carboxylase can complement the phenotypic effects of PC deficiency in yeast [4]. The anaplerotic role of PC has been shown to be essential for normal growth in *Rhizobium etli* [5].

In *Saccharomyces cerevisiae*, the two principal pathways for the replenishment of oxaloacetate levels are via the carboxylation of pyruvate by PC and from the glyoxylate cycle [6]. During growth on glucose, the enzymes of the glyoxylate cycle are repressed [7] and thus PC catalyses the only known reaction to replenish the tricarboxylic acid cycle under these conditions. The highest activity of PC was found in glucose-grown cells under anaerobic conditions [8].

Plants have also been shown to contain PC, which may provide an alternative gluconeogenic pathway to the photosynthetic process during germination [9].

In mammals, PC is expressed in a tissue-specific manner, with its activity found to be highest in the liver and kidney (gluconeogenic tissues), in adipose tissue and lactating mammary gland (lipogenic tissues), and in pancreatic islets. Activity is moderate in brain, heart and adrenal gland, and least in white blood cells and skin fibroblasts [2,10,11]. The roles of PC in these different tissues are described below.

Gluconeogenesis

In fasting conditions, gluconeogenesis accounts for up to 96% of total glucose production [12]. The presence of very high PC activity, together with high activities of other gluconeogenic enzymes including phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase and glucose-6-phosphatase in liver and kidney cortex, suggests that a primary role of PC is to participate in gluconeogenesis in these tissues. During fasting or starvation when endogenous glucose is required for certain tissues (brain, white blood cells and kidney medulla), expression of PC and other gluconeogenic enzymes has been shown to be elevated [13].

Lipogenesis

Although considered at first to be solely a gluconeogenic enzyme, PC was soon recognized also to be expressed at a very high level during the differentiation of adipocytes [14]. PC appears to be

Abbreviations used: PC, pyruvate carboxylase; ACC, acetyl-CoA carboxylase, PCC, propionyl-CoA carboxylase; MCC, methylcrotonoyl-CoA carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; CPS, carbamoyl-phosphate synthase.

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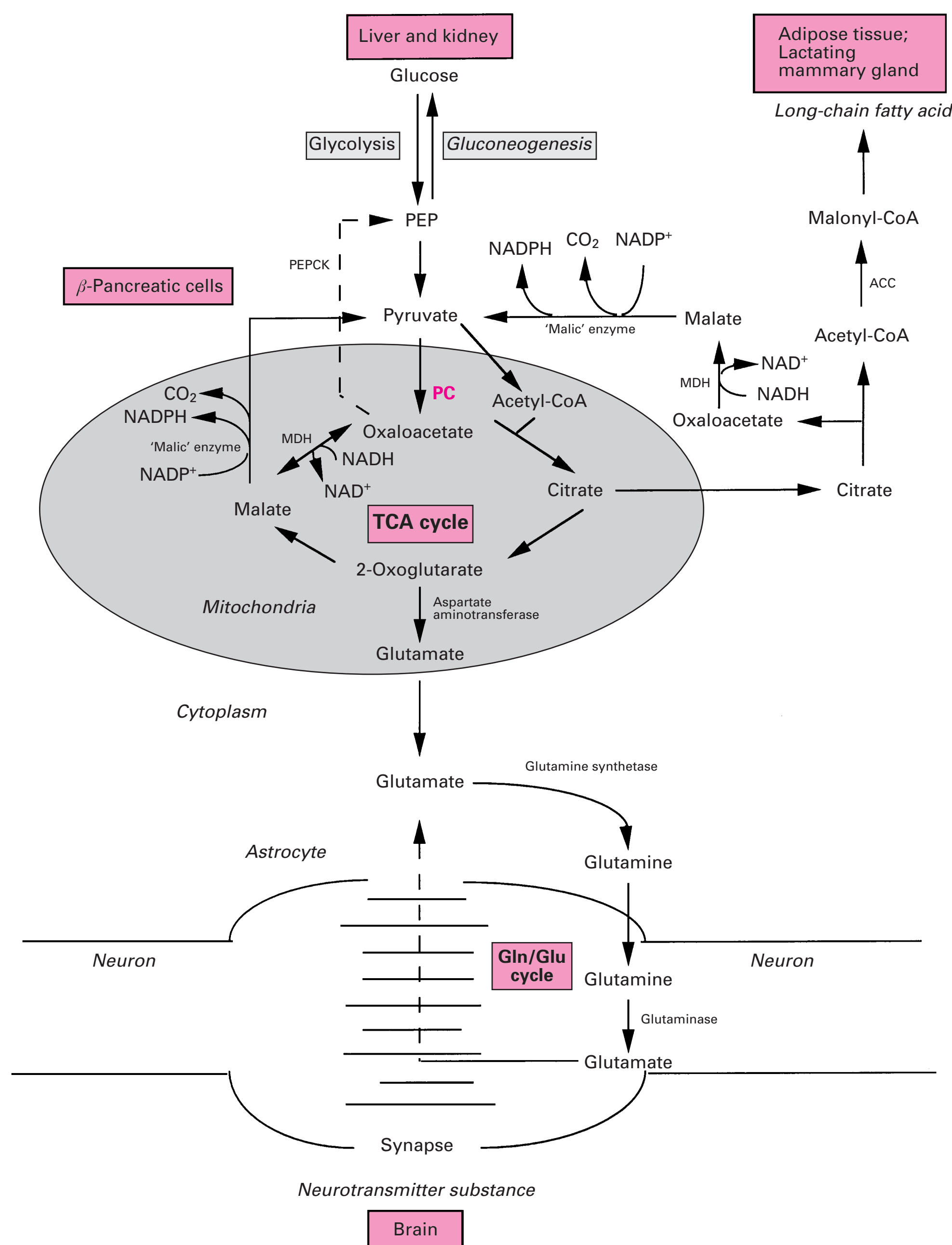

Scheme 1 Schematic representation of the anaplerotic functions of PC in mammalian tissues in relation to the biosynthetic pathways in which oxaloacetate is utilized

Four major biosynthetic pathways are shown: gluconeogenesis in liver and kidney, fatty acid synthesis in adipose tissue and lactating mammary gland, synthesis of neurotransmitter precursors in astrocytes, and the generation of metabolic coupling factor NADPH in pancreatic islets. Abbreviations: TCA, tricarboxylic acid; MDH, malate dehydrogenase; PEP, phosphoenolpyruvate.

particularly important in adipose tissue, where it contributes to the generation of a substantial proportion of the NADPH required for lipogenesis [15]. As shown in Scheme 1, the generation of NADPH is coupled to the transport of mitochondrial acetyl groups into the cytosol for fatty acid synthesis. Acetyl-CoA is generated in the mitochondria by the oxidative decarboxylation of pyruvate, and, after condensation with oxaloacetate, acetyl groups are transported to the cytoplasm as citrate, which undergoes ATP-dependent cleavage to yield acetyl-CoA and oxaloacetate. This pathway requires a continuous supply of oxaloacetate, which is produced by the activity of PC. Acetyl-CoA, a building block for the synthesis of long-chain fatty acids, is then converted into malonyl-CoA by acetyl-CoA carboxylase (ACC). Meanwhile, the oxaloacetate generated in the cytosol from citrate is reduced with NADH to malate, which in turn is oxidatively decarboxylated in a reaction catalysed by $NADP^+$-dependent malate dehydrogenase ('malic enzyme'; EC 1.1.1.40). The pyruvate thereby produced is taken up by the mitochondria and carboxylated to give oxaloacetate, while the NADPH generated is used in the pathway of fatty acid synthesis.

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Role of PC in insulin signalling in pancreatic islets

Glucose is a potent stimulator of insulin secretion from β-pancreatic cells when extracellular levels are greater than 3 mM. Secretion of insulin in response to a high concentration of glucose results in the rapid uptake of glucose by pancreatic β-cells more than by other cell types [16,17]. This is a feature of pancreatic β-cells, but not α-cells [18]. Signalling for glucose-induced insulin release is believed to require aerobic glycolysis plus tricarboxylic acid cycle activity [19,20]. The activities of two mitochondrial enzymes, pyruvate dehydrogenase and PC, have been shown to be elevated when islets are grown in higher-than-physiological concentrations of glucose, suggesting that both enzymes are involved in the regulation of glucose-induced insulin release [21]. It is known that pancreatic islets contain a concentration of PC equivalent to that in gluconeogenic tissues, but lack PEPCK activity and mRNA. This suggests that PC is not present for the purpose of gluconeogenesis [22,23]. The rapid uptake of glucose is thought to be mediated through the glucose-sensing enzyme glucokinase, which is rate-limiting for overall glucose utilization in β-cells [24,25]. This glucose undergoes oxidation to pyruvate [18], which is subsequently carboxylated by PC [18,26]. Higher concentrations of glucose also up-regulate the levels of PC protein [27] and mRNA [21,27,28].

It has been demonstrated that a pyruvate/malate shuttle operates across the mitochondrial membrane, as shown in Scheme 1. The high level of PC permits the rapid formation of oxaloacetate, which is subsequently converted into malate; this crosses the mitochondrial membrane to the cytosol, where it is decarboxylated to pyruvate by malate dehydrogenase, producing a putative coupling factor, NADPH [26]. Since this pathway occurs as a cycle, this shuttle can generate far more NADPH than the pentose phosphate pathway. Although the above pathway has been shown to be linked to insulin secretion, the metabolic signalling mechanism leading to insulin release remains unknown. However, it has been shown that an increase in glucose metabolism, which causes a rise in the ATP/ADP ratio in islets, results in the closure of the ATP-sensitive potassium channel, resulting in an influx of potassium; this in turn causes depolarization of the plasma membrane and an influx of calcium [29]. The calcium from external and intracellular sources activates contractile proteins, which propel insulin granules to the plasma membrane for extrusion [29].

Role of PC in astrocytes

Although four gluconeogenic enzymes, i.e. glucose-6-phosphatase [30], fructose bisphosphatase [31], PEPCK [32] and PC [33], have been reported to be present in the brain, their specific activities are too low to ensure gluconeogenesis. However, a few reports have demonstrated that lactate, alanine, aspartate or glutamine could be converted into glycogen in astrocyte culture [34,35], but not in neurons due to the absence of PC [36].

The anaplerotic role of PC has been proposed to be necessary for the production of glutamine, the precursor of excitatory amino acid neurotransmitters, via the operation of the glutamate/glutamine cycle [37,38]. As indicated in Scheme 1, when glutamate, a neurotransmitter substance, is released from the nerve endings of neurons, it is taken up by astrocytes. Subsequently, glutamate is converted into glutamine by astrocytic glutamine synthetase and secreted into the extracellular fluid, from which it is taken up by neurons for conversion into glutamate, aspartate and γ-aminobutyric acid [36,38,39]. Oxaloacetate, produced by PC, can also participate in this glutamate/glutamine cycle [40], and a recent study has shown that PC can alter the rate of *de novo* astrocytic synthesis of glutamate by increasing the amount of tricarboxylic acid cycle intermediates [39].

SYNTHESIS, DEGRADATION AND INTRACELLULAR LOCALIZATION OF PC

In *S. cerevisiae* there are two PC isoenzymes (PC1 and PC2) encoded by separate genes [41,42], while in mammals no tissue-specific isoenzymes have been reported. The newly synthesized enzyme undergoes a post-translational modification whereby one biotin moiety is covalently attached to the side chain of a specific lysine residue located near the C-terminus of each protomer. This reaction is catalysed by biotin protein ligase [also known as holocarboxylase synthetase (EC 6.3.4.15)] [43]. The intracellular site of the biotinylation reaction catalysed by holocarboxylase synthetase is not clear. In 3T3-L1 mouse adipocytes, which contain very high levels of PC and ACC (EC 6.4.1.2), most holocarboxylase synthetase activity was detected in the cytosol, and only 30% of activity was detected in the mitochondrial fraction [44]. A study with rat liver yielded similar results [45]. In lower organisms, such as *S. cerevisiae* [46], *Methanobacterium thermoautotrophicum* [47], *Bacillus stearothermophilus* [48] and *Rhizobium etli* [5,49], the availability of biotin in the medium has been shown to greatly enhance PC activity, and this effect is thought to be mediated through biotinylation of apoenzyme to holoenzyme, rather than by gene induction. In contrast, changes in PC specific activity of *Rhodobacter capsulatus* under different growth conditions are mediated at the level of enzyme synthesis [50]. L-Aspartate is known to inhibit PC activity and biotinylation in yeast through an allosteric effect [46].

In vertebrates, newly synthesized enzyme contains a leader sequence at the N-terminus comprising several positively charged and several hydroxylated amino acids, but no acidic amino acids [11]. Upon translocation to the mitochondria, this targeting sequence undergoes cleavage, resulting in a decrease in the subunit molecular mass [51]. No further post-translational modification (including phosphorylation [52]) has been reported except for biotinylation.

In confirmation of earlier studies on the intracellular localization of PC [53,54], Rohde et al. [55] employed an immunoelectron microscopic approach to show that vertebrate PC is located exclusively in the mitochondrial matrix, close to the mitochondrial inner membrane. Several studies have indicated that the glycolytic and gluconeogenic enzymes present in the cell are associated in the form of multienzyme complexes [56,57]. PC is also found to be specifically associated with other mitochondrial enzymes: in binary complexes with mitochondrial aspartate transferase or malate dehydrogenase, in a ternary complex with aspartate aminotransferase and glutamate dehydrogenase, and in a quaternary complex with aspartate aminotransferase, glutamate dehydrogenase and malate dehydrogenase [58]. These interactions among PC and other mitochondrial enzymes are likely to profoundly influence their characteristics and kinetic properties [56], but such effects are yet to be defined for PC.

The difference in the subcellular localization of PC between vertebrates (mitochondria) and yeast (cytosol) implies that these enzymes have different regulatory features that control the flow of metabolites for gluconeogenesis, lipogenesis and anaplerosis in these organisms. The vertebrate enzyme is known to be activated by short-chain derivatives of CoA, preferably acetyl-CoA. In contrast, the yeast enzyme is most effectively activated by long-chain acyl-CoA derivatives, such as palmitoyl-CoA, and

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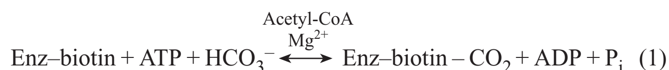
is inhibited by aspartate and 2-oxoglutarate, whereas the mitochondrial enzyme is not [59].

The half-life of PC in rat liver is about 4.6 days [60,61], which is slightly longer than the average turnover time of 3.8 days for mitochondrial proteins. However in a human cell line [HE(39)L] [62] and in 3T3-L1 mouse adipocytes [63], PC has been shown to have a shorter half-life. The degradation of PC in these cell lines has been suggested to be mediated via the mitochondrial autophagic/lysosomal degradative pathway [62].

REACTION MECHANISM

The biotin-dependent enzymes comprise a diverse group of enzymes, which includes carboxylases, i.e. PC, ACC, propionyl-CoA carboxylase (PCC; EC 6.4.1.3), methylcrotonoyl-CoA carboxylase (MCC; EC 6.4.1.4), urea carboxylase (EC 6.3.4.6), geranoyl-CoA carboxylase (EC 6.4.1.5) and transcarboxylase (methylmalonyl-CoA carboxyltransferase; EC 2.1.3.1), and decarboxylases, e.g. oxaloacetate decarboxylase (EC 4.1.1.3), glutaconyl-CoA decarboxylase (EC 4.1.1.70) and methylmalonyl-CoA decarboxylase (EC 4.1.1.41) [64]. Each of these enzymes contains the prosthetic group biotin, which is covalently bound to the ϵ-amino group of a specific lysine residue. In mammals only four biotin-dependent carboxylases, i.e. ACC, PC, PCC and MCC, have been identified so far [65]. The biotin carboxylase enzymes, comprising three functional components (i.e. biotin carboxylase, biotin carboxyl carrier protein and carboxyltransferase), have been found in a wide variety of organisms. Although these enzymes are involved in diverse metabolic pathways, such as gluconeogenesis, lipogenesis and the breakdown of five amino acids, they share a common reaction mechanism. The reactions involve the ATP-dependent carboxylation of biotin, which serves as a 'swinging arm' in transferring CO_2 to different acceptor molecules [66].

The reaction mechanism of PC has been extensively reviewed by Attwood [67]. The overall reaction catalysed by PC occurs in two spatially separated subsites, and can be summarized in two partial reactions (eqns. 1 and 2), as follows (where Enz denotes enzyme):



$$\text{Enz–biotin} + \text{ATP} + HCO_3^- \xleftrightarrow[\text{Mg}^{2+}]{\text{Acetyl-CoA}} \text{Enz–biotin} - CO_2 + \text{ADP} + P_i \quad (1)$$

$$\text{Enz–biotin} + CO_2 + \text{pyruvate} \longleftrightarrow \text{oxaloacetate} + \text{Enz–biotin} \quad (2)$$

The first partial reaction involves the formation of the enzyme–carboxybiotin complex, most probably via the formation of a very labile carboxyphosphate intermediate [66]. Evidence in support of this comes from studies demonstrating that the biotin carboxylase subunit from *E. coli* ACC can phosphorylate ADP from carbamoyl phosphate, which is an analogue of carboxyphosphate, to form ATP [68]. The observation that PCs from sheep kidney and chicken liver are also capable of catalysing the phosphorylation of ADP from carbamoyl phosphate provides further support for such an intermediate [69,70]. Support for this proposed mechanism was provided by the isolation of a putative enzyme-bound carboxyphosphate intermediate [71]. In the presence of acetyl-CoA, the carboxy group is subsequently transferred to biotin to form carboxybiotin, which is the product of the first partial reaction [71,72]. Further evidence favouring the formation of a carboxyphosphate intermediate is to be found in the similarity of the primary structure of biotin carboxylase to that region of carbamoyl-phosphate synthase (CPS; EC 6.3.5.5) shown by site-directed mutagenesis to be involved in the reaction mechanism for the synthesis of carboxyphosphate [73,74].

PCs from most species are allosterically activated by acetyl-CoA. PC isolated from chicken is absolutely dependent on acetyl-CoA [75], while PCs isolated from rat liver [76], sheep [77] and *Bacillus stearothermophilus* [78] are also highly dependent on acetyl-CoA. In contrast, yeast PC is less dependent on acetyl-CoA [79]. Unlike PCs from other sources, those from *Pseudomonas citronellolis* [80], *Aspergillus niger* [81] and *M. thermoautotrophicum* [47] are acetyl-CoA-independent.

PCs from many sources possess a reactive lysine residue whose integrity is essential for full enzymic activity. Modification of the enzyme with an amino-group-selective reagent causes the preferential loss of the catalytic activity that is stimulated on allosteric activation by acetyl-CoA [82,83]. The loss of acetyl-CoA-dependent activity is due to the modification of a single lysine residue per active site [82]. Acetyl-CoA has also been shown to protect against the loss of enzyme activity caused by this modification [82–84]. This essential lysine residue has been suggested to form part of the acetyl-CoA-binding site, rather than being protected by a conformational change following activator binding [84]. Sequence comparisons of yeast PC with other biotin-dependent enzymes that bind acetyl-CoA (i.e. ACC and the β-subunit of PCC) or propionyl-CoA (i.e. the 12 S subunit of transcarboxylase), as well as with other acyl-CoA-binding enzymes, have so far been unable to identify any putative acetyl-CoA-binding site on PC. This may reflect the fact that ACC and PCC bind acetyl-CoA as a substrate, whereas PC binds it as an allosteric ligand [85].

A number of ionizable groups are proposed to form part of the active site of PC involved in enolizing biotin [86,87]. Modification of the side chains of a lysine (-NH_3 group) and a cysteine (-SH group) residue by *o*-phthalaldehyde results in inactivation of the first partial reaction [88], supporting their crucial role as an ion pair [11].

In addition to its role in complexing with ATP, Mg^{2+} is an essential cofactor of the reaction, as revealed by kinetic studies of PC [76,89–91]. Evidence in favour of there being two extrinsic binding sites for bivalent cations, in addition to the intrinsic bivalent cation (see below), was obtained using EPR spectroscopy to show that extrinsic Mn^{2+} binds to PC in the presence of CrATP, a potent competitive inhibitor of MgATP [92]. Recently, EPR has again been used to demonstrate that two equivalents of the bivalent oxyvanadyl cation, VO^{2+}, bind at the first subsite of PC: one is involved in nucleotide substrate binding, while the other interacts strongly with bicarbonate [93]. These authors have suggested that the roles of this second extrinsic cation could include orientation of the bicarbonate for attack on the γ-phosphoryl group of ATP, as well as minimizing charge repulsion between these anionic substrate species.

Kinetic studies [77,94] have previously shown that certain univalent cations (K^+, NH_4^+, Rb^+, Cs^+ and Tl^+) are effective (3–7-fold) activators of PC, having an apparent equilibrium-ordered binding interaction with HCO_3^-, which binds first. Recently, direct evidence supporting this relationship was obtained when binding constants for K^+ and Tl^+, measured by their quenching of intrinsic protein fluorescence, were shown to agree well with the activator constants, and HCO_3^- was shown to enhance the affinity of chicken PC for Tl^+ by 2-fold [93]. Together these data suggest the univalent cation binds in the vicinity of bicarbonate in the first subsite. However, it was concluded from a study of the superhyperfine coupling between the electron spin of VO^{2+} and the nuclear spin of Tl^+ that this activating univalent cation is unlikely to share a ligand with either the enzymic or the nucleotide VO^{2+} cation [93].

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The second partial reaction involves the transfer of the carboxy group from carboxybiotin to pyruvate, to form oxaloacetate. It was proposed that the binding of pyruvate induces the carboxybiotin to move into the second subsite, where it is destabilized [95]. Goodall et al. [96] confirmed this proposal, and also showed that a number of pyruvate analogues can induce the translocation of carboxybiotin to the second subsite. As shown in eqn. (2), this process requires the removal of a proton from pyruvate, a carboxy-group transfer and the reprotonation of biotin. There is kinetic evidence for the involvement of another cysteine–lysine ion pair in the second subsite [97]. Werneberg and Ash [88] reported the presence of a second cysteine–lysine pair, as revealed by the modification of such an ion pair by *o*-phthalaldehyde resulting in the loss of the second partial reaction. On the basis of these results, Attwood [67] proposed the detail of the second partial reaction: the cysteine–lysine pair stabilizes the enol form of biotin and participates in the proton transfer between biotin and pyruvate, via the -SH group of cysteine.

STRUCTURE

Native PC from a number of sources, including bacteria, yeast, insects and mammals, consists of four identical subunits (α_4) of approx. 120–130 kDa [98]. However, for the enzymes from *Pseudomonas citronellolis* [99], *Azotobacter vinelandii* [100] and *Methanobacterium thermoautotrophicum* [47], each protomer consists of two polypeptide chains, with 75 kDa (α) and 52 kDa (β) subunits arranged as an $(\alpha\beta)_4$ structure. Although primary structures of PCs from a number of organisms have been reported in recent years, a high-resolution three-dimensional structure of this enzyme has yet to be established. Sequencing of cDNA or genes encoding PC, limited proteolysis and primary structure comparisons have shown that PCs from different species, including *S. cerevisiae* [42,85,101,102], various bacteria [5,47,103,104], mosquito [105], mouse [106], rat [11,107] and human ([10,108]; M. E. Walker, S. Jitrapakdee, D. L. Val and J. C. Wallace, GenBank accession number U30891), contain three functional domains, i.e. the biotin carboxylation domain (N-terminal region), the transcarboxylation domain (central region) and the biotin carboxyl carrier domain (C-terminal region).

Since biotin-dependent carboxylases share common reaction mechanisms, it is not surprising that the various regions of these enzymes exhibit sequence similarities with each other, especially within the biotin carboxylation and the biotin carboxyl carrier domains, which are both involved in the first partial reaction. Indeed, Lim et al. [85] and Samols et al. [110] identified a number of regions of sequence similarity between different members of the biotin carboxylases, in addition to similarities with some other proteins. The multiple sequence alignment of the N-terminal region of PCs from different species in our previous reports [11,85] has been extended and is shown in Figure 1(a). A number of highly conserved amino acid residues that are likely to play a role in either structure or catalysis are highlighted. Analysis of this region in comparison with the crystal structure of the biotin carboxylase subunit of *E. coli* ACC [111] has identified 11 highly conserved residues within the biotin carboxylation domain [11] that are likely to play an important role in catalysis. Notably, a cysteine–lysine pair located in the biotin carboxylation domain of different biotin carboxylases is invariant, and has been suggested on the basis of chemical modification studies [88] to form an ion pair in the enolization of biotin in the first partial reaction [11]. Mutation of these cysteine and lysine residues individually to alanine in the yeast PC1 isoenzyme dramatically diminished its activity, confirming the crucial role of these two residues in the catalytic reaction (M. G. Nezic and J. C. Wallace, unpublished work).

Analysis of the secondary-structure elements of the *E. coli* biotin carboxylase subunit compared with the biotin carboxylase domain of PC has suggested that the overall folding of these two enzymes within this region is likely to be conserved [11]. Recent analyses have revealed very extensive similarities between the folds of the biotin carboxylase subunit of *E. coli* ACC, of other members of the family of ATP-binding ligases [129] and of CPS [130]. Interestingly, the last enzyme catalyses the formation of carbamoyl phosphate from bicarbonate, glutamine and two molecules of MgATP through the formation of a carboxyphosphate intermediate, which is considered to be the intermediate in the PC reaction [110,131]. The first three domains in each half of the large subunit, where the formation of carboxyphosphate occurs, show a remarkably similar structure to those observed in the biotin carboxylase subunit of *E. coli* ACC [130] (Figure 2). This part of the molecule also displays a structure similar to those of other ATP-binding proteins, e.g. D-alanine ligase [135], glutathione synthase [136] and succinyl-CoA synthetase [137]. Not only is the structure of the large subunit of CPS similar to that of the biotin carboxylase subunit of *E. coli* ACC, but some residues forming part of the active site, including Arg^{303}, Asn^{301} and Glu^{299}, are also identical to those found in the biotin carboxylase subunit of *E. coli* ACC. These residues are equivalent to Arg^{292}, Asn^{290} and Glu^{288} respectively in the biotin carboxylase subunit of *E. coli* ACC, where they have been shown to surround a phosphate molecule [111]. These residues are also equivalent to Arg^{328}, Asn^{326} and Glu^{324} in mammalian PC [10,11,106–108] (see Figure 1a). In addition to these identical residues, there are other positions where the substitution are conservative, e.g. Lys or Arg; Asp or Glu; Val, Leu, Ile or Met, etc.

Except for the sequence similarities between the lipoyl domain and biotinyl domains (see below), there are no other significant sequence similarities between PC and either pyruvate dehydrogenase (EC 1.2.4.1) or pyruvate decarboxylase (EC 4.1.1.1). However, there is extensive identity within the transcarboxylation domains of PC and other biotin-dependent pyruvate-binding enzymes, e.g. the 5 S subunit of transcarboxylase and oxaloacetate decarboxylase, as shown in Figure 1(b). The conserved motif EXWGGATXDXXXRFLECP<u>W</u>XRL has been identified in PCs and oxaloacetate decarboxylases from different species [110]. Chemical modification of Trp^{73} of the 5 S subunit of *Propionibacterium shermanii* transcarboxylase (corresponding to the underlined Trp within the above motif) has indicated that this residue is either directly involved in or near the pyruvate-binding site of the enzyme [138]. Each subunit of all PCs studied so far contains a tightly bound bivalent metal ion, either Mn^{2+} (vertebrate enzyme) [139] or Zn^{2+} (yeast enzyme) [140], which appears to play a structural rather than a catalytic role [141]. Chelation of Mn^{2+} by 1,10-phenanthroline causes loss of enzymic activity, as a result of destabilization of the active tetrameric structure of the enzyme [141]. A putative metal-binding motif (HXHXH), similar to that found in the protein kinase C inhibitor [142], has been proposed to bind these metal ions and to stabilize the active conformation of PC [11,143] (see Figure 1b). ψ-BLAST searches [222] also reveal a significant degree of similarity with hydroxymethylglutaryl-CoA lyase.

A number of residues within the biotin carboxyl carrier domain of all known PCs show significant identity with other biotin-containing enzymes, as shown in Figure 1(c), suggesting that they fold to a similar structure [43]. The crystal structure of the C-terminal 80 residues of the biotin carboxyl carrier subunit of

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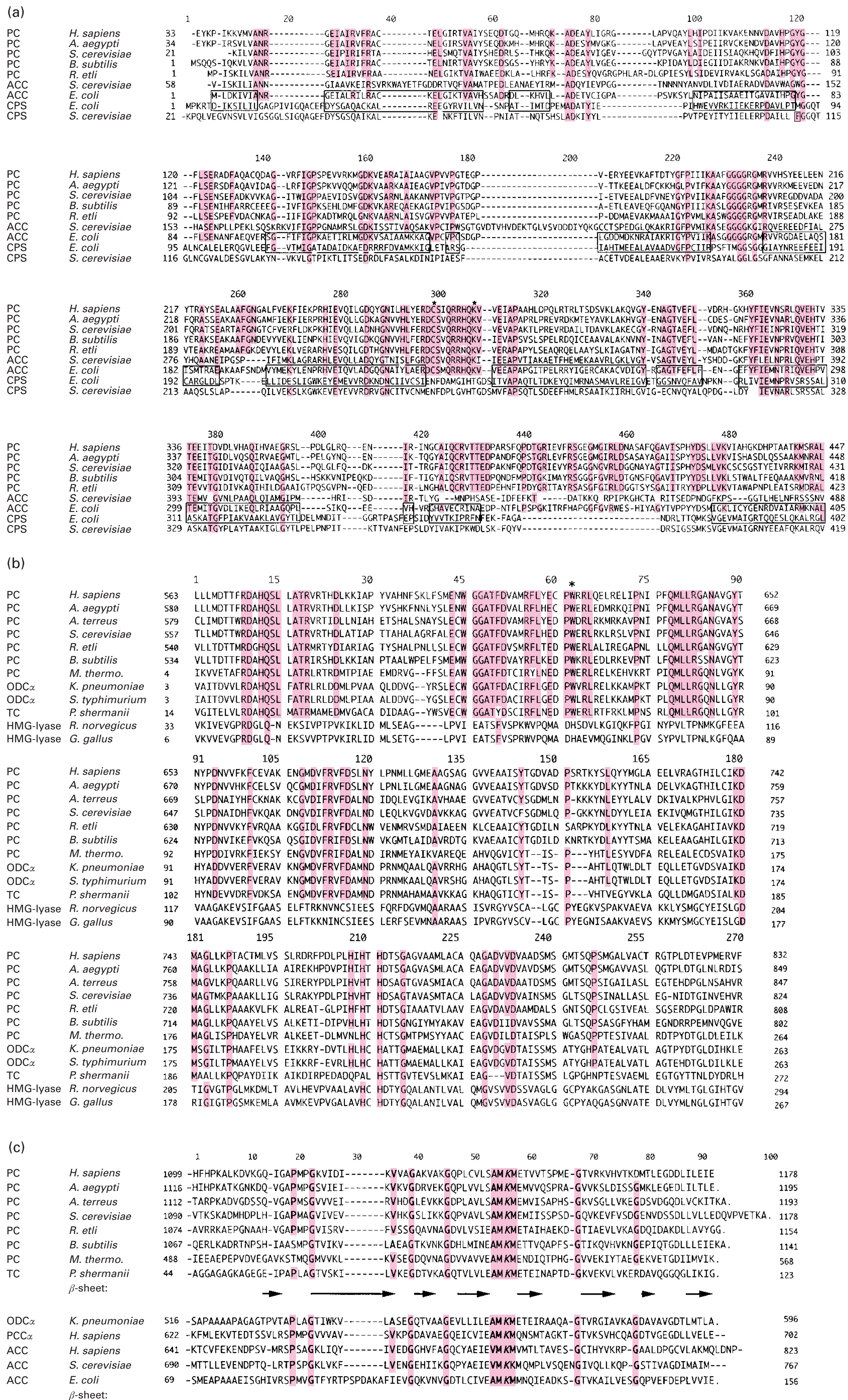

Figure 1 For legend see opposite page

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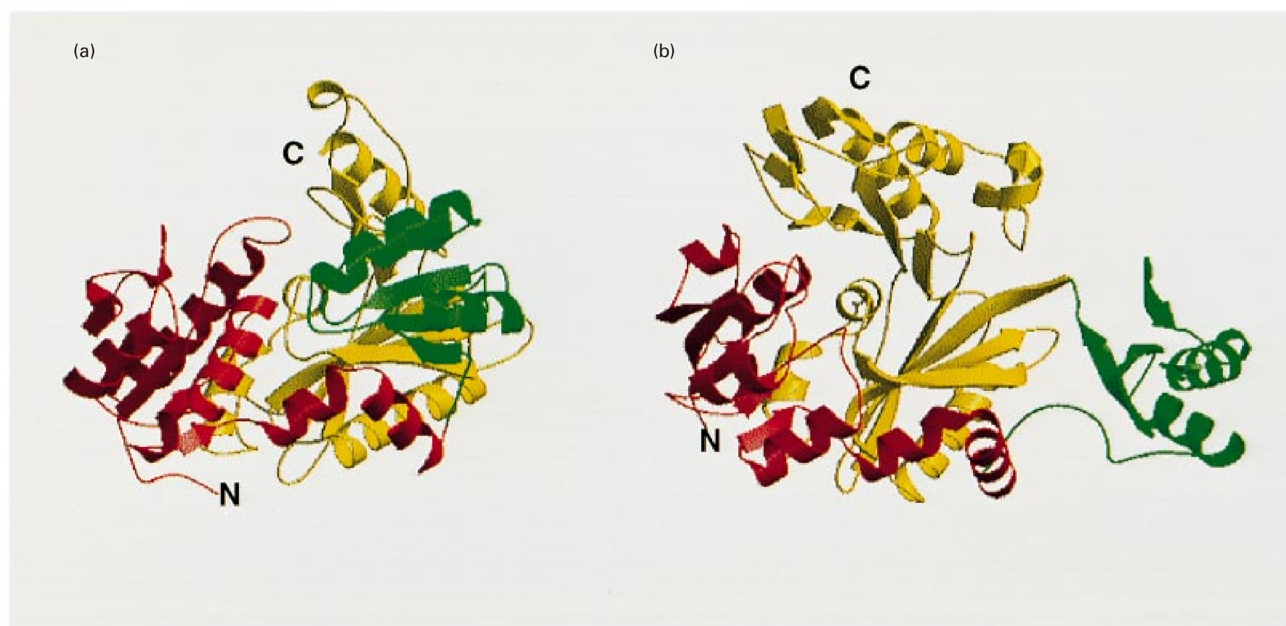

Figure 2 X-ray crystal structure of *E. coli* CPS and the biotin carboxylase subunit of *E. coli* ACC

Shown are schematic representations of the three-dimensional structure of the large subunit of *E. coli* CPS [130] (**a**) and the biotin carboxylase subunit of *E. coli* ACC [111] (**b**), showing the arrangement of β-strands and α-helices generated with MOLSCRIPT [132] and Raster3D [133,134]. Regions of structural similarity between these two enzymes, determined by superimposition using Homology/Insight (Molecular Simulations Inc.), are indicated by the same colours: red (corresponding to the residues 1–142 of CPS and residues 1–130 of *E. coli* ACC, as shown in Figure 1a), green (residues 143–211 and residues 131–206 respectively) and yellow (residues 212–401 and residues 207–406 respectively).

E. coli ACC (holoenzyme) [113] revealed that this domain adopts the same basic fold as the lipoyl domains of *E. coli* pyruvate dehydrogenase [144,145]. The structure of the holoprotein is very similar to that of the apoprotein, determined by NMR [146], with small local conformational changes observed in the β-turn that contains the lysine residue modified in the biotin ligation reaction. Chemical modification and proteolysis studies of the apo- and holo-enzymes also indicated that a conformational change accompanies biotinylation [147]. The recent determination by NMR of the three-dimensional structure of the entire 1.3 S subunit of *P. shermanii* transcarboxylase, which functions as the carboxyl group carrier of this enzyme, also showed that the C-terminal half of this subunit is folded into a compact domain, consistent with the fold found both in the carboxyl carrier protein of *E. coli* ACC and in the lipoyl domains, to which this domain exhibits only 26–30% sequence similarity [114]. Therefore it is predictable that the biotin carboxyl carrier domain of yeast PC would fold to a similar structure as the lipoyl domains [148], as there are remarkable sequence similarities between these two families of proteins [85].

A number of highly conserved residues flanking the biotin attachment site of different biotin-dependent enzymes are highlighted in Figure 1(c). Since it has long been known that the holocarboxylase synthetase from mammals can biotinylate bacterial apocarboxylases [149] and that mammalian apocarboxylases are biotinylated by bacterial biotin ligase *in vitro* [150], this sequence conservation might reflect a molecular mechanism common to all of the biotin-dependent enzymes that interact with biotin ligase or holocarboxylase synthetase. Although an Ala-Met-Lys-Met motif is highly conserved across biotin-dependent enzymes, substitution of either methionine residue flanking the biotinylated lysine of the 1.3 S biotinyl subunit of *P. shermanii* transcarboxylase [151] or of the α-subunit of human PCC [152] had no effect on biotinylation efficiency. However, substitution of the methionines flanking the targeted lysine of the biotin carboxyl carrier protein of *E. coli* ACC did affect the biotinylation reaction [153].

In all PCs with an α_4 subunit composition, the transcarboxylation domain is connected to the N-terminal biotin carboxylation domain and to the C-terminal biotin carboxyl carrier domain by

Figure 1 Multiple sequence alignment of PC with other biotin-dependent enzymes

The amino acid sequences from a representative selection of eukaryotic and prokaryotic PCs, and similar residues from other biotin-dependent enzymes and other enzymes shown by a ψ-BLAST search [231] to be related, were compared using Clustal W [112]. (**a**) Biotin carboxylation domain. The highly conserved amino acid residues found in different groups of enzymes are showed by pink shading. The open boxes represent the residues of ACC and CPS of *E. coli* superimposed using the Homology/Insight program (Molecular Simulations Inc., San Diego, CA, U.S.A.) (see Figure 2). The cysteine–lysine pair is indicated by asterisks. (**b**) Transcarboxylation domain. The highly conserved residues are indicated by pink shading. The putative pyruvate-binding site is indicated by an asterisk. (**c**) Biotinyl domain. The highly conserved residues are indicated by pink shading. The biotinylated lysine residue is italicized. Also shown are the β-strands (arrows) observed in the crystal structure of the biotin carboxyl carrier protein of *E. coli* ACC [113] and in the NMR structure of the 1.3 S subunit of *Propionibacterium shermanii* transcarboxylase [114]. Sources: PC *Homo sapiens* (human) [10,108,109]; PC *Aedes aegypti* (mosquito) [105]; PC1 *Saccharomyces cerevisiae* [85]; PC *Aspergillus terreus* (Y. F. Li, M. C. Chen, Y. H. Lin, C. C. Hsu and Y. C. Tsai, GenBank accession number AF097728); PC *Rhizobium etli* [5]; PC *Bacillus subtilis* [116]; PC *Methanobacterium thermoautotrophicum* (*M. thermo.*) [47]; ACC human [117]; ACC *S. cerevisiae* [118]; ACC *E. coli* [119]; CPS *E. coli* [120], CPS *S. cerevisiae* [121]; transcarboxylase (TC), 5 S subunit [122] and 1.3 S subunit [123] of *P. shermanii*; ODCα (oxaloacetate decarboxylase α-subunit) *Klebsiella pneumoniae* [124]; ODCα *Salmonella typhimurium* [125]; PCCα (PCC α-subunit) human [126]; 3-hydroxyl-3-methylglutaryl-CoA lyase (HMG-lyase) rat (*Rattus norvegicus*) [127]; HMG-lyase chicken (*Gallus gallus*) [128].

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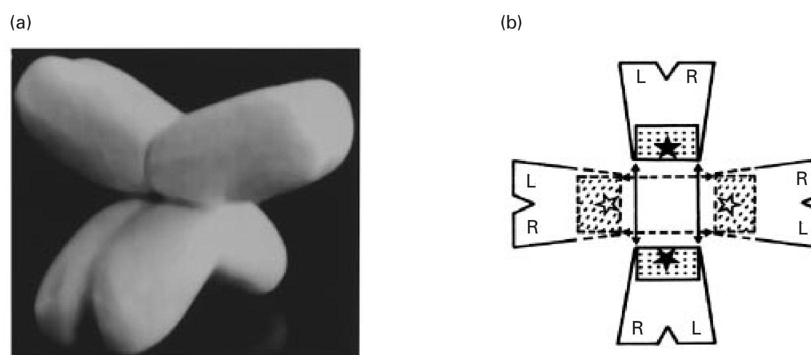

Figure 3 Quaternary structure of vertebrate PC derived from electron microscopic studies

(**a**) PC consists of four identical subunits arranged in a tetrahedron-like structure, with a midline cleft separating two distinct domains and running along the longitudinal axis of each monomer (reproduced, with permission, from Mayer et al. [154]). (**b**) Exploded-face view of the enzyme tetramer with an indication of the bound avidin molecule (shaded), with the sites of the biotin-binding areas indicated (solid star, on the avidin bound to upper pair of PC subunits; open star, on the avidin bound to the lower pair of PC subunits (reproduced, with permission, from Johannssen et al. [155]).

proline-rich sequences. These unusual sequences have been suggested to form 'hinge-like structures' that allow the three domains of PC to fold together to form a single active site [11]. The motif Pro-Xaa-(Pro/Ala), found approx. 30 residues upstream of the biotin-binding site (except in *M. thermoautotrophicum* and *Bacillus subtilis* PCs), has been proposed to provide flexibility for movement of the biotin prosthetic group between the catalytic centres in a manner analogous to the highly mobile Pro-Ala sequences in lipoylated proteins [110]. In the α-subunit of human PCC, it has also been shown that the Pro-Met-Pro motif (26 residues N-terminal of the target lysine) is critical for biotinylation. Deletion of this motif abolished biotinylation [152].

The quaternary structure of PC has so far only been obtained by electron microscopic studies, which have revealed that the PCs from chicken, rat and sheep are indistinguishable tetrahedron-like structures, composed of two pairs of subunits in different planes orthogonal to each other [154] (Figure 3a). The opposite pairs contact each other on their convex surfaces, with a midline cleft separating two distinct domains and running along the longitudinal axis of each monomer. Since this midline cleft becomes less visible in the presence of acetyl-CoA, it has been suggested that this cleft area might be the active site of the enzyme [154]. Using avidin as a structural probe, Johannssen et al. [155] have shown that the biotin moieties are localized in the midline cleft on the external surface of each subunit, close to the inter-subunit junction (see Figure 3b). PCs from *Aspergillus nidulans* [156], *S. cerevisiae* [157] and even *Pseudomonas citronellolis* [158], in which PC is arranged as an $(\alpha\beta)_4$ configuration, all appeared to be tetrahedron-like structures with a midline cleft similar to that in the vertebrate enzymes. Dilution of sheep [77,159] and chicken [160] PCs resulted in inactivation of PC activity, accompanied by dissociation of the active tetramers into inactive dimers and monomers, as revealed by electron microscopic and high-resolution gel-filtration studies. Acetyl-CoA was shown to prevent both the dissociation of the tetrameric enzyme and the associated loss of activity. Addition of acetyl-CoA to partially dilution-inactivated enzyme prevented further loss of enzymic activity and of tetrameric structure [159,160]. This ligand was similarly effective in preventing the cold-induced loss of both activity and tetrameric structure of chicken PC [161]. Apart from stabilizing the quaternary structure of PC, addition of acetyl-CoA was also shown to cause conformational changes in PC, as revealed by spectrophotometric [162], ultracentrifugal [163] and electron microscopic [164] studies.

THE GENE ENCODING PC

A gene encoding the *S. cerevisiae* PC1 isoenzyme was first cloned by Lim et al. [85]. Walker et al. [41] and Stucka et al. [42] independently discovered that, in fact, this yeast contains two genes encoding two isoenzymes (PC1 and PC2). The *PC1* gene is located on chromosome VII, while the *PC2* gene is located on chromosome II [41,42]. Neither *PC1* nor *PC2* contains an intron. Walker et al. [41] found that disruption of the *PC1* gene reduced the PC activity of DBY 746 yeast to 10–20 %. In contrast, Stucka et al. [42] found that disruption of either *PC1* or *PC2* in the W303 strain resulted in retention of 50 % of total PC activity. However, disruption of both genes resulted in complete loss of enzyme activity [42,165]. It was found later that there is a polymorphism of the *PC2* gene in these different yeast strains used by the two groups of investigators, as indicated by amino acid differences in the PC2 protein [101]. The most significant difference is a single-base substitution near the 3′-end of the gene, which alters the reading frame encoding the biotin domain of the enzyme. This C-terminal variant has been shown to affect biotinylation of the enzyme *in vitro* [101].

In the mosquito, there is evidence for the presence of two PC isoforms of similar size, i.e. 133 and 128 kDa. These two isoenzymes exhibit tissue-specific expression [105]. However, it is uncertain whether these PC isoforms are the products of two separate genes, or of a single gene with allelic polymorphism in the genome [105].

In the rat, a single PC gene has been mapped to chromosome 1q43 [166], and consists of 19 coding exons and four 5′-untranslated region exons [167] spanning over 40 kb, as indicated in Figure 4. Alternative transcription from two tissue-specific promoters is responsible for the production of different transcripts, which undergo differential splicing at the 5′-end. This alternative splicing yields five different mature transcripts which contain the same coding region but differ in their 5′ non-coding sequences [168].

In humans, the PC gene has been mapped to the long arm of chromosome 11 by the somatic cell hybrid technique [169], and to the 11q13.4 position by fluorescence *in situ* hybridization

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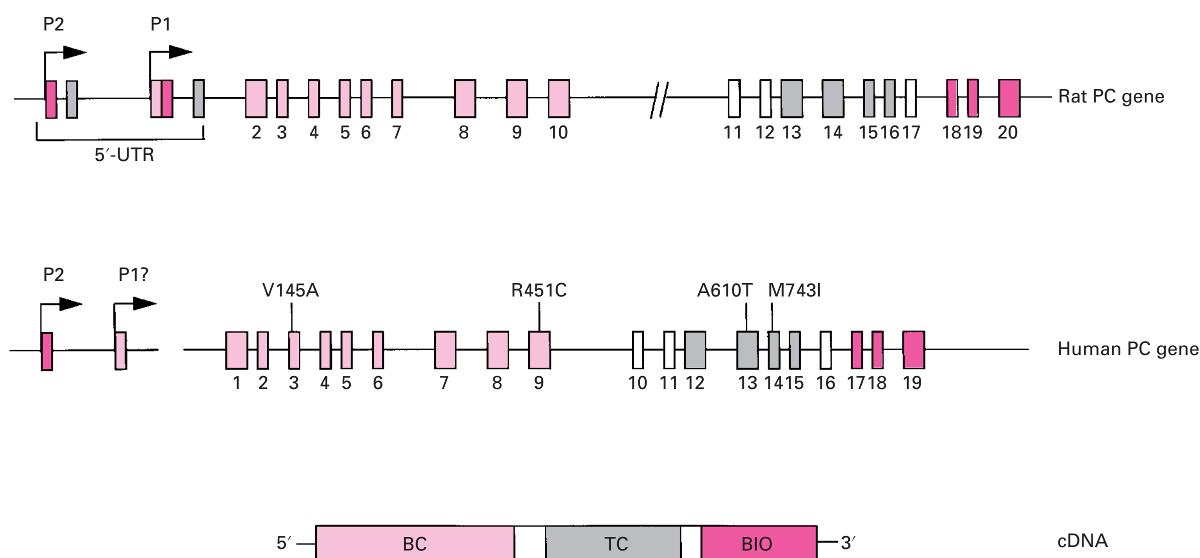

Figure 4 Structural organization of mammalian PC genes and point mutations associated with PC deficiency, identified within different coding exons of the human PC gene

The human PC gene consists of 19 coding exons [171] and is organized in the same manner as in the rat gene, which includes two alternative promoters (P1 and P2) [167]. Two putative alternate promoters (P1 and P2), located upstream from the first coding exon of the human PC gene, are likely to control alternative transcription of a single PC gene [168]. Different point mutations on the human PC gene, reported to be responsible for some forms of PC deficiency [171,228], are also shown. Boxes represent exon sequence. The cDNA structure is also shown: BC, biotin carboxylation domain; TC, transcarboxylation domain; BIO, biotin carboxyl carrier domain. 5′-UTR, 5′-untranslated region exon.

[170]. The human PC gene also contains 19 coding exons, spanning over 16 kb [171] (Figure 4). Two alternative transcripts, having the same coding region but differing in the 5′-untranslated regions, have also been reported in liver, and are likely to be transcribed from two alternative promoters [168].

EVOLUTION OF BIOTIN-DEPENDENT CARBOXYLASE ENZYMES

Clearly, there are many proteins for which some structural part of the molecule resembles a part of other proteins in the same family, or even in an unrelated family. This appears to have resulted from gene duplication and rearrangement, thus facilitating the reconfiguration of protein domains with different functions [172]. In the case of the biotin carboxylase family, it has long been proposed that this group of enzymes has evolved into complex multifunctional proteins from smaller mono-functional precursors through successive gene fusions [173], perhaps via recombination and rearrangement of primordial genes encoding different functional domains of the biotin carboxylases. In Eubacteria such as *B. stearothermophilus* and *R. etli*, or in a lower eukaryote such as yeast, there would seem to have been a fusion of the genes encoding the biotin carboxylase, transcarboxylase and biotin carboxyl carrier components into one gene encoding a single polypeptide. In Archaebacteria such as *M. thermoautotrophicum*, which is distantly related to the Eubacteria, there appears to have been a fusion of the genes encoding the trancarboxylase and biotin carboxyl carrier components to give a gene encoding a 75 kDa biotinylated subunit (PYCB or β-subunit). However, the gene encoding a 52 kDa non-biotinylated subunit (PYCA or α-subunit) is located approx. 727 kb, or approximately half a genome away, from the gene encoding PYCB. The amino acid sequence of the PYCA subunit corresponds to that of the biotin carboxylase domain, whereas the amino acid sequence of the PYCB subunit corresponds to those of the transcarboxylation and biotin carboxyl carrier domains of PCs from a number of species that possess a single polypeptide (see Figure 1) [47].

However, in higher eukaryotes, there may have been an interruption by introns of these primordial genes encoding different components of biotin carboxylases, as is believed to have occurred with other eukaryotic genes [174] during evolution. It is widely accepted that the introns that are present in eukaryotic genes can enhance the rate of evolution through recombination events between intron sequences of different genes followed by divergence of the duplicated gene, thereby creating new combinations of independently folded protein domains [175].

REGULATION OF PC GENE EXPRESSION

Only for yeast *S. cerevisiae* and rat have the promoter regions of the PC genes been investigated thus far. In yeast, although the PC1 and PC2 isoenzymes exhibit high sequence similarity at both the amino acid and nucleotide levels, their 5′-non-coding regions are markedly different [42,85,101], suggesting that the two genes are regulated differently. Two copies of TATA boxes located at positions -117 and -110 relative to the initiation codon were found in the PC1 promoter [85], but only the downstream TATA box is functional and responsible for transcribing PC1 mRNA with distinct transcription initiation sites [176]. The basal promoter of PC1 is located within the first 330 bp of the 5′-non-coding region, which consists of a TATA element and a UAS1 transcription factor binding site, whereas the basal promoter of PC2 is located within the first -291 bp of the 5′-non-coding region [177]. PC1 and PC2 appear to carry out different metabolic functions. PC1 expression has been shown to be relatively constant throughout the main growth phase during growth on glucose minimal media, while PC2 expression is characterized by a high level of transcript production in the early growth phase. Both genes are repressed throughout the latter stages of growth. During growth on ethanol minimal media, PC1 and PC2

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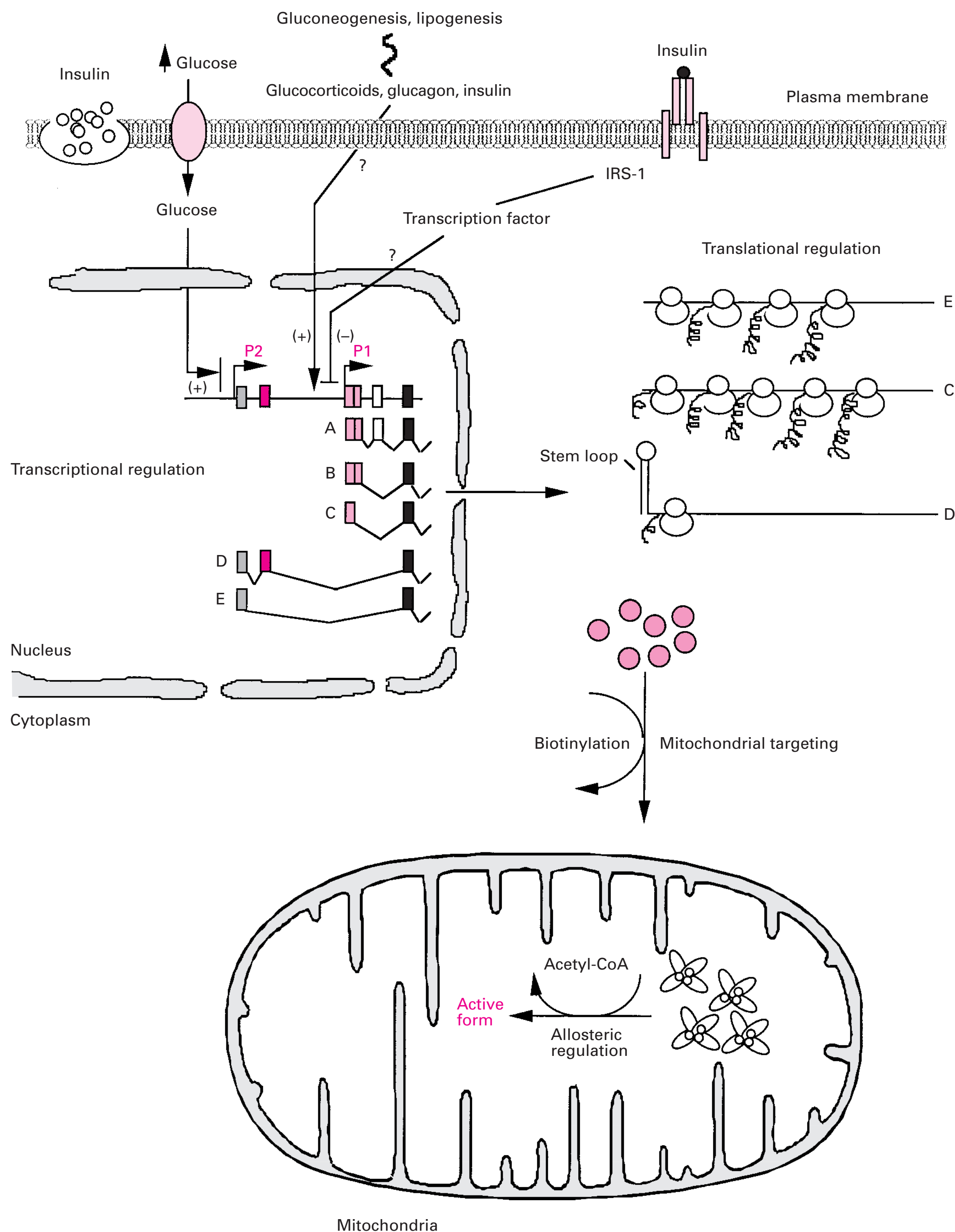

Figure 5 Co-operative regulation of rat PC expression by short-term and long-term mechanisms

Different metabolic signals outside the cells (i.e. glucose-induced insulin release in pancreatic β-cells, gluconeogenesis and lipogenesis), mediated through the hormonal changes that can affect PC expression, are shown to alter the activity of two alternative promoters (P1 and P2) of the rat PC gene at the transcriptional level (+, stimulation; –, inhibition). This results in the generation of alternative transcripts with 5′-end heterogeneity, i.e. liver/adipose-specific PC transcript C and 'housekeeping' PC transcripts D and E, which can be controlled at the translational level through the formation of a stable secondary structure (stem loop) in the 5′-untranslated region. Short-term regulation of PC activity is achieved post-translationally through allosteric activation by acetyl-CoA following targeting of the enzyme to the mitochondria. Different coloured boxes shown in the nucleus represent the different 5′-untranslated regions of different PC transcripts derived by alternative splicing. Abbreviation: IRS-1, insulin receptor substrate-1.

expression exhibits a similar pattern, i.e. decline from early to mid-exponential phase. However, in the DBY 746 strain the level of PC1 expression is 10-fold above that of PC2 during this fermentative growth [165]. Further studies with PC1 or PC2 null mutants have also indicated that the lack of either PC gene has little effect on the level and pattern of expression of the other PC

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gene, suggesting that the two genes are regulated differently. However, the PC1 null mutant or double null mutant of DBY 746 showed a strong requirement for L-aspartate in ethanol minimal media [165]. This strongly suggests that the PC1 isoenzyme plays a crucial role in maintaining growth on ethanol media, and more specifically in the establishment of glucose-dependent growth on glucose minimal media. In contrast, the role of the PC2 isoenzyme remains unclear, but it is believed to support growth on a glycolytic carbon source [165]. Different carbon sources have also been shown to affect PC1 and PC2 expression differently [165,177].

Regulation of PC expression in mammals appears to be more complicated than in yeast. In the rat, two distinct promoters have been shown to be responsible for alternative transcription from a single gene. As shown in Figure 5, alternative transcription from these two promoters results in the production of two distinct primary transcripts, which undergo differential splicing of the 5′-untranslated region exons and give rise to five transcripts, of which three are predominant with distinct 5′-non-coding regions [167,168]. The proximal promoter (P1) is only active in gluconeogenic (liver and kidney) and lipogenic (adipose tissue) tissues, and is responsible for the production of the liver/adipose-specific transcript (transcript C). This is probably due to the presence of tissue-specific transcription factors that interact with *cis*-acting elements in the proximal promoter (presumably hepatic nuclear factor-4 in liver, and fat-specific element-1 in adipose tissue). In contrast, the distal promoter (P2) is active in most tissues, resulting in the production of 'housekeeping' transcripts (transcripts D and E) [167,168]. The proximal promoter lacks a canonical TATA or CAAT box, but contains a motif resembling a housekeeping initiator (HIP-1) binding site, while the distal promoter contains three copies of the CCAAT box. Deletion analysis has demonstrated that the 153 bp and 187 bp preceding the transcription start site of the proximal and distal promoters respectively are required for basal transcription [167].

As indicated in Figure 5, alterations in plasma insulin, glucagon and glucocorticoid levels during the postnatal gluconeogenic period [178] and during lipogenesis [179] have been shown to affect PC expression. These hormonal changes may involve up-regulation of transcription from the proximal promoter to supply the demands of the cells under these conditions. An *in vivo* study has shown that insulin down-regulates PC expression in diabetic rats [61], but the mechanism by which insulin works remains unclear. Indirect evidence, obtained from a reporter gene study, has shown that insulin inhibited transcription from the proximal promoter through an insulin-responsive element which is currently unidentified [167]. Binding of insulin to its receptor is known to result in a signalling cascade triggered by the tyrosine-specific protein kinase activity of the insulin receptor. This signalling event leads to the phosphorylation of insulin receptor substrate-1, which in turn activates several downstream effectors, including transcription factors [180]. On the other hand, the distal promoter appears to have a housekeeping function in other tissue types. Interestingly, this promoter seems to play an anaplerotic role in insulin-secreting cells, i.e. pancreatic islets and insulinoma cells. This promoter is induced if these cell types are grown in higher-than-physiological concentrations of glucose [28]. The presence of alternative promoters which are activated under different physiological conditions is an important mechanism to allow an independent regulation.

In the rat, post-translational control also appears to be another important mechanism for the long-term regulation of PC expression. Different PC transcripts produced from two tissue-specific promoters exhibit different translational efficiencies. This has been shown to be mediated through a sequence in the 5′-untranslated regions of certain PC mRNAs (transcript D) which has the potential to form a secondary structure that could block ribosomal access to the cap site [28]. Therefore the rate of enzyme synthesis would depend on which mRNA species is being produced at the time. Transcriptional and post-transcriptional regulation appear to be the important mechanisms that cells use to modulate PC expression for the long term during different physiological states. The newly synthesized PC then undergoes post-translational modification by biotinylation, followed by translocation into the mitochondrial matrix. However, there is no evidence yet to suggest that the biotinylation of PC is a regulatory step, as it is in some bacteria. Short-term control is known to be via allosteric regulation by acetyl-CoA [181]. The β-oxidation of fatty acids is known to generate a large amount of acetyl-CoA, which acts as a physiological regulator of PC. Allosteric activation of PC by acetyl-CoA enhances the production of oxaloacetate in the short term [182].

PHYSIOLOGICAL STATES THAT ALTER PC EXPRESSION

PC is one of a number of important metabolic enzymes whose expression is regulated in a differential manner between particular tissues in order to achieve an appropriate response to various physiological and pathological stimuli. Long-term regulation involves changes in the total amount of PC through alterations in the rate of enzyme synthesis in liver, kidney and adipose tissue [182]. Different physiological conditions have been shown to alter the level of PC expression: these include nutritional alterations, diabetes, hormonal changes, neonatal development, adipogenesis and lactation.

Nutrition and xenobiotics

Fasting in rats has been shown to induce 2–3-fold increases in hepatic PC activity [183]. Similar results have also been reported in other animals, i.e. cow [184], guinea pig [185] and sheep [54,186]. Little information is available on the effects of refeeding starved rats. However, a small increase in total PC activity has been detected in kidney. The hormonal mechanisms that regulate the total amount of PC activity during fasting and refeeding still remain unclear. Increases in the level of PC activity during starvation have been correlated with increases in the plasma concentrations of glucagon and glucocorticoids [187]. PC activity has also been shown to be reduced by 50 % in diabetes-prone BHE/cdb rats fed a diet containing 6 % menhaden oil, which is rich in long-chain highly unsaturated fatty acids [188].

A study in rats has also shown that both in liver degeneration caused by carbon tetrachloride administration and in alloxan-induced diabetes, there is an increase in PC protein and its activity [189]. Chronic administration to rats of lipoic acid, a chemical that has structural similary to biotin, lowered the activities of biotin-dependent carboxylases, including PC and MCC, to 28–36 % of those of control animals. The decrease in these carboxylases was thought to be due to competition for biotin transport into cells caused by lipoic acid binding to the biotin transporters in the cell membrane or displacing biotin from holocarboxylase synthetase, and was reversed by dietary biotin supplementation [190].

Cadmium has long been known to increase the activities of gluconeogenic enzymes, including hepatic and renal PC in rats, possibly via an elevation in cAMP levels [191,192]. A recent study demonstrated that the PC transcript was up-regulated 2–4-fold in response to cadmium treatment in *Caenorhabditis elegans*. The mechanism by which cadmium induces PC expression has

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not yet been elucidated, although it has been suggested that cadmium could act via either a cAMP- or a calcium-mediated pathway [193].

Diabetes

The rate of hepatic gluconeogenesis is increased dramatically in the diabetic state, concomitant with increases in the activities of all gluconeogenic enzymes, i.e. PEPCK, fructose-1,6-bisphosphatase, glucose-6-phosphatase [183,194] and PC [61]. In rats with streptozotocin-induced diabetes, the hepatic PC activity was increased 2-fold over that of control rats. This increase in enzymic activity, which resulted from an increased amount of protein due to an enhanced rate of synthesis, is thought to be mediated by a high plasma glucagon/insulin ratio [61]. Administration of insulin to diabetic rats brought the amount of PC and its activity back to the control levels.

Apart from being both a gluconeogenic and a lipogenic enzyme, PC also plays an important role in glucose-induced insulin secretion in pancreatic islets, as described above. In the Goto–Kakizaki rat, a genetic model of type II (non-insulin-dependent) diabetes in which glucose-induced insulin secretion in pancreatic β-cells is impaired, it has been found that PC activity was 45 % of that in the normal rat islets, due to a decrease in the amount of PC protein. However, administration of insulin to Goto–Kakizaki rats resulted in the recovery of PC activity to that of normal rats [195]. A low level of pancreatic PC activity was also concomitant with a decrease in the levels of the glucose transporter GLUT2 [196] and of mitochondrial glycerol phosphate dehydrogenase, another enzyme believed to play a role in glucose-induced insulin secretion [195]. Down-regulation of these three proteins in the pancreatic β-cell in type II diabetes is proposed to be an adaptive response by the cell to protect itself from a high glucose concentration by modulating glucose metabolism [195].

Hormonal alterations

It has long been known that thyroid hormone affects the hepatic gluconeogenic rate in rats by increasing the activity of gluconeogenic enzymes, including PC [197]. Experiments carried out by Weinberg and Utter [60] showed that hepatic PC activity was increased 2-fold in hyperthyroid rats, whereas in hypothyroid rats PC was decreased 2-fold. Inhibition of *de novo* protein synthesis with actinomycin D reduced PC activity in thyroxine-treated thyroidectomized rats, suggesting that thyroid hormone increases the rate of PC synthesis [197]. The mechanism of action of thyroid hormone on PC expression remains unclear, as analysis of the promoter regions of the rat PC gene did not reveal any potential thyroid-responsive element within the first 1 kb region upstream from the transcription initiation sites [167].

Glucocorticoids have been shown to acutely stimulate gluconeogenesis [198,199] and to result in an increased glucose output in rat hepatocytes [200]. It was suggested that glucocorticoids induce the gluconeogenic enzymes PC and PEPCK. Short-term treatment of rats with dexamethasone, an analogue of glucocorticoids, caused an increase in PC activity. It has been suggested [201] that glucocorticoids act by relieving the restraint on PC by altering the substrate supply and the intramitochondrial concentrations of effectors [202], perhaps via a Ca^{2+}-influx-mediated mechanism. It has also been found that glucocorticoids are not necessary to maintain basal metabolic gluconeogenic rates in adrenalectomized rats [202,203].

Glucagon has long been demonstrated to increase the rate of pyruvate carboxylation in mitochondria isolated from rat hepatocytes, without changing the level of PC [204,205]. The effect of glucagon could be detected within 6 min, and reached a maximum within 10 min, after liver or hepatocytes were exposed to the hormone [205]. The precise mechanism by which glucagon acts on PC is not well understood. Initially it was suggested that glucagon causes an increase in the transmembrane pH gradient, which in turn stimulates the rate of pyruvate transport into the mitochondria [206,207]. Subsequent experiments using a more potent inhibitor of the pyruvate transporter have shown that, in fact, glucagon does not exert its effect by this mechanism, but rather it stimulates the respiratory chain, leading to an activation of pyruvate carboxylation [208]. Further evidence to support the later hypothesis was obtained from studies using an inhibitor of the respiratory chain in mitochondria isolated from rat hepatocytes. This led to the conclusion that glucagon stimulates respiratory-chain activity via a Ca^{2+}-influx mechanism [209]. The increase in respiratory-chain activity (O_2 uptake) stimulates gluconeogenesis by generating ATP and by providing reducing equivalents to the cytosol. The increase in O_2 uptake therefore indirectly stimulates pyruvate uptake into the mitochondria [210,211]. The mechanism(s) by which glucagon affects pyruvate metabolism have recently been reviewed [201].

Adrenaline is also known to stimulate pyruvate carboxylation by isolated liver mitochondria [212]. Little is known about the mechanism by which adrenaline acts on pyruvate metabolism, although it has been shown that adrenaline also acts via Ca^{2+}-mediated pathways, similar to glucagon.

Postnatal gluconeogenesis

As the maternal circulation provides glucose for the developing fetus, gluconeogenesis does not occur in fetal liver, but is triggered rapidly soon after birth [213]. An increase in PC activity is accompanied by increases in the activities of other gluconeogenic enzymes, confirming that the gluconeogenic pathway begins to function [214,215]. In rats, PC activity is highest at day 7 after birth (suckling period), and begins to decline in the weaned rat to adult levels [215]. The marked increase in PC activity during the suckling period has recently been shown to be concomitant with increases in PC protein and its transcripts [28]. The liver/adipose-specific PC transcript C, of high translational efficiency, was generated from the proximal promoter of the rat PC gene and has been shown to accumulate during such a period. Alterations in plasma glucagon and insulin levels during the weaning period have been proposed to involve up-regulation of hepatic PC expression [178]. Down-regulation of the liver/adipose-specific PC transcript C, concomitant with an increase in housekeeping transcripts of lower translation efficiency, accompanied a decrease in PC protein and its activity during the weaning period and in adults [28].

PC and genetic obesity

In genetically obese Zucker fatty rats (*fa/fa*), PC expression has been shown to be elevated 2–5-fold at the onset of obesity [179], concomitant with an increased level of the liver/adipose-specific PC transcript C [28]. This increase in PC levels is also accompanied by increases in the levels of other lipogenic enzymes, i.e. ACC, fatty acid synthase and ATP-citrate lyase [179]. Given the lipogenic role of PC, as mentioned above, it has been proposed that oxaloacetate is consumed copiously in obesity, thus contributing to the hypertrophy of adipose tissue during the development of obesity [179].

It has also been shown that, during the *in vitro* differentiation of mouse 3T3-L1 preadipocytes into mature adipocytes, this

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conversion is accompanied by increases in the lipogenic enzymes, including fatty acid synthase [216,217] and PC [14,218,219]. The increase in PC activity is concomitant with increases in the rate of enzyme synthesis [218–220] and mRNA level [169,219,221]. The induction of PC in this cell line is consistent with its role in lipogenesis. It has been shown that cAMP down-regulates PC mRNA and PC activity by decreasing the transcription rate of the PC gene and/or PC mRNA stability [219]. Despite these decreases in its mRNA level and enzyme activity, the level of PC protein was not affected. The inactivation of PC did not involve the loss of biotin from the holoenzyme, but was suggested to be due to loss of the active tetrameric form of the enzyme. The mechanism by which this is effected has not been elucidated. Thus cAMP not only exerts its effects by inactivating the protein, but also affects the transcription rate of PC or the stability of its mRNA [219].

PC DEFICIENCY

Given the diverse functions of PC described above, it is apparent that this enzyme plays very significant roles in metabolism. This conclusion is supported by the effects of PC deficiency, whether it occurs in yeast or human. In *S. cerevisiae*, a number of mutants have been reported and shown to affect the growth phenotype [41,165,222]. Furthermore, defects in both the *PC1* and *PC2* genes resulted in a failure to grow on glucose minimal media [42,165]. The same effect has been reported when the PC gene locus was disrupted in the yeast *Pichia pastoris* [102].

In humans, PC deficiency is an autosomal, recessively inherited disease. Patients who suffer from the disease have less than 5 % of normal PC activity when assayed in skin-fibroblast cultures [223]. The main clinical features associated with a PC deficiency are congenital lactic acidosis [223] and deterioration of the central nervous system [224]. Lactic acidosis is associated with the deficit in both gluconeogenesis and tricarboxylic acid cycle activity, leading to an accumulation of alanine, lactate and pyruvate and a decrease in oxaloacetate and glucose [225]. Two groups of patients have been reported. The first group of patients suffer from mild-to-moderate lactic acidaemia, delayed development and psychomotor retardation, but may survive for many years. These patients have some residual immunoreactive PC and mRNA, as detected by Northern blot analysis (known as CRM^{+ve} phenotype; patients exhibit material that is cross-reactive with anti-PC antibodies) [226]. The second group of patients represent a more serious disease, with a severe lactic acidaemia accompanied by hyperammonaemia, citrullinaemia and hyperlysinaemia, and they rarely survive longer than 3 months after birth. In contrast with the first group, these patients lack immunoreactive PC and its mRNA (CRM^{-ve} phenotype) [226,227]. The two forms of the disease have distinct ethnic groups in which they occur [226,227]. The CRM^{+ve} phenotype has been reported among North American native peoples, whereas the CRM^{-ve} pheotype has been reported in the U.K. and France [226]. To date, four different single point mutations, i.e. the substitutions $Val^{145} \rightarrow Ala$, $Arg^{451} \rightarrow Cys$, $Ala^{610} \rightarrow Thr$ and $Met^{743} \rightarrow Ile$, in both alleles, have been shown to be responsible for some forms of the disease. The first two mutations are found within the exons encoding the biotin carboxylation domain [228], whereas the last two mutations [171] were identified within the exons encoding the transcarboxylation domain of the enzyme (see Figure 4). The first case ($Val^{145} \rightarrow Ala$) resulted in barely detectable levels of immunoreactive PC and activity, suggesting that this mutation affects protein stability [228]. In contrast, the other cases resulted in a normal level of immunoreactive PC, but lower PC activity, suggesting that these mutations affect the catalytic activity of the enzyme [171,228]. The carriers who contain heterozygous alleles of these mutations ($Val^{145} \rightarrow Ala$, $Arg^{451} \rightarrow Cys$) are able to survive, but PC activity detected in skin fibroblasts was about 50 % of normal [228]. The genotypes of the CRM^{-ve} phenotype patients have not been identified. It has been suggested that the mutation that is responsible for this form of the disease may be due to splicing mutations that result in the absence of PC mRNA [171]. The severity of PC deficiency may also be influenced by environmental factors, such as stress and fasting [227]. Another group of patients who also show PC deficiency are those suffering multiple carboxylase deficiency due to a defect in biotin metabolism. This group of patients shows elevated levels of organic acids, which are metabolites of acetyl-CoA, propionyl-CoA and 3-methylcrotonyl-CoA, as well as lactic acidaemia.

The first report on the overexpression of recombinant human PC in mammalian cells [229] provides an alternative source of human PC that replaces the native material previously derived from liver obtained at autopsy [230]. Most importantly, this system will allow the creation of mutant forms of PC, mimicking those found in humans, for more detailed *in vitro* characterization.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

PC has been of particular interest to our research laboratory in the past four decades since it was first discovered. This enzyme catalyses the first regulated step in the conversion of pyruvate into oxaloacetate, a tricarboxylic acid cycle intermediate that is utilized as the substrate for many biosynthetic purposes. Early work focused on the characterization of the physical properties and the kinetics of the enzyme. Since the development of recombinant DNA technology, information on the structure of the enzyme has been enormously enhanced by the cloning and sequencing of the genes and cDNA encoding this enzyme. Accumulation of sequence information on PC derived from different organisms, together with the three-dimensional structures of known related biotin-dependent enzymes, should allow one to investigate the role of highly conserved amino acid residues by site-directed mutagenesis. The availability of various expression vectors should also facilitate the production of recombinant PC on a large scale for structure determination by X-ray crystallography. This information should allow us to fully understand the relationship of structure to function for PC. The availability of cloned promoters of yeast and mammalian PC genes will also provide an excellent opportunity to investigate the role of regulatory proteins that mediate transcriptional regulation. In terms of clinical importance, PC deficiency has brought attention to the need for an understanding of the molecular biology of this defect in humans. Mouse models of PC deficiency have not been created, as the mouse gene encoding PC has not yet been isolated. However, with the information on the genomic organization of both rat and human PC, the way is clear to proceed with the isolation of the mouse gene. The availability of embryonic stem cell technology should allow one to create a mouse model of PC deficiency by mimicking mutations that occur naturally in humans, or to investigate other physiological roles of PC by the gene knock-out approach.

We gratefully acknowledge our colleagues in the PC field who sent their interesting recent reprints. While we have tried to be objective in our selection and discussion of papers included, numerous very interesting articles on the enzyme have not been cited due to a limitation of space. We apologize in advance for any inadvertent oversight in the selection of relevant work. Research in J. C. W.'s laboratory has been supported over the years by grants from the Australian Research Council. S. J. was the recipient of a Royal Thai Government Scholarship. We thank Dr. Terry Mulhern

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