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Review

A fourth level of regulation of PEP synthetase in *Escherichia coli*; implications of ADP-dependent inactivation

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ABSTRACT

In *Escherichia coli*, phosphoenolpyruvate synthetase (PEPS) catalyzes the conversion of pyruvate to phosphoenolpyruvate (PEP). The enzyme is regulated at four levels: it is regulated at the level of transcription by the carbon source available for growth, by regulation of mRNA stability, by metabolic intermediates, and, by a recently discovered phosphorylation/dephosphorylation mechanism. This manuscript reviews our current understanding of the four levels of regulation of the *peps* gene and its gene product and reports on recent advances made in our understanding of the post-translational regulation of the protein. A model is proposed that summarizes the control of pyruvate metabolism in *E. coli* (and other bacterial species possessing PEP synthetase or pyruvate, orthophosphate dikinase); whether pyruvate is used for the generation of ATP or whether pyruvate is converted to PEP for use in gluconeogenesis, aromatic amino acid synthesis and other anabolic pathways. Finally, we provide some suggestions regarding the use of PEPS in the biotechnology industry based on our understanding of the regulation of the enzyme.

KEYWORDS: glycolysis, pyruvate, orthophosphate dikinase, PEP synthetase, phosphorylation/ dephosphorylation, bifunctional enzymes

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INTRODUCTION

Most organisms, regardless of whether they are aerobic or anaerobic, utilize the glycolytic pathway for the conversion of glucose to pyruvate. The pathway involves a series of ten separate reactions that serve to harvest the energy of glucose in the reduction of NAD and the phosphorylation of ADP. Most organisms utilize a common glycolytic pathway, however, there are several variants of the pathway and these metabolic variations generally involve reactions catalyzed by kinases (hexokinase or glucokinase, phosphofructokinase and pyruvate kinase) (see Figure 1). Some members of the Archaea possess hexokinases that use ADP in place of ATP (*Pyrococcus furiosus* [1, 2] and *Archaeoglobus fulgidus* [3]). Similarly phospho-fructokinase 1, the enzyme that catalyzes the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate, differs in some organisms according to the donor of the high energy phosphate group. In eukaryotes the phosphate donor is ATP while in some prokaryotes the donor may be inorganic pyrophosphate [4-6], or ADP [7]. Interestingly, the hyperthermophilic Archaeon *Methanococcus jannaschii* possesses a bifunctional ADP-dependent glucokinase/phosphofructokinase [8, 9]. And finally, the last reaction of glycolysis involving the conversion of PEP to pyruvate may be catalyzed by either of two enzymes depending on the organism. In most organisms the reaction is catalyzed by pyruvate kinase whilst in others the reaction is catalyzed by pyruvate, orthophosphate dikinase (PPDK) [10, 11]. So, depending on the

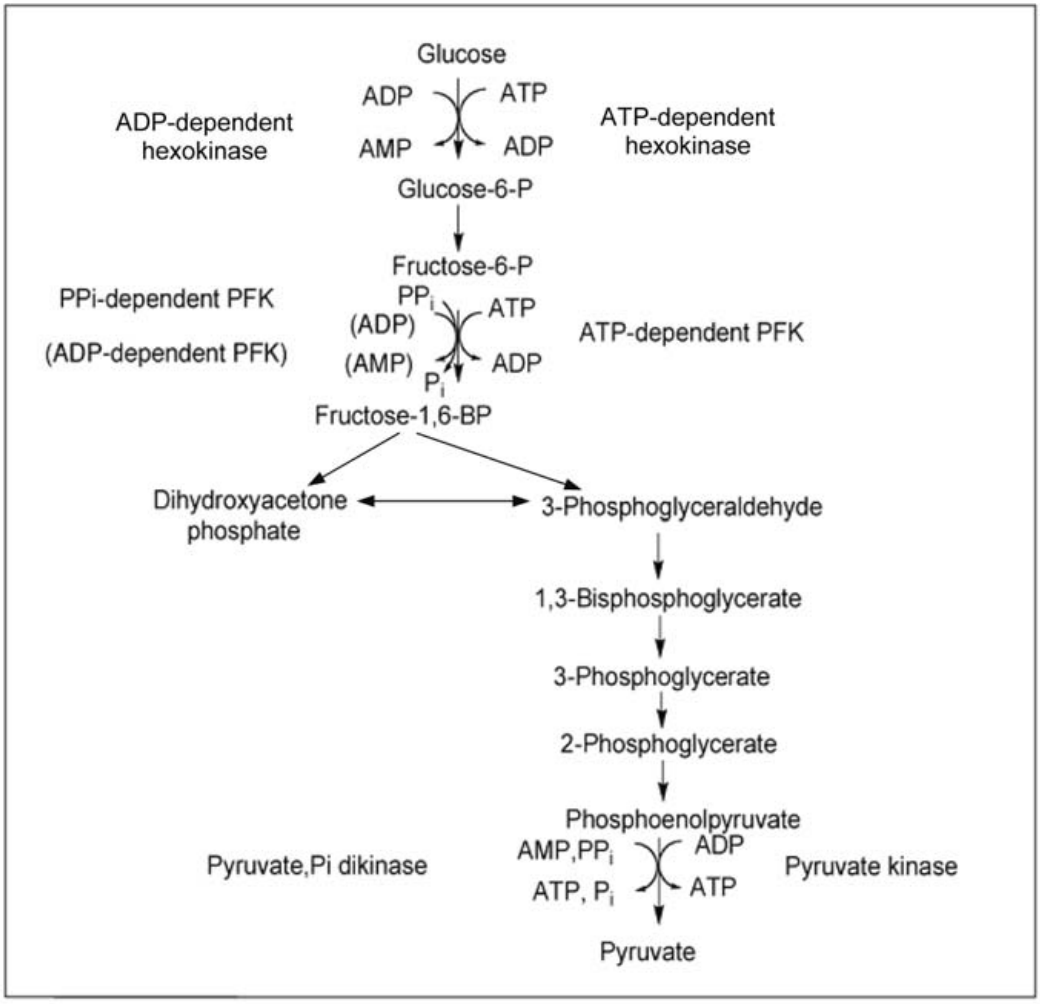

Figure 1. Glycolytic pathway showing the variation of enzymes, substrates and products of kinase-catalyzed reactions.

specific glycolytic pathway used the pathway can produce different yields of ATP. In the case of organisms utilizing the ATP-dependent PFK1 coupled with pyruvate kinase the net yield is 2 ATPs per glucose while glycolysis in organisms possessing PPi-dependent PFK and PPDK have a net yield of 5 ATPs per glucose.

In the reverse, gluconeogenic or anabolic direction (pyruvate to glucose) the three kinases mentioned above are replaced by separate enzymes while the other glycolytic enzymes operate in the reverse direction. In most organisms, the conversion of pyruvate to PEP is achieved by two separate enzymes (pyruvate carboxylase and PEP carboxy-kinase) that operate together to bypass the essentially irreversible pyruvate kinase-catalyzed reaction. In organisms possessing PPDK, pyruvate can be readily converted to PEP due to the fact that the reaction catalyzed by PPDK is reversible under physiological conditions. In some bacteria, the conversion of pyruvate to PEP may be catalyzed by another enzyme, phosphoenolpyruvate synthetase (PEPS). Following its synthesis PEP is converted to fructose-1,6-bisphosphate by reversing the glycolytic enzymes however, the conversion of F16BP to F6P and glucose-6-P to glucose are catalyzed by specific phosphatases.

This review focuses on the enzymes that catalyze the inter-conversion of phosphoenolpyruvate and pyruvate (PPDK and PEPS) with emphasis on the regulatory mechanism that controls their activity.

The inter-conversion of PEP and pyruvate

Most, but not all, organisms use pyruvate kinase to catalyze the conversion of PEP to pyruvate, however, examination of completely sequenced genomes reveals the presence of genes that encode both pyruvate kinase and either PPDK or PEPS. In some organisms, for example members of the Archaea, sequences of all three enzymes are present (see ref [12]). These three enzymes, pyruvate kinase, PPDK and PEPS differ in the preferred direction of catalysis. Pyruvate kinase catalyzes the conversion of PEP to pyruvate and is essentially irreversible under physiological conditions. PPDK catalyzes the reversible inter-conversion of pyruvate to PEP with the direction of catalysis influenced by pH; under acidic conditions the enzyme prefers to catalyze the conversion of PEP to pyruvate while at alkaline conditions the enzyme converts pyruvate to PEP. And under physiological conditions the conversion of pyruvate to PEP catalyzed by PEPS is essentially irreversible. Pyruvate kinase will not be discussed further in this review.

The gene sequence for PEPS has been found in a large number of species mainly bacterial, however, the kinetic characteristics of the enzyme have been determined in a very limited number of species including *Escherichia coli* [13], *Thermococcus kodakarensis* [14], *Thermoproteus tenax* [12] and *Pyrococcus furiosus* [14]. PPDK and PEPS are often misidentified, however there are three regions of the protein that allows correct identification of the two enzymes. Signature sequences (VAVRSSATAEDQEASFAGQQDTY LNV for PEP synthetase and PLLVSVRSG AAVSMPGMMDTVLNLGL for PPDK) located towards the N-terminal end of the proteins identified by Tjaden *et al*. [12] are the most rigorous means of discriminating between the two enzymes. A less rigorous diagnostic means of identifying the two proteins is the three amino acid sequence around the regulatory and catalytically-important phosphorylation sites (TCH for PEPS and TSH for PPDK). And an alignment of the pyruvate binding site at the C-terminal region of the PEPS and PPDK segregates the two enzyme species into two distinct clades; these clades may be indicative of the α-ketoglutarate (α-KG) sensitivity of pyruvate binding sites of the two enzymes: PEPS is inhibited by α-KG while PPDK is not.

The central role of pyruvate in metabolism

Pyruvate occupies a central location in carbon metabolism in a cell (see Figure 2). In aerobic respiration it sits between glycolysis and the Krebs cycle (via pyruvate dehydrogenase). Pyruvate may also be transaminated to form alanine (alanine aminotransferase), converted to oxaloacetate and PEP by pyruvate carboxylase and PEP carboxykinase or directly to PEP using PEP synthetase. PEP is essential for the synthesis of aromatic amino acids; it is a substrate for reactions catalyzed by both 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHP synthase) and 5-enolpyruvyl-shikimate-3-phosphate synthase (ESPS). PEP synthesis is also required for the synthesis of sugars required in a significant number of pathways. When bacterial cells are grown on small carbon sources (less than four carbons) the conversion of pyruvate to PEP assumes a critical regulatory step in the metabolism of a cell. The partitioning of pyruvate between anabolism and catabolism is crucial to the efficient growth of a cell and there is growing evidence to support the involvement of both the adenylate energy charge and the pyruvate:PEP ratio in controlling the metabolic fate of pyruvate or acetyl CoA when cells are grown on pyruvate or acetate, respectively [15]. When cells are grown on glucose as the sole carbon source the rate of flow of glucose through glycolysis is controlled by the demand for ATP; increased rates of ATP hydrolysis increase the glycolytic flux [16]. However, one critical energy-utilizing step in glucose metabolism in *E. coli* is the involvement of PEP in supplying the high-energy phosphate group used to convert glucose to glucose-6-phosphate in the phosphotransferase-dependent transport system (PTS).

PEP-pyruvate phosphotransferase system

In *E. coli*, PEP is a phosphoryl donor for the phosphorylation cascade involving the proteins EI, HPr, EIIA and EIIB in addition to providing the energy for the transport step catalyzed by the membrane-bound EIIC. Not only are the PTS proteins involved in sugar transport but they also have regulatory functions. The PTS components

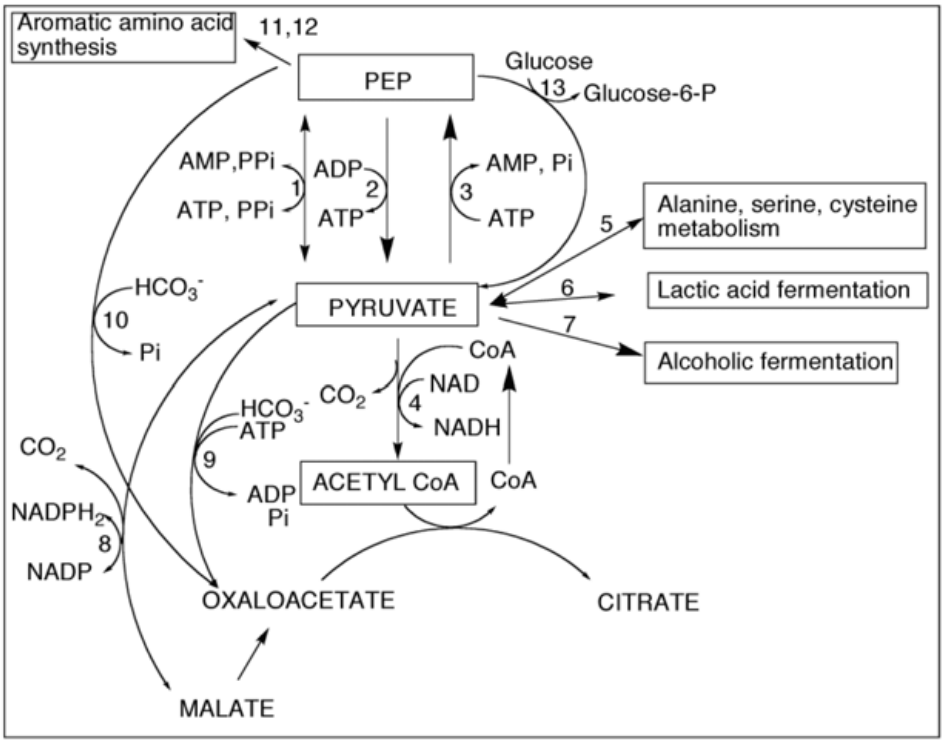

Figure 2. Enzyme catalyzed reactions involving phosphoenolpyruvate and pyruvate. 1. PPDK; 2. Pyruvate kinase; 3. PEP synthetase; 4. Pyruvate dehydrogenase; 5. Alanine aminotransferase; 6. Lactate dehydrogenase; 7. Alcohol dehydrogenase; 8. Malic enzyme; 9. Pyruvate carboxylase; 10. PEP carboxylase 11. 3-Deoxy-D-arabino-heptulosonate-7-phosphate synthase; 12. 5-Enolpyruvyl-shikimate-3-phosphate synthase; 13. PTS- phosphotransferase-dependent transport system.

are present in the cell in either their dephosphorylated form (when the PEP: pyruvate ratio is low and/or the uptake of the PTS substrate is high) or in the phosphorylated form (when the PEP: pyruvate ratio is high and/or no PTS substrate is available). In *E. coli* the phosphorylated form of $EIIA^{glc}$ stimulates adenylate cyclase forming cAMP that in turn is a co-activator of CRP (cAMP receptor protein). The phosphotransferase system in bacteria has been extensively reviewed [17] and will not be discussed further.

Integration of carbon metabolism in acetate grown cells

When *E. coli* is grown on acetate as the sole carbon source, the glyoxylate bypass involving isocitrate lyase and malate synthase facilitates the conversion of acetate to malate without the loss of carbon as carbon dioxide [18, 19]. The partitioning of isocitrate between the TCA cycle and the glyoxylate bypass is controlled by regulation of the activity of isocitrate dehydrogenase. Isocitrate dehydrogenase (ICH) activity is controlled by a phosphorylation/dephosphorylation mechanism that involves a single bifunctional kinase/phosphatase [20]. The growth of a cell on acetate will not only be dependent on the partitioning of isocitrate between the TCA cycle and the glyoxylate bypass but it will also be dependent on the partitioning of pyruvate to gluconeogenic intermediates. Expression studies have shown that acetate induces the up-regulation of glyoxylate cycle enzymes (isocitrate lyase and malate synthase), malic enzyme and PEP synthetase with confirmation of malate conversion to PEP demonstrated by mutant studies [21]. Two malic enzymes have been identified in *E. coli* (MaeB and SfcA); both MaeB and SfcA are induced when *E. coli* are grown on acetate [21].

Regulation of PEP synthetase

Up until very recently, it was recognized that PEP synthetase activity was regulated on three levels;

at the transcriptional level, at the post-transcriptional level and by metabolic effectors at the enzyme activity level. With the elucidation of a reversible phosphorylation-dephosphorylation control mechanism [22] it is now apparent that the enzyme is controlled on four levels. These regulatory mechanisms are discussed below.

Transcriptional regulation

In *E. coli*, a number of transcription factors are known to regulate *ppsA* expression. Two global metabolic regulators CRP (the cyclic AMP receptor protein) and Cra (catabolite repressor/ activator) are integrated in a complex regulatory network. In a model of carbon regulation in *E. coli* proposed by Hardiman [23] cAMP and fructose-1,6-bisphosphate function as important signalling molecules that induce repression or activation of genes by cAMP-CRP and Cra, respectively.

Transcriptional regulation by CRP

CRP exerts its effect on transcription when it is bound to cAMP in an active complex. When a cell's access to glucose is low, cAMP levels increase stimulating the levels of cAMP-CRP complexes within the cell. These complexes bind to palindromic sequences with the consensus sequence (5'-TGTGANNNNNNTCACA-3') in the promoter region of regulated genes/operons [24, 25]. Both positive and negative regulation by cAMP-CRP complexes has been reported. In the case of *ppsA*, the binding of cAMP-CRP to its recognition site results in the activation of transcription. CRP binding sites have been identified upstream of genes encoding other gluconeogenic enzymes, PEP carboxykinase and fructose-1,6-bisphosphatase. Positive regulation of both *ppsA* and *pckA* promoters have been demonstrated using promoter-LacZ fusions [25].

In many bacteria, *ppsR* (also known as *ydiA*) that encodes the PEPS regulatory protein (PSRP) is located adjacent to *ppsA*. In the *E. coli* genome the genes are located next to each other but are transcribed in opposite directions (divergently transcribed). The proposed CRP-binding site upstream of *ppsA* is located between *ydiA* and *ppsA* and the next gene, *aroH* [25]. *aroH* encodes 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHP synthase), the enzyme that catalyzes the first committed step in aromatic amino acid synthesis involving the condensation between PEP and erythrose-4-phosphate.

Transcriptional regulation by Cra

Cra (the catabolite repressor/activator) is a global transcription regulator involved in the regulation of expression of enzymes in a number of metabolic pathways [27]. Initially identified as a repressor of the PEP: fructose phosphotransferase system (PTS) and named FruR due to its regulation of the *fru* operon [26] subsequent identification of further regulatory targets prompted its renaming to Cra. Cra binds to an imperfect palindrome with the consensus sequence GCTGAAACGTTTCA [27]. In relation to the control of genes involved in central carbon metabolism, glycolysis and gluconeogenesis, Cra functions primarily as a repressor, however, it has also been shown to positively affect gene transcription [27]. Cra represses transcription of specific genes by binding to its target sequence in the gene promoter. Effectors such as fructose-6-phosphate and fructose-1,6-bisphosphate can bind to Cra preventing its interaction at the promoter region allowing transcription to proceed resulting in the synthesis of enzymes that metabolize the effectors.

In contrast to CRP that controls only a few glycolytic genes, potential Cra binding sites have been identified in the promoter regions of most glycolytic genes and only a few gluconeogenic genes [27].

ppsA has a weak promoter that is characteristic of positively regulated promoter regions and needs help for efficient initiation of transcription. It has been proposed that Cra stimulates induction of *ppsA* expression by binding to its recognition site in the promoter causing the bending of DNA that allows the interaction of an alpha C-terminal domain with DNA upstream of the -45 FruR binding site thus stabilizing the RNA polymerase-DNA complex [28].

Gene transcription of glycolytic enzymes is mainly controlled by Cra binding to its target sequences and preventing transcription. The presence of inducers (F6P and F16BP) during high sugar provision to the cell detaches Cra from

the promoter allowing gene transcription [27]. The binding sites of the Cra repressor are generally located near and downstream of their target promoters while the Cra activator binds upstream of the target promoters which is the case of the transcription activation of the PEPS promoter. So lack of glucose would lead to the accumulation of the phosphorylated form of EII^{glc} in the PTS, which stimulates adenylate cyclase thus increasing the concentration of cAMP. cAMP will bind to CRP and cAMP-CRP represses glycolysis and the TCA cycle. Cra acts in the opposite manner while stimulating gluconeogenesis (PEPS and PCK).

Post-transcriptional regulation

PEPS is negatively regulated by CsrA. CsrA is a carbon storage regulator [29] that binds mRNAs and elicits its activity by stimulating mRNA degradation. CsrA increases glycolysis and represses gluconeogenesis and glycogen metabolism. The post-transcriptional regulation by CsrA in bacteria has recently been reviewed [30] and will not be discussed further.

Biochemical regulation

E. coli PEPS is a homo-dimer composed of 87.4 kDa monomeric subunits. The enzyme preferentially catalyzes the conversion of pyruvate to PEP and has a pH optimum for the conversion of pyruvate to PEP of 8.4 [31]. It can also catalyze the reverse reaction and the conversion of PEP to pyruvate has a pH optimum of 6.8 [32]. At pH 8.0 the K_m[ATP] is 28 micromolar and the K_m[pyruvate] is 83 micromolar. In the PEP-forming direction the enzyme is inhibited by AMP (a product), ADP and α-KG. In *E. coli* and a number of other organisms α-KG inhibition is competitive with respect to pyruvate [12, 33]. As mentioned above, α-KG inhibition can be used to distinguish between PPDK and PEPS. And given its role in the conversion of pyruvate to PEP the inhibition of PEPS by α-KG may be physiologically important in maintaining the balance between anabolic and catabolic metabolism of pyruvate and this is discussed further below.

When cells are grown on small carbon sources the conversion of pyruvate to PEP via PEPS represents an important regulatory step in carbon metabolism. PEP synthetase activity is regulated not only by a variety of metabolic intermediates including AMP, ADP, oxaloacetate and α-KG but it is also controlled by a rare phosphorylation-dephosphorylation mechanism [22] previously found to regulate PPDK activity in plants (see [34, 35] for reviews). The regulation of PEPS activity by the PSRP is illustrated in Figure 3.

This regulatory mechanism is unique in three specific details. The first unique feature of the regulatory mechanism is that the PEPS must be catalytically phosphorylated before it can be inactivated. The second feature is that the phosphate donor for inactivation is ADP as opposed to ATP and the third unique feature is that the activation of inactivated PEPS involves a phosphate-dependent phosphorolytic cleavage reaction that results in the formation of an inorganic pyrophosphate as opposed to a simple phosphatase reaction. A fourth feature of the PEPS regulatory mechanism that is unusual, but not unique, is the fact that a bifunctional enzyme catalyzes both the ADP-dependent inactivation and the Pi-dependent activation of PEPS [22].

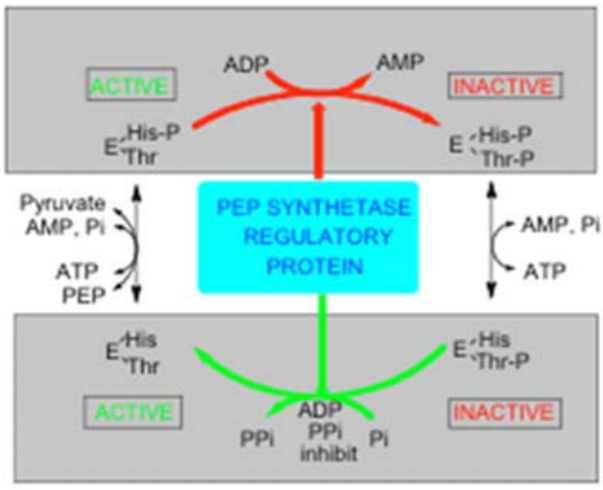

Figure 3. Regulation of PEPS by PSRP.
PEPS must be phosphorylated on a catalytic histidine (His) residue prior to phosphorylation by ADP and either PEP or ATP can act as the source of the catalytic phosphate. PEPS is inactive when a threonine (Thr) residue is phosphorylated by ADP. Both ADP-dependent inactivation and Pi-dependent activation are catalysed by PSRP.

It is relevant to consider these unique features in a little more detail as it appears that they may be important to the overall mechanism of controlling the flow of carbon during growth of *E. coli* on two and three carbon compounds.

1. Catalytic phosphorylation prior to inactivation

PEPS catalyzes the conversion of pyruvate to PEP via a two step reaction that involves the formation of a phosphohistidine intermediate according to the two partial reactions:

ATP + E-His $\Leftrightarrow$ AMP + Pi + E-His-P

E-His-P + pyruvate $\Leftrightarrow$ PEP + E-His

Examination of these two partial reactions reveals that a phosphorylated intermediate may be formed in either of two ways - by reacting with either ATP or PEP - and a stable phosphorylated intermediate can be isolated following incubation of enzyme with either ATP or PEP [36]. It seems more likely that the substrate responsible for the catalytic phosphorylation of the enzyme prior to inactivation is PEP as opposed to ATP as it makes more sense, from a biochemical point of view, that the activity of PEPS should be down regulated when PEP accumulates within the cell as opposed to ATP.

2. ADP as the phosphate source for inactivation

In contrast to almost every other regulatory mechanism involving a phosphorylation reaction, the inactivation of PEPS involves the transfer of a phosphate group from ADP. During inactivation the β-phosphate from ADP is transferred to a threonine residue that is located two amino acids distant from the histidine residue that is phosphorylated during catalysis. (Site-directed mutagenesis studies have shown that in *E. coli* PEPS T419 is phosphorylated during inactivation - Burnell 2011, unpublished results) The use of ADP rather than ATP as the phosphate donor indicates that the PEPS inactivation will only occur when the adenylate energy charge within the cell decreases as the ADP concentration increases. Historically, the ADP-dependent inactivation of PEPS (and PPDK) has been measured by adding both ADP and ATP to initiate inactivation - ATP to catalytically phosphorylate the enzyme substrate and ADP to provide the phosphate required to inactivate the enzyme, however, it is more likely that PEP is the phosphate donor prior to ADP-dependent inactivation. ATP, as opposed to PEP, has been added to inactivation assays due to the fact that adding PEP to inactivation assays interferes with the enzyme-linked assay used to monitor PEPS activity as PEPS activity is routinely measured in an enzyme-coupled assay containing PEP carboxylase and malate dehydrogenase; PEP formed as a result of PEPS activity is converted to oxaloacetate via PEP carboxylase and oxaloacetate production followed by monitoring the oxidation of NADH in the presence of an NAD-dependent malate dehydrogenase. Pyruvate is a potent inhibitor of ADP-dependent inactivation due to the removal of the phosphate from the catalytic histidine. During growth on a two, three or four carbon source PEP concentrations would be expected to increase if there were low rates of conversion of 3-phosphoglycerate to 1,3-bisphosphoglycerate when ATP levels are low; accumulation of 3-PGA would lead to the accumulation of both 2-PGA and PEP.

One corollary of the fact that the substrate for ADP-dependent inactivation is a catalytically phosphorylated form of the enzyme is that pyruvate is a potent inhibitor of ADP-dependent inactivation; pyruvate will remove the catalytic phosphate with the concomitant formation of PEP. Therefore it is logical to suggest that the activity status of PEPS will be influenced not only by the energy status of the cell as reflected by the adenylate energy charge but it will also be influenced by the PEP: pyruvate ratio within the cell.

3. Dephosphorylation via a P_i-dependent phosphotransferase

The dephosphorylation of *E. coli* PEPS during activation differs from almost all other regulatory dephosphorylation reactions which employ a simple phosphatase-catalyzed dephosphorylation. In contrast, the dephosphorylation of the regulatory phosphothreonine during activation of *E. coli* PEPS is dependent on the presence of phosphate [22] and, by analogy with the mechanism of plant PPDK, results in the formation of inorganic pyrophosphate [37]. The involvement of a phosphotransferase as opposed to a simple phosphatase may allow for greater control of

activation of the level of PEPS activity as P_i-dependent activation is inhibited by ADP, AMP and PP_i ([22] and unpublished results).

4. PSRP is a bifunctional regulatory protein

Both the ADP-dependent inactivation and the Pi-dependent activation of PEPS are catalyzed by a single bifunctional protein [22]. Although this is unusual it is not unique among regulatory enzymes. Other bifunctional enzymes involved in enzyme regulation include isocitrate dehydrogenase kinase/phosphatase [38] and glutamine synthetase [39].

Regulation of PEPS activity

Elevated PEP: pyruvate ratios would increase the level of catalytically-phosphorylated PEPS that would, in turn, with increased ADP levels, favour inactivation of PEPS. It is also important to recognise that the effect of ADP on PEPS activity levels is three-fold: ADP is a competitive inhibitor of PEPS activity *per se*. In addition ADP is not only the phosphate donor for inactivation but it also an inhibitor of Pi-dependent activation. Therefore, the carbon flux in *E. coli* cells grown on acetate will be controlled by two separate phosphorylation-dephosphorylation mechanisms catalysed by bifunctional enzymes, one of which uses ATP as the phosphate donor (isocitrate dehydrogenase) while the second one uses ADP (PEPS). This use of different phosphate donors may allow greater control of carbon fluxes through the TCA cycle/glyoxylate cycle and gluconeogenesis.

When cells are grown on pyruvate (or substrates that can be converted to pyruvate - alanine or lactate), the catabolism of pyruvate via the TCA cycle and the electron transport chain to generate ATP is controlled by flow through pyruvate dehydrogenase. When ATP is required, pyruvate will be utilized for ATP synthesis via the Krebs cycle and the electron transport chain, and when ATP levels are sufficient, pyruvate may be utilized in anabolic pathways such as aromatic amino acid synthesis and gluconeogenesis.

Regulation by phosphorylation/ dephosphorylation

One of the properties of the PPDK/PEPS regulatory system that remains to be fully characterized relates to the preferred substrate for Pi-dependent activation of inactivated PPDK/PEPS. Studies using maize leaf PDRP and PPDK showed that higher rates of Pi-dependent activation were achieved if PPDK that had been inactivated was pre-treated with AMP and PPi prior to activation. This pre-treatment would have the effect of dephosphorylating the catalytically-phosphorylated histidine residue of the PPDK. These experiments were conducted following the demonstration that PPDK that had been inactivated by ADP-dependent phosphorylation could catalyze [^{14}C]-ATP-AMP exchange but could not catalyze [^{14}C]pyruvate-PEP exchange [37]. This indicated that PPDK that had been inactivated by ADP-dependent phosphorylation was still able to catalyze the first partial reaction but that it could not catalyze the second partial reaction.

PEPS is an important member of the pyruvate-PEP-OAA node that should probably be expanded to the pyruvate-PEP-OAA-malate node given the importance of these metabolic intermediates in anabolic metabolism in *E. coli*. When *E. coli* is grown on acetate the probable metabolic pathway followed would be the conversion of acetate to acetyl CoA, the condensation of acetyl CoA with oxaloacetate to form citrate that is then converted to isocitrate. The isocitrate may be cleaved to form succinate and glyoxylate and the glyoxylate, together with a further molecule of acetyl CoA, converted to malate by malate synthase. Malate can be converted to OAA that may then be converted to PEP via PEP carboxykinase. However, malate may also be converted to pyruvate by an NADP-dependent malic enzyme with the concomitant production of $NADPH_2$, required during anabolism. The pyruvate formed could be converted to PEP via PEPS. It is interesting to note that genes that encode isocitrate lyase and malate synthase are located together in the *E. coli* genome and they also lie next to the gene that encodes isocitrate dehydrogenase kinase/phosphatase (the bifunctional enzyme that regulates the phosphorylation status and, therefore, the activity of isocitrate dehydrogenase).

Closer examination of the likely pathway to be followed by acetate (see Figure 4) also helps to explain why PEPS is sensitive to α-ketoglutarate.

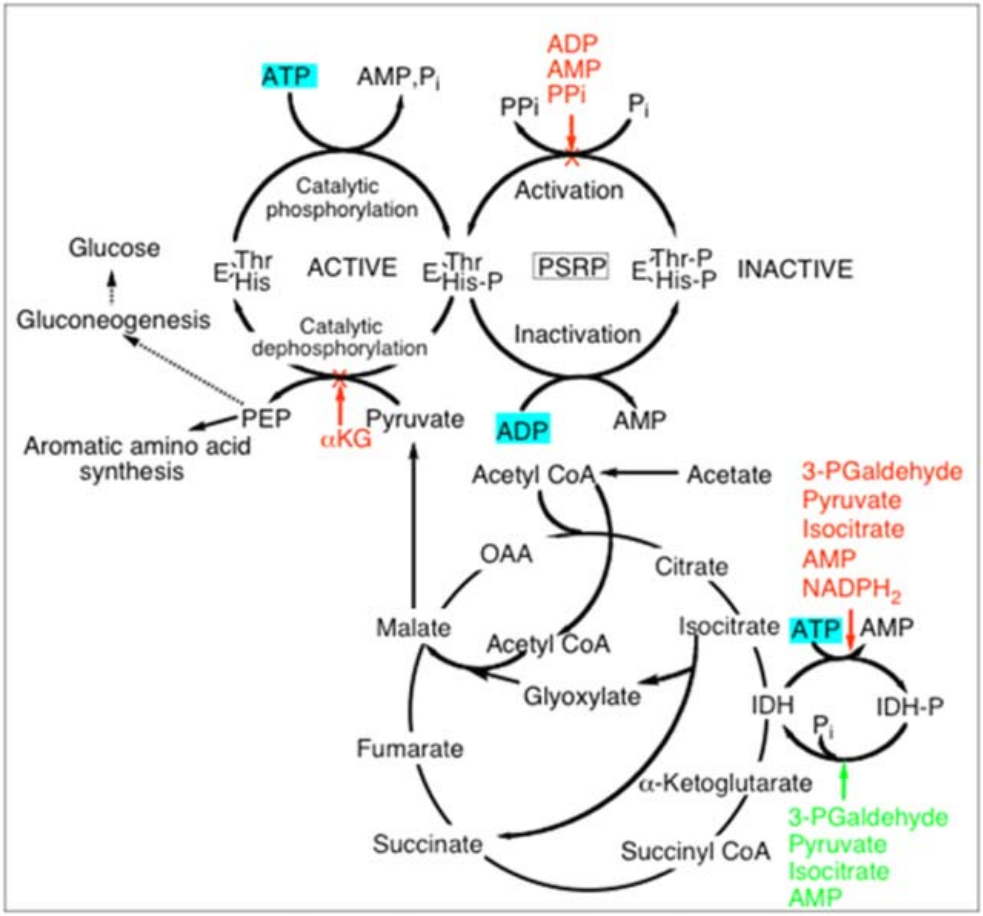

Figure 4. Integration of the regulation of acetate metabolism via the glyoxylate cycle and gluconeogenesis. The regulation of both isocitrate dehydrogenase and PEP synthetase by phosphorylation/dephosphorylation mechanisms by metabolic intermediates is included. Compounds in red are inhibitors and compounds in green stimulators of regulation.

After acetate is converted to acetyl CoA and enters the TCA cycle, the amount of isocitrate that is converted to succinate and glyoxylate will largely be controlled by how much isocitrate accumulates due to the inactivation of isocitrate dehydrogenase by the isocitrate dehydrogenase kinase/phosphatase in response to the ATP concentration. If ATP levels are sufficiently high, isocitrate dehydrogenase will be inactivated diverting isocitrate through the glyoxylate shunt resulting in the formation of malate and succinate. The malate can be either converted to oxaloacetate depending upon the NAD/NADH ratio or converted directly to pyruvate via NADP-malic enzyme with the accompanying reduction of NADP. When isocitrate dehydrogenase activities are low it is expected that α-KG concentrations would also be low and PEPS activity would not be inhibited by α-KG. Alternatively, if the ATP concentrations are low, isocitrate dehydrogenase would be active (non-phosphorylated) generating α-KG that may inhibit PEPS. So the regulation of PEPS activity may play an important role in controlling the partitioning of carbon compounds between energy production (via the TCA cycle and the oxidative phosphorylation) and biosynthetic pathways (via the glyoxylate cycle and gluconeogenesis). It may be significant that α-KG inhibits PEPS competitively with respect to pyruvate. α-KG could inhibit PEPS activity directly by competing with pyruvate but it would also influence PEPS activity by inhibiting the pyruvate-

dependent dephosphorylation of catalytically-phosphorylated PEPS, the substrate for ADP-dependent inactivation, increasing levels of catalytically-phosphorylated PEPS leading to greater rates of inactivation in the presence of sufficiently high ADP concentrations. Therefore in *E. coli*, the activity of PEPS will be influenced on the one hand by the ATP/ADP/AMP levels and on the other hand by the PEP/pyruvate/α-KG levels with ATP, PEP, pyruvate and α-KG controlling the catalytic phosphorylation status of PEPS and the ADP concentration controlling the phosphorylation state of the regulatory threonine residue of PEPS. Using this mechanism, the TCA cycle and gluconeogenesis could be coordinated to maximise the efficient balance of energy production and energy consumption.

It may be significant that the regulation of both PEP synthetase and isocitrate dehydrogenase (see discussion below) is controlled by regulatory mechanisms that involve single bifunctional proteins that catalyze reversible phosphorylation reactions. Although these control mechanisms involve phosphorylation/dephosphorylation mechanisms the two mechanisms differ significantly. The phosphorylation of isocitrate dehydrogenase is largely controlled by the adenylate energy charge with high adenylate energy charge stimulating the phosphorylation (and therefore the inactivation) of isocitrate dehydrogenase, while a low adenylate energy charge, signified by increased AMP concentrations, results in inhibition of the kinase activity and stimulation of the phosphatase activity. So a decrease in ATP concentrations would cause greater flow of isocitrate though the TCA cycle generating more reduced NADH and FADH that would, in turn, result in oxidative phosphorylation and increased ATP concentration. Simultaneously, low adenylate charge levels would reflect increased ADP levels that would favour phosphorylation (and therefore inactivation) of PEPS and inhibition of Pi-dependent activation of PEPS.

It would be interesting to repeat the *in vitro* experiments of Chulavatnatol and Atkinson [40] using purified PEPS and pyruvate dehydrogenase complex from *E. coli* with the addition of purified PSRP; especially given the requirement to catalytically phosphorylate PEPS prior to ADP-dependent inactivation. And these types of experiments may provide an indication as to the importance (or otherwise) of the catalytic dephosphorylation of inactivated PEPS.

Similarities and difference between isocitrate dehydrogenase and PEPS regulatory proteins

The PEPS regulatory protein shares some similarities with isocitrate dehydrogenase (ICDH). It is a bifunctional enzyme that both phosphorylates and dephosphorylates its substrate in response to changing intracellular concentrations of metabolic intermediates. However, the mode of inhibition of the proteins by AMP appears to differ. ICDH possesses an allosteric site to which AMP binds [38] while the inhibition of Pi-dependent activation of PEPS by AMP is competitive with respect to Pi indicating that the AMP and the Pi bind at the same site. ADP inhibition of Pi-dependent activation of PEPS is also competitive with respect to Pi indicating that the intracellular concentration of ADP is probably the most important regulatory compound in controlling the level of PEPS activity in the cell. Site-directed mutagenesis studies have shown that both the phosphorylating and the dephosphorylating activities occur at or very close to the same sites with ADP-dependent inactivation and ADP-dependent inhibition of Pi-dependent activation being affected by identical mutations (Burnell JN, unpublished results). Isocitrate dehydrogenase regulatory protein has two separate domains with an AMP-mediated conformational change acting as a switch between the kinase and phosphatase activities [38].

PEPS in biotechnology

A wide range of chemicals can be synthesized using microbial catalysis and the shikimate pathway [41]. These include adipic acid [42], phenol [43], vanillin [44], indigo [45], gallic acid [46] and pyrogallol [47]. The efficient operation of the shikimic acid pathway is dependent on the supply of PEP. A number of biochemical modifications have been made to *E. coli* used to synthesize chemicals that depend on an efficient supply of PEP. These have included:

1. Altering the starting substrate

In *E. coli* the PTS consumes more than half of the glucose-derived PEP and this competes with both

DAHP synthase and ESPS for PEP [48]. To increase product yields non-glucose sources of energy have been used that do not use a PTS for uptake.

2. Decreasing the utilization of PEP

This has included decreasing PEP carboxylase activity [49] pyruvate kinase (*pykA* and pykF) [50, 51] and inactivating the PTS [52].

3. Over-expression of key genes

Genes that have been over-expressed that have increased the efficiency of the shikimic acid pathway include feedback insensitive DAHP synthase and chorismate-mutase prephenate dehydratase, transketolase *(tktA),* and PEPS.

Recent experiments (Burnell unpublished results) have demonstrated that heterologous over-expressed PEPS can be inactivated by endogenous PSRP activity in *E. coli.* This observation was made following the use of PEPS isolated from bacteria that had been overgrown. Reaction mixtures containing "over-expressed" PEPS (PEPS isolated from cells that had been grown for three days post-induction), purified PSRP and Pi activated to levels that were more than 215% of the initial activity of PEPS. This may have significant implications to industrial cultures containing over-expressed PEPS used to increase the synthesis of products based on the shikimic acid pathway. Precautions may have to be taken to ensure that the PEPS is maintained in an active state. To this end, the regulatory threonine of *E. coli* PEPS has been mutated to either an alanine or a valine residue without loss of PEP synthetic activity. The use of a T419V PEPS mutant would eliminate any potential loss of PEPS activity during the industrial production of compounds utilizing the shikimic acid pathway.

Expression levels in *E. coli*

Several studies have reported on the regulation of metabolic pathways of *E. coli* grown on different carbon sources [21, 53, 54]; some of these studies have measured transcript levels and some have measured enzyme activity. For those studies that have reported enzyme activity there is no evidence to suggest that the expressed PEPS has been activated prior to measuring enzyme activity; this is a precaution routinely taken in measuring PPDK activity expressed in plant leaves (see [55]).

Regulation of PEPS and PPDK in bacteria

Examination of bacterial genomes revealed the presence of DUF299 gene sequences in most bacterial species. In many species the DUF299 (*ppsR*) gene lies adjacent to either the *ppdk* or the *peps* genes. And in a significant number of species (including *E. coli*) these two genes were located adjacent to the gene encoding DAHP synthase. A recent study has demonstrated that the PPDK from *Listeria monocytogenes* is regulated by an ADP-dependent inactivation and a Pi-dependent activation mechanism involving a bifunctional PDRP indicating that regulation of PPDK and PEPS by a phosphorylation/ dephosphorylation is widespread in nature. These findings are further evidence to the complexity of regulatory mechanisms that serve to control the flow of carbon within a cell and regulate the allocation of resources between anabolic and catabolic pathways.

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