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Original Communication

Identification of critical residues in the bifunctional phosphoenolpyruvate synthetase kinase/phosphotransferase of *Escherichia coli*

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ABSTRACT

In bacteria, phosphoenolpyruvate synthetase (EC 2.7.9.2) catalyses the conversion of pyruvate to phosphoenolpyruvate during gluconeogenesis. The enzyme is regulated by an unusual bifunctional serine/threonine kinase-phosphotransferase (PEP synthetase regulatory protein) that involves an ADP-dependent phosphorylation and a Pi-dependent dephosphorylation mechanism. Site-directed mutagenesis studies have revealed that two separate regions of *Escherichia coli* PEP synthetase regulatory protein are involved in catalysis; a central P-loop that is probably critical for the binding of the protein substrate (PEP synthetase) and a C-terminal region that interacts with the P-loop and is required to bind ADP and Pi. In addition, our findings are consistent with the P-loop and the C-terminal region responsible for ADP and Pi binding being juxtaposed in the functioning enzyme. Given the high degree of sequence similarity between bacterial PEP synthetase regulatory protein and plant pyruvate, orthophosphate dikinase regulatory protein, it is highly likely that there are two active sites involved in the ADP-dependent inactivation and the Pi-dependent activation of both PEP synthetase and pyruvate, orthophosphate dikinase and they are very close together.

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KEYWORDS: *Escherichia coli*, enzyme regulation, PEP synthetase, gluconeogenesis, phosphorylation/dephosphorylation

ABREVIATIONS

PPDK, pyruvate orthophosphate dikinase; PEP, phosphoenolpyruvate; PEPS, PEP synthetase; PSRP, PEP synthetase regulatory protein

INTRODUCTION

Phosphoenolpyruvate synthetase (EC 2.7.9.2.) also known as pyruvate, water dikinase catalyzes the conversion of pyruvate to phosphoenolpyruvate (PEP) according to the reaction:

$ATP + pyruvate + H_2O \Rightarrow AMP + Pi + PEP.$

In *Escherichia coli* the enzyme is essential for gluconeogenesis during growth on pyruvate, lactate or alanine [1]. In addition the enzyme lies very strongly towards PEP synthesis [2] and enzyme catalysis involves the formation of a phosphorylated intermediate [3]. We recently reported the enzyme is regulated on four levels; at the transcriptional level, the post-transcriptional level, by metabolic effectors and by post-translational modification [4]. The post-translational modification involving a phosphorylation/dephosphorylation mechanism has been reported [5] and is very similar to the mechanism reported for the regulation of pyruvate, orthophosphate dikinase in plants that involves

an ADP-dependent phosphorylation and a Pi-dependent dephosphorylation [6, 7].

Over the last 15 years there have been a number of attempts to increase photosynthetic rates in a variety of plant species by introducing enzymes involved in the C_4 photosynthetic pathway (see [8] for a review). One of the most common combination of enzymes used in these studies has been PEP carboxylase and pyruvate, orthophosphate dikinase; the former to catalyze the carboxylation of PEP to oxaloacetate and the latter to convert pyruvate to PEP. However, the choice of PPDK as a suitable candidate to convert pyruvate to PEP may be flawed in that C_3 plants lack sufficient levels of inorganic pyrophosphatase (PPase) and adenylate kinase (AK) activity required to maximize the rates of conversion of pyruvate to PEP. An alternate approach may be to replace PPDK with PEP synthetase, an enzyme that favours PEP synthesis without the assistance of either PPase or AK. In light of the possibility of using PEPS as part of a mechanism to increase a C_4 photosynthetic pathway into plants we have been investigating the regulation of the PEPS from *E. coli* and, specifically, the phosphorylation/dephosphorylation mechanism.

The phosphorylation/dephosphorylation regulatory mechanism of *E. coli* PEPS appears to be very similar to the regulatory mechanism described for plant PPDK [6, 7]. The mechanism involves an unusual phosphorylation/dephosphorylation mechanism that is catalysed by a unique kinase/phosphotransferase. Like the PDRP from maize, the *E. coli* PSRP regulatory protein is a bifunctional enzyme that catalyzes the phosphorylation and dephosphorylation of the gluconeogenic enzyme, PEPS. It uses ADP instead of ATP as the Pi donor in a kinase-type reaction that results, by analogy with plant PPDK, in the phosphorylation of a threonine residue (T419) in PEPS. The enzyme also catalyses the Pi-dependent dephosphorylation of T419 via a phosphorolysis reaction [5]. The regulation of PEPS is summarised in Figure 1.

The recent cloning and stable expression of *E. coli* PSRP [5] presented an opportunity to investigate the catalytic mechanisms involved in both the kinase and phosphotransferase activities of the bifunctional regulatory protein. Using site-directed mutagenesis

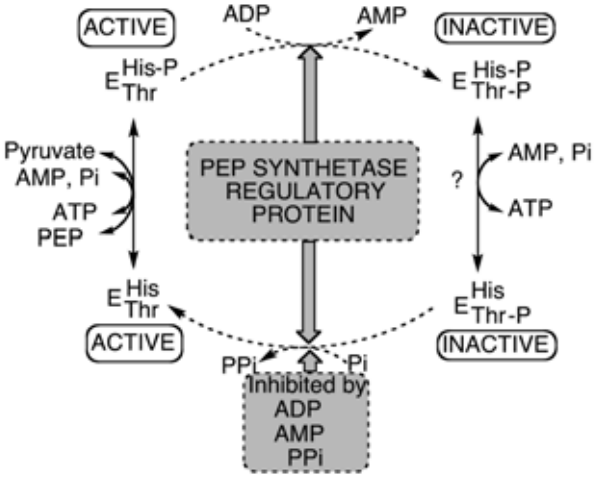

Figure 1. Scheme for the regulation of *E. coli* PEP synthetase catalysed by the PEP synthetase regulatory protein.
His and Thr represent amino acids located at the active site of PEPS that are involved in catalysis and regulation, respectively.

and by monitoring not only the ADP-dependent inactivation and the Pi-dependent activation activities of the mutated PSRP, but also the ADP-dependent inhibition of Pi-dependent activation, we have identified two regions within *E. coli* PSRP that are involved in catalytic activity. We have concluded that in *E. coli* PSRP there are two separate regions within the protein that are involved in binding ADP and Pi and that the sites are located very close together in the active protein. These findings are highly significant given the recent interest in the robust control of PEP formation in C_4 plants by PPDK [9].

METHODS

Site-directed mutagenesis of *E. coli ppsAR*

Mutations to the *psrp* sequence were introduced using paired mutagenic PCR primers in reaction mixtures containing 70 ng plasmid DNA (*E. coli pps* inserted into the *EcoR*I and *Spe*I sites in pPROEX HTa expression vector), 20 pmol of forward and reverse mutagenic primers, 20 nmol dNTPs, 4% DMSO, 10 µL Phusion buffer HF (5X), 1 unit Phusion High-Fidelity DNA Polymerase and DNase- RNase free H_2O in a total vol of 50 µL. A PCR protocol consisting of heating at 98°C for 30 sec, followed by 35 cycles of 98°C for 15 sec,

55°C for 30 sec and 72°C for 4 min was employed. After incubation at 72°C for 10 min PCR products were immediately treated with 20 units of *Dpn*I at 37°C for 90 min following addition of 5 µL of NEB Buffer 4. Competent *E. coli* NM522 cells were transformed with *Dpn*I-treated PCR product by heat shock at 42°C for 60 sec and cell transformation mixes plated directly onto LB plus ampicillin (100 $\mu g.mL^{-1}$) agar plates and incubated at 37°C overnight. Five mL LB media containing ampicillin (100 $\mu g.mL^{-1}$) in 15 mL Falcon tubes were inoculated with single colonies. Following shaking overnight at 37°C glycerol stocks were prepared and plasmid DNA isolated from the remaining culture. Plasmid DNA was isolated and purified using a Machery Nagel miniprep kit according to the kit instructions and the DNA sequence of the plasmid DNA determined commercially (Macrogen, Korea).

Protein expression and purification

PEPS and PSRP were expressed at 25 and 16°C, respectively, and proteins extracted and purified by Ni-affinity purification as described previously [5].

Assay of PEP synthetase activity

PEP synthetase activity was assayed using an enzyme-coupled spectrophotometric assay in which PEP synthesis was coupled to NADH oxidation via PEP carboxylase and malate dehydrogenase [5].

Assay of PEP synthetase inactivation

The ability of PSRP to inactivate PEPS was measured by incubating PSRP and PEPS together and, following the addition of ADP and ATP (2 and 0.1 mM, respectively), monitoring the activity of PEPS at various time intervals. At time zero, ADP/ATP was added and an aliquot (20 µL) of the inactivation assay removed and transferred to a cuvette to measure PEPS activity. PEPS assay mixtures contained 20 µL linking enzymes (PEP carboxylase and malate dehydrogenase) and 0.94 mL reaction cocktail. PEPS activity was determined by measuring the decrease of absorbance at 340 nm. Measurements were recorded at each time point (0, 2, 5 10 and 20 min) in $\Delta AU.min^{-1}$ and the percentage of activity was calculated for each time point with 100% being the activity at time zero.

Assay of Pi-dependent reactivation of PEP synthetase and ADP-dependent inhibition of Pi-dependent reactivation

The reactivation of PEPS as catalysed by PSRP, and the ADP-dependent inhibition of Pi-dependent reactivation of inactive PEPS, was measured in cuvettes used to measure PEPS activity following inactivation of PEPS in inactivation reactions. This was achieved by comparing the increase in PEPS activity in cuvettes containing no Pi with those containing 2 mM Pi (final concentration). Similarly, the potential ADP-inhibition of Pi-dependent activation was measured by comparing the increase in PSRP activity in cuvettes containing no Pi, 2 mM Pi, 2 mM Pi plus 1 mM ADP and 1 mM ADP alone. Activity measurements were recorded at each time point in $\Delta AU.min^{-1}$ and the percentage of initial activities calculated for each time point with 100% being the activity at time zero of the inactivation assay.

For those mutants in which ADP-dependent inactivation was lost, 0.2 units of purified PEPS synthetase and 50 µL of purified inactivated PSRP were added directly to cuvettes used to assay PEPS synthetase activity and changes in PEPS activity recorded for at least five minutes. Reaction rates in cuvettes containing no additions, plus 2 mM Pi, plus 1 mM ADP and plus both 2 mM Pi and 1 mM ADP were compared to reveal those mutants that were capable of Pi-dependent activation and to determine whether the mutants were sensitive to ADP-dependent inhibition.

PEP synthetase reaction mixtures contained 50 mM Tris pH 8.0, 2.5 mM pyruvate, 8 mM $MgCl_2$, 10 mM $NaHCO_3$, 10 mM DTT, 0.2 mM NADH, 1 mM glucose-6-phosphate, 1 mM ATP, PEP carboxylase (2.5 units) and malate dehydrogenase (5 units) in a total volume of 0.94 mL.

Inactivation reactions made up in a 1.5 mL microcentrifuge tube contained 25 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$ 10 mM 2-mercaptoethanol, purified *E. coli* PEP synthetase (0.5 units) and varying volumes of PSRP in a total volume of 200 µL. Inactivation reactions were initiated by adding 20 µL 20 mM ADP/1 mM ATP, mixed rapidly and 20 µL aliquots removed and added to a cuvette containing PEPS reaction mixture, and the rates of NADH oxidation recorded spectrophotometrically.

PEPS reactivation activity was measured by following the change in the A340 nm per minute in assays measuring PEPS activity as part of inactivation assays following the addition of phosphate to a final concentration of 2 mM. The effect of ADP on Pi-dependent inactivation was measured by comparing the rate of Pi-dependent PEPS activation in the presence with the rate on activation in the absence of 1 mM ADP.

RESULTS AND DISCUSSION

Identification of highly conserved amino acid residues

To facilitate the identification of potentially important functional amino acid residues in *E. coli* PSRP, more than 200 bacterial and plant DUF299 sequences were downloaded from bacterial and plant genome sequencing databases (http://img.jgi.doe.gov) and aligned using the ClustalW algorithm (MacVector 9.0) and more than a dozen highly conserved amino acid residues identified (see Figure 2). In addition, more than a dozen PSRP amino acid sequences were analysed by the PredictProtein software [10] and a number of putative domains identified (including a tyrosine kinase binding site, a protein kinase C phosphorylation site, a Pi-binding site, an ADP-binding site and three protein-protein binding sites) (see Figure 2). A combination of highly conserved amino acids and amino acids located in specific protein domains were selected and altered by site-directed mutagenesis and the regulatory properties of the expressed proteins characterised.

Site-directed mutagenesis

Twenty-six amino acid residues were altered by site-directed mutagenesis and the mutations verified by nucleotide sequencing (see Figure 2). *E. coli* cells (NM522) were transformed with the mutated plasmids and transformation of the cells verified by plasmid isolation and nucleotide sequencing. Following growth of transformed cells the solubility of expressed proteins was assessed by analysing samples of the crude supernatants and the protein pellets obtained following centrifugation of French Press extracts. Of the mutants generated, only five mutations resulted in the production of insoluble protein (S196A, L214A, Y227A, E239A and a double mutant D140A/D141A) (Table 1). It was assumed that these residues were critical for correct folding of the protein. All other mutations resulted in the production of soluble protein (Table 1).

The successful purification of PSRP was achieved only if care was taken to ensure that the protein eluted from nickel-affinity column was diluted as it eluted from the column; high concentrations of PSRP tended to precipitate regardless of temperature (room temperature, 16°C or 0°C), the addition of reducing agent (10 mM DTT) or the addition of glycerol (up to 15% by vol.).

Mutations made in putative tyrosine kinase sites (I56A and Y60F) and putative protein kinase C sites (S144A and S229A) did not affect the rates of ADP-dependent inactivation, the Pi-dependent activation activities or the ADP-inhibition of Pi-dependent activation. In addition, mutations to

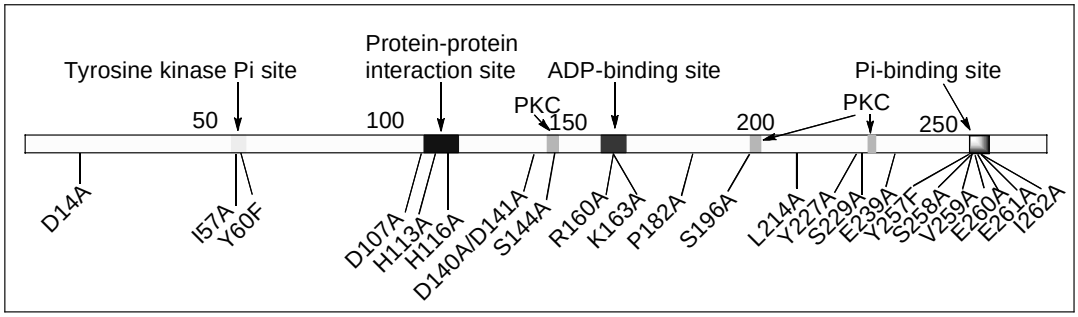

Figure 2. Location of specific domains and sites of amino acid mutations made in *E. coli* PSRP.
Amino acids are numbered according to the *E. coli* PDRP amino acid sequence. More than 200 bacterial and plant species were aligned using ClustalW software. Conserved amino acid residues common to either or both PPDK- or PEPS-containing species are listed.

Table 1. A summary of the effect of mutations introduced into *E. coli* PSRP on the solubility, ADP-dependent inactivation, the Pi-dependent activation and the ADP-dependent inhibition of Pi-dependent activation of PEPS.

[1]Putative domain	Mutation	[2]Protein solubility	[3]ADP-dependent inactivation relative to wild type	[4]Pi-dependent activation relative to wild type	[5]ADP-dependent inhibition of activation relative to wild type
	Wild type	Soluble	90 - 100	90 - 100	>90
Tyrosine kinase Pi-site	I57A	Soluble	90 - 100	90 - 100	>90
	Y60F	Soluble	90 - 100	90 - 100	>90
Protein kinase C site	S144A	Soluble	90 - 100	90 - 100	>90
	S196A	Insoluble	[6]c.n.d	c.n.d	c.n.d
	S229A	Soluble	90 - 100	90 - 100	>90
ADP-binding site	S159A	Soluble	0	[7]0	c.n.d
	R160A	Soluble	0	0	c.n.d
	K163A	Soluble	0	0	c.n.d
Pi-binding site	Y257F	Soluble	90 - 100	90 - 100	0
	S258A	Soluble	0	90 - 100	0
	V259A	Soluble	90 - 100	0	c.n.d
	E260A	Soluble	90 - 100	0	c.n.d
	E261A	Soluble	90 - 100	90 - 100	>90
	I262A	Soluble	0	90 - 100	0
Highly conserved amino acids	D14A	Soluble	90 - 100	90 - 100	>90
	A17S/T19A	Soluble	90 - 100	90 - 100	>90
	D140/D141A	Insoluble	c.n.d	c.n.d	c.n.d
	P182A	Soluble	0	90 - 100	>90
	L214A	Insoluble	c.n.d	c.n.d	c.n.d
	Y227A	Insoluble	c.n.d	c.n.d	c.n.d
	E239A	Insoluble	c.n.d	c.n.d	c.n.d
Protein-protein interaction sites	D107A	Soluble	90 - 100	90 - 100	>90
	H113A	Soluble	35 - 45	35 - 45	>90
	H116A	Soluble	30 - 40	30 - 40	>90

[1]Putative domains were identified using the PredictProtein software.
[2]Protein solubility was assessed by observing Coomassie Blue staining of proteins on SDS-PAGE gels following lysis of cells using a French Press.
[3]Rates of ADP-dependent inactivation are expressed in terms of the initial rate of inactivation as percentage of the original PPDK activity inactivated per minute
[4]Activation rates were measured by following the rates of in-assay activation in the absence of ADP.
[5]ADP inhibition was determined by measuring the rates of in-assay activation in the presence of 2 mM ADP and the level of inhibition given as a percentage of the activation rate in the absence of ADP.
[6]c.n.d - could not determine.
[7]Activation was measured using PEPS that had been partially inactivated during bacterial expression of the protein - see text.

amino acid residues highly conserved across both PPDK and PEPS species (D14A or A17S/T19A) failed to alter PSRP-dependent activities. In contrast, however, PSRP activities were affected by mutations in three specific regions; in the potential protein-protein interaction sites (D107A, H113A and H116A), in the ADP/ATP-binding P-loop (residues 157-164) and within a Pi-binding site (Y257-I262). Mutations to both H113 and H116 produced soluble protein that was able to catalyze both inactivation and activation; however, the rate of both activities was less than half the rate

observed compared to wild type enzyme. Mutations within the highly conserved P-loop (S159A, R160A and K163A) produced soluble protein but the mutated protein lacked both inactivation and activation activities. This contrasted with mutations made within the Pi-binding region where enzyme activities were differentially affected. In particular, two mutants (S258A and I262A) were unable to catalyse ADP-dependent inactivation activity, were able to catalyse Pi-dependent activation but were insensitive to ADP-dependent inhibition of Pi-dependent activation. The changes introduced by these two mutations destroyed the ability of the mutants to bind ADP involved in both ADP-dependent inactivation and ADP-dependent inhibition of Pi-dependent activation. Collectively, these mutations suggest that there is a single ADP binding site that affects both inactivation and activation and a separate Pi-binding site.

In contrast, mutants V259S and E260A both retained ADP-dependent inactivation activity but lost Pi-dependent activation activity. (Since activation activity was lost the effect of ADP on activation could not be investigated). These results indicated that there were separate sites involved in binding ADP and Pi.

Partial inactivation of expressed PEPS

A critical part of these studies involved the expression and purification of *E. coli* PEPS; this was achieved by subcloning the *E. coli ppsA* into pROEx and expressing the protein with an N-terminal 6- His tag that facilitated rapid purification of the expressed protein by nickel affinity column chromatography. During this study PEPS was expressed and purified three times under slightly different conditions. In one case, the expression of PEPS was extended by 24 hours following the addition of IPTG (48 rather than 24 hours). When this protein was used as substrate in inactivation assays and then used in subsequent in-assay activation assays, levels of activity achieved following in-assay activation exceeded initial activity levels prior to inactivation. This suggested that the PEPS expressed in *E. coli* had been partially inactivated by endogenous PSRP during expression. Subsequent experiments in which purified PSRP was incubated with Pi and PEPS activity levels measured indicated that almost 50% of the expressed PEPS was inactivated; maximum activity levels could be recovered by incubating the purified PEPS with Pi and purified expressed PSRP. Activation of H113A, H116A, S159A, R160A, K163A, P182A. S258A and I262A mutants was assessed using this enzyme.

Secondary structure analysis

Analysis of the putative P_i-binding site of *E. coli* PSRP using the Chou-Fasman secondary structure prediction algorithm [11] identified an alpha helix between residues 257 and 264. Given that 3.6 amino acid residues are required for a single turn within an alpha helix, the mutation studies suggest that S258 and I262, which would be located along one side of an alpha helix, may be involved in ADP binding. Furthermore, given that S258A and I262A destroyed both the ADP-dependent inactivation and the ADP-dependent inhibition of Pi-dependent activation it is logical to conclude that this single ADP-binding site is common to the ADP-dependent inactivation activity and the ADP-dependent inhibition of PEPS activation. In addition, the results are consistent with the suggestion that the P-loop is involved in both the activation and activation activities of PSRP and that the P-loop binds ADP in close proximity to S258 and I262 that are exposed to the P-loop. Given the loss of both inactivation and activation activities with changes to the S159, R160 and K163 we suggest that these residues may be involved in binding directly to the regulatory T419 residue of PEPS. Therefore during ADP-dependent inactivation the P-loop and a C-terminal alpha helix are involved in binding ADP and transferring the beta-phosphate of ADP to T419 of PEPS. Similarly, these two regions are also involved in removing the phosphate group from T419 of PEPS during Pi-dependent activation. However, if ADP is bound to PSRP, Pi is unable to interact with T419 on PEPS and activation is inhibited. For this to occur the two binding sites (the ADP- and the Pi-binding sites), although separate, must be very close together.

These studies support and extend the results obtained in site-directed mutagenesis studies conducted with *Arabidopsis thaliana* PPDK regulatory protein [12] and provide strong evidence that the regulatory

mechanism found in plants is the same as that present in almost all bacteria.

CONCLUSIONS

These studies, involving the site-directed mutagenesis and the expression and purification of *E. coli* PSRP, provide greater insight into the location of regions involved in catalysis within the enzyme. The region identified as a P-loop appears to be directly involved in binding PEPS to the regulatory protein while amino acids located at the C-terminal region of the regulatory enzyme are critical to the binding of both ADP and Pi. Furthermore, there is only one ADP-binding site involved in donating a phosphate group during inactivation and inhibiting Pi-dependent activation.

It is anticipated that protein crystallization and subsequent X-ray diffraction studies will confirm the results presented in this report and may, importantly, reveal structural similarities the *E. coli* PSRP regulatory protein may have with other proteins. However, the inherent protein solubility problems of the regulatory protein will have to be overcome prior to crystallization of the protein being achievable.

ACKNOWLEDGEMENT

SB and JS conducted the site-directed mutagenesis, protein expression and enzyme purification and JB assayed the PSRP activities and drafted the manuscript. All authors read and approved the final manuscript. The authors wish to thank the School of Pharmacy and Molecular Sciences at James Cook University for research funding.

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