IN VIVO PHOSPHORYLATION OF BACTERIAL–TYPE PHOSPHOENOLPYRUVATE CARBOXYLASE FROM DEVELOPING CASTOR OIL SEEDS AT THREONINE-4 AND SERINE-451

by

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Abstract

Phosphoenolpyruvate carboxylase (PEPC) is a tightly controlled anaplerotic enzyme situated at a pivotal branchpoint of plant C-metabolism. Plant genomes encode several closely related plant-type PEPC (PTPC) isozymes, and a distantly related bacterial-type PEPC (BTPC). Two physically and kinetically distinct oligomeric classes of PEPC occur in the endosperm of developing castor oil seeds (COS). Class-1 PEPC is a typical homotetramer composed of 107-kDa PTPC subunits, whereas the novel 910-kDa Class-2 PEPC hetero-octameric complex arises from a tight interaction between Class-1 PEPC and 118-kDa bacterial-type PEPC (BTPC) subunits. BTPC functions as a catalytic and regulatory subunit of the allosterically-desensitized Class-2 PEPC, hypothesized to support PEP-flux to malate for leucoplast fatty acid synthesis. Previous studies established that BTPC: (i) subunits of COS Class-2 PEPC are phosphorylated at multiple sites in vivo and (ii) phosphorylation at Ser^{425} provides a new tier of enzyme control in developing COS. LC MS/MS and LTQ-FT MS identified Thr^{4} and Ser^{451} as additional in vivo phosphorylation sites of immunopurified COS BTPC (corresponding to acidophilic and basophilic protein kinase consensus sequences, respectively). Immunoblots probed with a phosphorylation-site specific antibody raised against a synthetic phosphopeptide indicated that Ser^{451} phosphorylation is promoted during seed development, becoming maximal in stage VII (full cotyledon) COS. Although several pThr^{4} containing BTPC peptides were non-immunogenic, the collective results indicate that Thr^{4} is also phosphorylated in vivo. Kinetic effects of each phosphorylation site were examined using phospho-mimetic mutants of heterologously expressed COS BTPC. BTPC’s phosphorylation at Ser^{451} appears to be inhibitory, as reflected by significantly increased
$K_m$(PEP) values, and reduced $I_{50}$(malate) and $I_{50}$(Asp) values of a S451D mutant. By contrast, kinetic characterization of a T4D phosphomimetic mutant indicated that Thr$^4$ phosphorylation is not regulatory in nature. However, Thr$^4$ exists in a conserved forkhead-associated (FHA) binding domain (pTXXD) that has received considerable prominence as a phospho-Thr dependent protein interaction module. These results further our understanding of multisite phosphorylation of BTPC in developing COS and its possible contribution to the control of Class-2 PEPC activity.
Co-Authorship

With the exception of mass-spectrometry, all experimentation was performed within the Plaxton lab. Dr. Yimin She (previously, Dept. of Chemistry, Queen’s University, currently Health Canada, Ottawa) oversaw all mass spectrometric experimentation. The work presented in Chapter 2 is representative of a few collaborators. Dr. Srinath Rao performed the cloning, mutagenesis and optimization of expression for all PEPC constructs. Brendan O’Leary performed the immunoblots pertaining to the characterization of the anti-pSer\textsuperscript{879} (Fig. 2.3), as well as all work done on characterization of the anti-pSer\textsuperscript{425} in (Figs. 2.3 and 2.6). Carolyne Brikis, an undergraduate thesis student, performed the initial dot blots and immunoblots pertaining to the characterization of the anti-pSer\textsuperscript{451} in (Fig. 2.3 and 2.4 A).
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# Table of Contents

Abstract .................................................................................................................................................. ii
Co-Authorship ......................................................................................................................................... iv
Acknowledgements ............................................................................................................................... v
Table of Contents ................................................................................................................................... vi
List of Figures ........................................................................................................................................ vii
List of Tables .......................................................................................................................................... viii
List of Abbreviations ............................................................................................................................. ix

## Chapter 1. Introduction and Literature Review

1.1 Physiological roles of PEPC ........................................................................................................... 1
1.2 Carbon partitioning in developing seeds ......................................................................................... 2
1.3 Post-translational control of plant-type PEPC .............................................................................. 4
  Allosteric effectors ................................................................................................................................ 4
  Regulatory phosphorylation .................................................................................................................. 4
  Monoubiquitination .............................................................................................................................. 6
1.4 Class-2 PEPC complexes ................................................................................................................ 8
  Discovery of the bacterial-type PEPC (BTPC) gene ........................................................................... 8
  Discovery of bacterial-type PEPC (BTPC) polypeptides .................................................................... 8
  Tissue-specific expression of bacterial-type PEPC (BTPC) in castor plant .................................... 11
  Post-translational modification of BTPC in castor oil seed ............................................................... 12
1.5 Thesis Objectives ............................................................................................................................ 13

## Chapter 2. In vivo phosphorylation of bacterial-type phosphoenolpyruvate carboxylase from developing castor oil seeds in at Thr<sup>4</sup> and Ser<sup>451</sup> ...................................................................................... 21

2.1 Abstract ........................................................................................................................................... 21
2.2 Introduction ..................................................................................................................................... 22
2.3 Materials and Methods .................................................................................................................. 25
  Plant material ...................................................................................................................................... 25
  Co-immunopurification and protein phosphatase treatments ............................................................ 25
  LC MS/MS analysis and phosphopeptide identification .................................................................. 25
  Site-directed mutagenesis and heterologous expression of recombinant PEPCs ........................ 26
  Purification of recombinant Class-2 PEPCs .................................................................................... 27
  Preparation of phosphosite specific antibodies against pThr<sup>4</sup>, pSer<sup>451</sup>, and pSer<sup>879</sup> of COS BTPC ........................................................................................................ 28
Electrophoresis and immunoblotting ................................................................. 29
Enzyme and protein assays and kinetic studies ...................................................... 29
Statistics .................................................................................................................. 30
2.4 Results and Discussion ...................................................................................... 30
BTPC phosphosite mapping ................................................................................... 30
Phospho-site specific antibodies confirm the phosphorylation of Ser^{451} in vivo..... 32
Monitoring Ser^{451} phosphorylation status throughout COS development and
following COS depodding ......................................................................................... 34
Characterization of phosphomimetic mutants suggests that Ser^{451} phosphorylation
inhibits BTPC within a Class-2 PEPC complex ......................................................... 35
2.5 Conclusion .......................................................................................................... 39

Chapter 3. General Discussion ............................................................................... 49

References ............................................................................................................... 53

Appendix A. NetPhos phospho-site prediction score and phosphomimetic mutant
purifications ................................................................................................................. 61

Appendix B. Biological Replicates of in vivo phosphorylation status of Ser^{451} in
developing COS endosperm ...................................................................................... 63
List of Figures

Figure 1.1 The phosphoenolpyruvate carboxylase reaction ................................. 15
Figure 1.2 The diverse functions of plant PEPC ............................................. 16
Figure 1.3 Models illustrating several metabolic functions of plant PEPC ............. 17
Figure 1.4 Amino acid sequence alignment of Arabidopsis and castor oil plant PEPC isoenzymes ................................................................. 18
Figure 1.5 Model illustrating the biochemical complexity of castor bean PEPC .... 20
Figure 2.1 COS BTPC MS/MS analysis and phosphorylation site mapping........... 41
Figure 2.2 Partial alignment of COS BTPC Thr^4 and Ser^{451} domains with other vascular plant BTPCs ................................................................. 42
Figure 2.3 Specificity of phosphosite-specific antibodies ................................. 43
Figure 2.4 In vivo phosphorylation status of Ser^{451} in developing COS endosperm ... 44
Figure 2.5 Purification of recombinant Class-2 PEPC mutants .......................... 45
Figure 2.6 Sensitivity of recombinant Class-2 PEPC mutants to effectors .......... 46
Figure 2.7 pH activity profile of recombinant Class-2 PEPC mutants ............... 47
List of Tables

Table 2.1 PEP saturation kinetics of wild-type and phosphomimetic mutants of heterologously expressed Class-2 PEPC .......................................................... 48
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>λ-P’tase</td>
<td>lambda phosphatase</td>
</tr>
<tr>
<td>A&lt;sub&gt;280&lt;/sub&gt;</td>
<td>absorbance at the 280 nanometer wavelength</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>BTPC</td>
<td>bacterial type phosphoenolpyruvate carboxylase</td>
</tr>
<tr>
<td>CAM</td>
<td>Crassulacean acid metabolism</td>
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<tr>
<td>COS</td>
<td>castor oil seed</td>
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<tr>
<td>CBB-R250</td>
<td>Coomassie Brilliant Blue 250</td>
</tr>
<tr>
<td>Co-IP</td>
<td>co-immunopurification</td>
</tr>
<tr>
<td>DDL</td>
<td>dawdle protein</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>FT-ICR-MS</td>
<td>Fourier transform ion cyclotron resonance mass spectrometry</td>
</tr>
<tr>
<td>FHA</td>
<td>forkhead associated</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
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<tr>
<td>Glu-6-P</td>
<td>glucose-6-phosphate</td>
</tr>
<tr>
<td>Gly-3-P</td>
<td>glycerol-3-phosphate</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>I&lt;sub&gt;50&lt;/sub&gt;</td>
<td>half maximal inhibition concentration</td>
</tr>
<tr>
<td>KAPP</td>
<td>kinase associated protein phosphatase</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>MDH</td>
<td>malate dehydrogenase</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>tandem mass spectrometry</td>
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<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>OAA</td>
<td>oxaloacetate</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PEP</td>
<td>phosphoenolpyruvate</td>
</tr>
<tr>
<td>PEPC</td>
<td>phosphoenolpyruvate carboxylase</td>
</tr>
<tr>
<td>P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>orthophosphate</td>
</tr>
<tr>
<td>PK&lt;sub&gt;c&lt;/sub&gt;</td>
<td>cytosolic pyruvate kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PK&lt;sub&gt;p&lt;/sub&gt;</td>
<td>plastidic pyruvate kinase</td>
</tr>
<tr>
<td>PP2A</td>
<td>protein phosphatase type 2A</td>
</tr>
<tr>
<td>PPCK</td>
<td>PEPC protein kinase</td>
</tr>
<tr>
<td>ProQ-PPS</td>
<td>Pro-Q Diamond Phosphoprotein stain</td>
</tr>
<tr>
<td>PTM</td>
<td>post-translational modification</td>
</tr>
<tr>
<td>qTOF</td>
<td>quadrupole time of flight</td>
</tr>
<tr>
<td>RT</td>
<td>retention time</td>
</tr>
<tr>
<td>SnRK1</td>
<td>sucrose non-fermenting related kinase</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid cycle</td>
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Phosphoenolpyruvate (PEP) carboxylase (PEPC; E.C. 4.1.1.31) is a ubiquitous, and tightly controlled cytosolic enzyme found in vascular plants, green algae and bacteria. It catalyzes the irreversible β-carboxylation of phosphoenolpyruvate (PEP) in the presence of HCO$_3^-$ to yield oxaloacetate (OAA) and P$_i$, using Mg$^{2+}$ as a cofactor (Fig. 1.1)[1]. PEPC plays pivotal roles in photosynthetic CO$_2$ fixation by C$_4$ and Crassulacean acid metabolism (CAM) leaves, as well as in the anaplerotic replenishment of tricarboxylic acid (TCA) cycle intermediates that are withdrawn for biosynthesis and N-assimilation (Fig. 1.2)[1,2]. There are two distinct types of PEPC isozymes in vascular plant and green alga: plant-type PEPC (PTPC) that belongs to a small multi-gene family in vascular plants, and a distantly related bacterial-type PEPC (BTPC)[3-7]. The well studied Class-1 PEPCs usually exist as 400-440 kDa homotetramers, consisting of 4 identical 100-110 kDa PTPC subunits [2,8]. PTPCs have been categorized as being either photosynthetic [C$_4$ and CAM PEPCs] or non-photosynthetic (C$_3$) isozymes [9]. Vascular plant and algal BTPCs, however, physically interact with a Class-1 PEPC homotetramer to form the novel hetero-octamer Class-2 PEPC complex which is discussed below in detail [10-12]

1.1 Physiological roles of PEPC

Photosynthetic PTPC of C$_4$ and CAM leaves reduce photorespiration and function as a CO$_2$ concentrating mechanism to improve overall photosynthetic efficiency by up to 2-fold compared to C$_3$ leaves [13-16]. In addition, PEPC has a wide range of non-photosynthetic roles including supporting carbon–nitrogen interactions, seed formation
and germination, fruit ripening, guard cell metabolism during stomatal opening, and provision of malate as a respiratory substrate for symbiotic N₂-fixing bacteroids of legume root nodules (Fig. 1.2)[17]. The role of PEPC in the control of carbon partitioning in developing oil seeds is the focus of this thesis. PEP metabolism via PEPC and cytosolic pyruvate kinase (PKc) and plastidic pyruvate kinase (PKp) isozymes plays a key role in partitioning photosynthate to either leucoplast fatty acid biosynthesis or mitochondrial production of carbon skeletons and ATP needed for storage protein biosynthesis (Fig. 1.3) [17, 93].

1.2 Carbon partitioning in developing seeds

In developing seeds the partitioning of imported photosynthate between starch, storage lipid (triglycerides), and storage protein biosynthesis is of major agronomic concern. Developing seeds act as metabolic sinks that import sugars and amino acids from leaves and subsequently metabolize them into starch, triglycerides and storage proteins. Photosynthate may either be imported into the leucoplast for fatty acid biosynthesis or the mitochondria for production of C-skeletons and ATP needed for storage protein biosynthesis (Fig. 1.3). The partitioning of carbon between the leucoplast and mitochondria is largely dependent on PEP metabolism via PEPC, PKc and PKp [93]. In particular, PEPC activity and storage protein biosynthesis have been linked due to the requirement for OAA and 2-oxoglutarate (2-OG) to assimilate N during formation of amino acids [17-20]. Additional C skeletons are required when seeds import N in the form of amino acids because (i) glutamine, a major component of the supplied amino acids, contains two amino groups per 2-OG skeleton, and (ii) amino acids such as alanine can be deaminated and respired through the TCA cycle to yield NH₄⁺ that must be re-
assimilated into glutamine or glutamate via glutamine synthetase and glutamate-oxoglutarate amino transferase (GS/GOGAT) (Fig. 1.2A).

In addition PEPC has been shown to contribute to fatty acid synthesis through generation of metabolic precursors and reducing power. Fatty acid synthesis within the leucoplast is ATP and NAD(P)H dependent and uses acetyl-CoA as a precursor (Fig. 1.3B). The pathway from sucrose-derived cytosolic hexose-Ps to plastidial acetyl-CoA is very flexible and varies within and between species (Fig. 1.3B) [21,22]. Studies of isolated leucoplasts from developing castor oil seed (COS) and Helianthus annus (sunflower) seeds showed that exogenous malate, compared to other precursors, supported maximal rates of fatty acid synthesis [23,24]. When malate is oxidized into acetyl-CoA by plastidic NADP-ME and isozymes of pyruvate dehydrogenase complex (PDC) it produces all the reductant required for carbon incorporation into fatty acids [24]. In addition (i) storage lipid accumulation is closely related to PEPC and NADP-ME activity and protein abundance [7,10,25,26], and (ii) in castor cytosolic malate is transported into the leucoplast via a malate/Pi antiport of the leucoplast envelope [27].

Metabolic flux studies on non-green developing maize and sunflower embryos demonstrated 30% and 10% of carbon flux into fatty acids was derived from PEPC generated malate [28,29] adding to the evidence for PEPC’s involvement in fatty acid synthesis in non green oil seeds. All of this evidence suggests that PEPC plays an important role in sucrose partitioning at the cytosolic PEP branchpoint to produce C-skeletons and reductant required for fatty acid biosynthesis in developing oil seeds such as COS (Fig. 1.3).
Pioneering work in the 1960’s by Harry Beevers (Univ. of Purdue) and colleagues has led to the wide spread use of COS as a model system for studies in oilseed development and germination, carbon metabolism and triacylglyceride storage and mobilization. The castor genome was recently sequenced and annotated providing an important foundation for further investigation of regulatory and metabolic networks of castor-oil biosynthesis [30]. Additionally, COS contains more triacylglycerides than any other seed examined to date (~64% of total seed weight) [31,32].

1.3 Post-translational control of plant-type PEPC

PTPCs are controlled by allosteric effectors, as well as by a variety of post-translational modifications (PTMs).

Allosteric effectors

Allosteric control of Class-1 PEPC is accomplished through both feed-forward activation and feedback inhibition. Hexose- and triose-phosphates such as glucose-6-phosphate (Glu-6-P) and glycerol-3-phosphate (Gly-3-P) are activators of PEPC, whereas L-malate, L-aspartate, and L-glutamate are feedback inhibitors (Figs. 1.1 and 1.3A) [1,10,17,33,34].

Regulatory phosphorylation

Regulatory PEPC phosphorylation was initially discovered with C₄ and CAM photosynthetic Class-1 PEPCs [35]. Their PTPC subunits are phosphorylated on a highly conserved Ser residue close to the N-terminus of the protein (Fig. 1.4) [1,35]. Phosphorylation at this site decreases PEPC’s sensitivity to allosteric inhibitors while increasing its affinity for PEP and allosteric activators [1,2,17,35,36]. A 30-33 kDa
calcium-independent PEPC protein kinase (PPCK) and protein phosphatase type 2A (PP2A) mediate the reversible phosphorylation of Class-1 PEPC [35-39]. PPCK is highly specific for the conserved N-terminal seryl phosphorylation site as it cannot phosphorylate N-terminal seryl mutant PEPCs [37,40,41]. Its high specificity may be due to the fact that PPCK lacks any regulatory domain and only contains a core kinase domain, making it the smallest protein kinase documented [2,36]. Class-1 PEPC phosphorylation appears to be largely controlled by changes in rates of PPCK synthesis and degradation, although allosteric effectors and dithiol-disulfide interconversion may also play a role [2,35,39].

Due to the lack of regulatory domains PPCK appears to be upregulated by a range of signals, including light in C4 leaves, a circadian rhythm in CAM leaves, light and N supply in C3 leaves, nutritional P_i deprivation in C3 plants and cell cultures, and photosynthate supply in legume root nodules and developing castor seeds [35,36,39,42-47]. Studies with developing COS and soybean root nodules showed that endogenous PPCK activity, transcripts and PTPC subunit phosphorylation disappeared in response to prolonged darkness of intact plants. Reillumination of these plants resulted in recovery of PPCK activity and PTPC phosphorylation [39,48-50]. It is clear that regaining of photosynthate supply to these non-photosynthetic sink tissues provides a direct link between sucrose and the upregulation of PPCK and PTPC phosphorylation. In addition, studies on the PP2A involved in dephosphorylating PEPC have shown constant levels throughout plant tissues lending to the idea that PEPC phosphorylation is largely controlled by PPCK activity [1,51,52]. However, further research is needed to assess the
possibility that the activity of the PP2A catalytic subunits are controlled by its associated regulatory subunits.

Several post-translational PPCK controls have been proposed including (i) malate inhibition of PPCK activity, by potentially interacting with PEPC [38,39,53], (ii) dithiol-disulfide interconversion [39,41,54], and (iii) a proteinaceous inhibitor [55]. However, further studies are needed to confirm these mechanisms in vivo.

In addition to controlling enzymatic activity protein phosphorylation can provide docking sites for other proteins to mediate protein-protein interactions. One of the more common protein groups known to mediate protein-protein interactions through protein phosphorylation is the 14-3-3 family. They are a highly conserved and abundant protein family that plays a central role in eukaryotic cells [56]. 14-3-3s have been implicated as a binding partner of the PTPC isozyme AtPPC1 following tandem affinity purification of 14-3-3 complexes from transgenic Arabidopsis seedlings [57]. Despite this finding, AtPPC1 does not contain a known 14-3-3 binding motif and attempts to demonstrate 14-3-3 binding to purified phospho- and dephosphorylated forms of native AtPPC1 using far western overlay assays have been unsuccessful [58, W. Plaxton and C. Mackintosh, unpublished]. Additional research is needed to determine whether or not 14-3-3s do in fact bind to AtPPC1 and if they exert an influence on the kinetic or regulatory properties of AtPPC1 or other plant PEPCs.

Monoubiquitination

Ubiquitin is small, highly conserved globular protein found in eukaryotic cells. Ubiquitin can be covalently attached to a target protein through an isopeptide bond between the C-terminal Gly residue of ubiquitin and the ε-amino group of a Lys residue
on the target protein. The attachment of ubiquitin to its target protein is mediated by activating (E1), conjugating (E2) and ligating (E3) enzymes. Polyubiquitination is well known as a PTM that targets proteins for degradation by the 26S proteasome. PTPC and PPCK have both been suggested to be degraded by the polyubiquitination and proteasome pathway [59,60].

Conversely, monoubiquitination is a reversible PTM that mediates protein:protein interactions and protein localization and is involved in several processes such as endocytosis, DNA repair, transcription and translation, and signal transduction [61,62]. Interestingly, Class-1 PEPC purified from germinating COS endosperm was a heterotetramer containing a 1:1 ratio 110- and 107-kDa PTPC polypeptides. Several lines of evidence (N-terminal microsequencing, mass spectrometry (MS) and immunoblotting) showed that both polypeptides arise from the same PTPC gene (RcPpc3), but the 110-kDa subunit is a monoubiquitinated form of the 107-kDa subunit (Fig. 1.5). Lys-628 [63], a residue conserved in all PTPCs and BTPCs located proximal to a PEP binding/catalytic domain, was identified as PEPCs monoubiquitination site by MS/MS. Characterization of this PTM included incubating pure germinated COS Class-1 PEPC with a deubiquitinating enzyme [USP-2 (Ubiquitin specific protease-2) catalytic core] resulting in cleavage of ubiquitin from the 110-kDa subunit. This led to a reduction in the enzyme’s $K_m$(PEP) and sensitivity to allosteric activators and inhibitors [63]. PTPC monoubiquitination has since been found in Lilium longiflorum (lily) pollen [6] and an immunoreactive doublet seen in germinating COS endosperm has also been observed on PTPC immunoblots from a broad variety of plants and tissues [64-67]. Additional
research is necessary to fully understand the prevalence and metabolic functions of PTPC monoubiquitination.

### 1.4 Class-2 PEPC complexes

The physical association of BTPC subunits with PTPC subunits to form Class-2 PEPC complexes (Fig. 1.5) adds to the already intricate regulation and modification of Class-1 PEPC.

*Discovery of the bacterial-type PEPC (BTPC) gene*

Annotation of *Arabidopsis* and rice genomes led to the discovery of a PEPC gene whose deduced amino acid sequence differed greatly from that of PTPCs [3]. In fact, these genes were more similar to those encoding PEPCs found in proteobacteria, and thus named BTPCs. BTPCs are thought to have evolved in green algae, and every sequenced plant genome contains at least one BTPC gene. Green algal BTPC genes encode an ~130 kDa polypeptide, whereas vascular plants BTPC genes encode polypeptides ranging from ~116 – 118 kDa [17].

*Discovery of bacterial-type PEPC (BTPC) polypeptides*

The discovery of BTPC polypeptides began in unicellular green algae (*Selenastrum minutum* and *Chlamydomonas reinhardtii*). Two distinct PEPC classes that exhibited different kinetic and physical properties but shared an identical PTPC subunit were purified and characterized [11,12,68-70]. These two classes were named Class-1 PEPC (consisting of a homotetramer of PTPC subunits) and Class-2 PEPC (high *M*₅ complexes of Class-1 PEPC tightly associated with an immunologically unrelated 130-kDa subunit, subsequently shown to be BTPC). This novel Class-2 PEPC demonstrated
enhanced thermal stability, a broader pH-activity profile, biphasic PEP saturation kinetics and a greatly reduced sensitivity to allosteric effectors [11,12,69]. Algal BTPC appears to be phosphorylated on unknown residues which may be involved in BTPC:PTPC subunit stoichiometry within Class-2 PEPC [70]. Studies of BTPC have led to several characteristics that distinguish it from PTPCs. The main criteria are: (i) C-terminal tetrapeptide is (R/K)NTG for BTPCs or QNTG for PTPCs, (ii) BTPCs lack the N-terminal seryl phosphorylation motif (Acid-Base-XX-SIDAQLR) that PTPCs contain [3,5,7] and (iii) all BTPCs contain an intrinsically disordered region which consists of a 10-kDa insertion that is unstructured and highly flexible (Fig. 1.4) [71].

In 2003 Blonde and Plaxton [10] subsequently purified two distinct oligomeric classes of PEPCs from developing COS endosperm and found that their respective physical and kinetic/regulatory properties were extremely similar to those of Class-1 vs. Class-2 PEPCs of unicellular green algae. COS Class-1 PEPC was determined to be a 410-kDa homotetramer of 107-kDa PTPC subunits, whereas COS Class-2 PEPC exists as a 910-kDa hetero-octameric complex in which the Class-1 PEPC homotetrameric core is tightly associated with four 118-kDa BTPC subunits (Fig. 1.5) [7,10]. Similar to algal Class-2 PEPCs, the COS Class-2 PEPC complex exhibited a broader pH activity profile, decreased allosteric sensitivity and greater thermostability than Class-1 PEPC [10]. Furthermore, Class-1 and Class-2 PEPC showed unique developmental profiles in developing COS, in which Class-2 PEPC was found to be more abundant in earlier stages, peaking at stage V (maximal oil synthesis) whereas Class-1 increased throughout development and peaked at stage VII-IX (mature cotyledon) [7,10]. In vitro techniques such as co-immunopurification (co-IP), non-denaturing PAGE of clarified extracts
coupled with in-gel PEPC activity staining, and parallel immunoblotting using BTPC- and PTPC-specific antibodies have further documented the presence of Class-1 and Class-2 PEPCs in developing COS [7,48,72].

Unfortunately, native BTPC subunits of green algal and vascular plant Class-2 PEPCs are highly susceptible to rapid in vitro proteolytic cleavage [7,11,12]. This occurs at a specific site located within their intrinsically disordered region by an endogenous thiol endopeptidase. Some protection from proteolysis can be achieved with the use of phenylmethylsulfonyl fluoride and the ProteCEASE 100 Cocktail marketed by G-Biosciences, making clarified COS extracts suitable for immunoblotting and co-IP of non-degraded BTPC [7,72]. This high susceptibility to proteolysis has been a major issue in studying native BTPC from developing COS endosperm. To overcome this obstacle, highly enriched native BTPC from developing COS endosperm was co-IP’d using an anti-(castor PTPC)-IgG immunoaffinity column [72]. A second approach was to purify recombinant COS BTPC (RcPPC4) from *Escherichia coli* cells in which it has been heterologously expressed. This approach was not ideal as purified RcPPC4 readily formed aggregates that tended to precipitate. However, by titrating purified RcPPC4 aggregate into recombinant *Arabidopsis* PTPC (AtPPC3), an active chimeric Class-2 PEPC was readily formed [73]. With this knowledge *E. coli* lysates containing wild-type versions of castor BTPC (RcPPC4) were mixed with lysates containing the *Arabidopsis* PTPC (AtPPC3) to create intact, stable chimeric Class-2 PEPCs in vitro, which were then purified and characterized [73]. This technique then allowed for analysis of: i) the kinetic properties of the BTPC subunits, ii) the regulatory effect of the BTPC subunits exert on PTPC subunits within a Class-2 PEPC, and iii) the effects of phosphomimetic mutations
on the kinetic and regulatory properties of BTPC subunits [71,73]. Several lines of evidence strongly suggest that BTPC does not exist on its own in vivo, but rather needs PTPC in order to form a stable Class-2 complex. For example (i) native BTPCs have only been seen in association with PTPC subunits in a Class-2 complex, (ii) BTPC subunits tightly associate with PTPC subunits during purification, (iii) heterologously expressed BTPC forms aggregates in E. coli lysates in the absence of PTPC subunits, and (iv) fluorescent protein-tagged COS BTPC subunits interact in vivo via its disordered region with COS PTPC during their transient co-expression in tobacco BY2 cells [6,7, 10-12, 17, 74].

_Tissue-specific expression of bacterial-type PEPC (BTPC) in castor plant_

Although most studies have focused on BTPC expression as a Class-2 PEPC subunit in the endosperm of developing COS, a recent study has expanded the search for BTPC to other tissues in the castor plant (*Ricinus communis*) [67]. The results of this study demonstrate that BTPC transcripts and polypeptides: (i) are abundant in inner integument, cotyledon and endosperm of developing COS but occur at low levels in roots and cotyledons of germinated COS, and (ii) show a unique developmental profile pattern in leaves such that they are present in leaf buds and young expanding leaves, but undetectable in fully expanded leaves [67]. Class-2 PEPC and its BTPC subunits therefore appear to be a characteristic feature of rapidly growing tissues that require a large anaplerotic flux from PEP to replenish TCA cycle intermediates consumed during biosynthesis.
Post-translational modification of BTPC in castor oil seed

In addition to COS BTPC acting as a catalytic and regulatory subunit in Class-2 PEPC, it is also subject to multisite in vivo phosphorylation (Fig. 1.5)[72]. Preliminary experiments with the phospho-protein stain Pro-Q Diamond revealed that co-IP’d BTPC from developing COS is highly phosphorylated in vivo [72]. In addition, P-i-affinity PAGE using Phos-Tag acrylamide in conjunction with λ-p’tase treatment demonstrated that COS BTPC is phosphorylated at multiple sites [72]. MS/MS was then used to determine one of the specific phosphorylation sites, Ser^{425} [71, 72]. In addition to this site a potential phosphorylation site (Ser^{879}) was proposed due to its presence in a precise recognition motif for by plant SNF-1 protein related kinase 1 (SnRK1) (Fig. 1.4) [72]. This motif requires hydrophobic residues at positions -5 and +4 and basic residues at – 3 and -6 and is conserved in vascular plant BTPCs.

The discovery of Class-2 PEPCs and their PTPC and BTPC subunits in developing COS have led to a better understanding of BTPCs biochemical and functional properties. Recent studies on the phosphorylation at Ser^{425} in developing COS have given some insight BTPC’s role in Class-2 PEPC. However, further studies are required to elicit more about its physiological functions [71].

Phospho-site specific antibodies were crucial for studying Ser^{425} phosphorylation of COS BTPC in vivo [71]. These antibodies were made by immunizing a rabbit with a short phosphopeptide corresponding to the sequence surrounding the phospho-site. A developing COS endosperm profile of BTPC’s pSer^{425} content demonstrated that phosphorylation increased from stage III – IX, coinciding with oil production. Removal of sucrose supply by depodding, induced a rapid increase in COS BTPC
Ser\textsuperscript{425} phosphorylation [71]. This differs from COS PTPC that demonstrated a rapid decrease in phosphorylation with removal of photosynthate, leading to the conclusion that PTPC phosphorylation at Ser\textsuperscript{11} and BTPC phosphorylation at Ser\textsuperscript{425} are controlled by different protein kinases and phosphatases. Lastly, BTPC’s pSer\textsuperscript{425} content appears to have a slight diel cycle where phosphorylation increases during the day compared to PTPC where Ser\textsuperscript{11} phosphorylation status does not change with regard to diurnal cycles [71].

Phosphomimetic mutants (Ser to Asp) were a second crucial tool used in studying the regulatory effects of phosphorylating COS BTPC at Ser\textsuperscript{425} [71]. It should be noted that although Class-2 PEPC was used for these studies, its PTPC subunits were inactive mutants (AtPPC3\_R644A) so only kinetics of BTPC would be observed. The introduction of an Asp at residue 425 increased COS BTPC $K_m$ (PEP) ~4 fold and lowered its $I_{50}$ (Asp and Mal) values by ~2.5 fold compared to wildtype dephosphorylated BTPC. These results indicated that COS BTPC is subject to regulatory inhibition when phosphorylated at Ser\textsuperscript{425} [71].

1.5 Thesis Objectives

Previous studies indicated that BTPC is subject to multi-site phosphorylation in developing COS [72]. However, only one site (Ser\textsuperscript{425}) has been mapped and studied in detail [71,72]. The objectives for my MSc thesis research were to: (i) map any additional in vivo phosphorylation sites of COS BTPC using MS, (ii) raise phospho-site specific antibodies to each site, (iii) use these antibodies to determine the influence of COS development and depodding on BTPC site-specific phosphorylation profiles, and (iv) deduce kinetic effect of each in vivo phosphorylation site using phospho-mimetic mutants.
of heterologously expressed COS BTPC. It is hypothesized that \textit{in vivo} multi-site phosphorylation of Class-2 PEPC’s BTPC subunits plays an important role in the control of C-partitioning to storage end-products in developing COS.
Figure 1.1: The phosphoenolpyruvate carboxylase reaction The conversion of PEP to oxaloacetate releasing $P_i$ requires the presence of $HCO_3^-$ and the metal cofactor $Mg^{2+}$. Glyceraldehyde-3-phosphate and glucose-6-phosphate act as allosteric activators whereas aspartate, glutamate, and malate act as allosteric inhibitors.
Figure 1.2 The diverse functions of plant PEPC. PEPC has a variety of functions including: CO$_2$ fixation in C$_4$ and CAM plants, C/N interactions, anaplerotic replenishment, N$_2$ fixation, carbon storage, energy production, abiotic stress acclimation, seed germination, seed development, and cell expansion (Taken from [17]).
Figure 1.3 Models illustrating several metabolic functions of plant PEPC. (A) Interactions between carbon and nitrogen metabolism involve three compartments in plant cells. This scheme highlights the important role of the two terminal enzymes of plant cytosolic glycolysis, PEPC and PK_c, in controlling the provision of the mitochondria with respiratory substrates, as well as for generating the 2-OG and OAA respectively required for NH_4^+-assimilation via GS/GOGAT in plastids and aspartate amino transferase in the cytosol. The co-ordinate control of PEPC and PK_c by allosteric effectors, particularly glutamate and aspartate, provides a mechanism for the regulation of cytosolic glycolytic flux and PEP partitioning during and following NH_4^+-assimilation. (B) This model illustrates the role of PEPC in controlling PEP partitioning to malate as a source of carbon skeletons and reducing power for leucoplast fatty acid synthesis. Abbreviations are as defined in the text, in addition to the following: AAT, Asp aminotransferase; E.T.C., electron transport chain; ME_m/PDH_m and ME_p/PDH_p, mitochondrial and plastidic isoenzymes of ME and PDH respectively (Taken from [17]).
Figure 1.4 Amino acid sequence alignment of *Arabidopsis* and castor oil plant PEPC isoenzymes. Identified *in vivo* phosphorylation sites of castor and *Arabidopsis* PTPCs (RcPPC3 and AtPPC1 respectively) and castor BTPC (RcPPC4), are highlighted in green, as well as the predicted BTPC (RcPPC4) Ser879 phosphorylation site. RcPPC3's conserved Lys628 monoubiquitination site is marked with a red font. BTPC's intrinsically disordered region is enclosed in a red rectangle. The *in vitro* proteolytic cleavage site (Lys 446) of RcPPC4 is marked with an X. Boxes I–III denote conserved subdomains essential for PEPC catalysis. The predicted pI, molecular mass and sequence identity (%) of the various PEPCs are shown. The deduced PEPC sequences were aligned using ClustalW software (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Colons and asterisks indicate conserved and identical amino acids, respectively (Adapted from [17]).
Figure 1.5 Model illustrating the biochemical complexity of castor bean PEPC

In developing COS endosperm the PTPC RcPPC3 (p107) exists as a Class-1 PEPC homotetramer (PEPC1) which is \textit{in vivo} phosphorylated at Ser\textsuperscript{11}. In addition it associates with BTPC RcPPC4 subunits (p118) to form the novel heteroctameric Class-2 PEPC. The BTPC subunit is also subject to multisite phosphorylation at Ser\textsuperscript{425} as well as other unidentified residues [72,73]. Finally, in germinating COS Class-1 PEPC (RcPPC3) is monoubiquitinated at Lys 628 (Adapted from [17]).
Chapter 2. *In vivo* phosphorylation of bacterial-type phosphoenolpyruvate carboxylase from developing castor oil seeds in at Thr$^4$ and Ser$^{451}$


**2.1 Abstract**

PEPC [PEP (phosphoenolpyruvate) carboxylase] is a tightly controlled anaplerotic enzyme situated at a pivotal branch point of plant carbohydrate-metabolism. Two distinct oligomeric PEPC classes were discovered in developing castor oil seeds (COS): Class-1 PEPC is a typical homotetramer of 107-kDa plant-type PEPC (PTPC) subunits, whereas the novel 910-kDa Class-2 PEPC hetero-octamer arises from a tight interaction between Class-1 PEPC and 118-kDa bacterial-type PEPC (BTPC) subunits. Mass spectrometric analysis of immunopurified COS BTPC indicated that it is subject to *in vivo* phosphorylation at Ser$^{425}$, Ser$^{451}$ and Thr$^4$. In addition a potential phosphorylation site (Ser$^{879}$) was predicted due to its presence in the known plant SNF1-related protein kinase 1 (SnRK1) phosphorylation motif. Here, immunoblots probed with phosphorylation site-specific antibodies demonstrated that Ser$^{451}$ phosphorylation is promoted during seed development, becoming maximal in stage VII. COS Ser$^{879}$ does not appear to be an *in vivo* phosphorylation site. Several synthetic pThr$^4$ containing phosphopeptides were non-immunogenic. Kinetic analyses of a heterologously expressed chimeric Class-2 PEPC containing phosphomimetic BTPC mutant subunits (S451D) indicated that Ser$^{451}$ phosphorylation caused significant BTPC inhibition by: (i) increasing its $K_m$(PEP) three-fold, (ii) reducing its $I_{50}$(L-malate and L-Asp) values by 2-fold, respectively, while (iii) decreasing its activity within the physiological pH range. By contrast recombinant Class-2 PEPC containing a Thr$^4$ phosphomimetic BTPC mutation did not show any difference...
in $K_m$(PEP), sensitivity to effectors or activity with varying pH compared to wild-type BTPC. A double phosphomimetic mutant of Ser$^{425}$ and Ser$^{451}$ (S425D+S451D) indicated that phosphorylation at both sites caused an intermediate level of BTPC inhibition. Collectively the results establish that BTPC’s pSer$^{451}$ content depends upon COS developmental and physiological status and that Ser$^{451}$ phosphorylation significantly attenuates the catalytic activity of BTPC subunits within a Class-2 PEPC complex. This study, along with a previous study on regulatory BTPC phosphorylation at Ser$^{425}$, provides further evidence of protein phosphorylation as a mechanism for the in vivo control of BTPC subunits of vascular plant Class-2 PEPC complexes.

2.2 Introduction

PEPC [PEP (phosphoenolpyruvate) carboxylase] (EC 4.1.1.31) is a tightly regulated cytosolic enzyme of vascular plants and green algae that catalyzes the irreversible β-carboxylation of PEP in the presence of HCO$_3^-$ to yield oxaloacetate and P$_i$. PEPC has been extensively studied with regards to its crucial role in catalyzing atmospheric CO$_2$ fixation in C$_4$ and CAM photosynthesis [1,2]. However, PEPC’s essential non-photosynthetic functions, particularly the anaplerotic replenishment of tricarboxylic-acid-cycle intermediates consumed during biosynthesis and N-assimilation, have received increased attention within the last decade [17].

Plant PEPCs belong to a small multigene family encoding several plant-type PEPC (PTPC) genes, along with at least one distantly related bacterial-type PEPC (BTPC) gene [3,5,7]. PTPC genes encode closely related 100-110-kDa polypeptides that: (i) contain a conserved N-terminal seryl-phosphorylation domain and critical C-terminal tetrapeptide QNTG, and (ii) typically exist in a homotetramer known as Class-1 PEPC
Plant BTPC genes encode 116-118-kDa polypeptides exhibiting low (<40%) sequence identity with PTPCs and that contain a prokaryotic-like (R/K)NTG C-terminal tetrapeptide [17]. BTPC genes and transcripts were discovered in rice and Arabidopsis in 2003 [3]. They have since been well documented in several vascular plants, but a clear expression pattern has not yet emerged [3,4,6,7,76-78]. Insights into the structure, function and location of BTPC polypeptides began earlier with their discovery in unicellular green algae [5,11,12,69,70,79], and have continued with their subsequent discovery in developing castor bean endosperm [10] and most recently in developing lily pollen [6]. Examination of PEPC from these algal and vascular plant sources revealed the presence of a high-$M_r$ Class-2 PEPC heteromeric complex composed of tightly associated PTPC and BTPC subunits that is remarkably desensitized to allosteric effectors relative to the corresponding Class-1 PEPCs [5,10-12]. Several lines of evidence strongly suggest that BTPCs only exist in vivo as part of a Class-2 PEPC complex [5,72,73].

Owing to its location at a pivotal branch point in primary carbon metabolism the activity of PEPC is tightly controlled in vivo. PTPCs have long been known to be controlled by a combination of allosteric effectors and reversible phosphorylation at their conserved N-terminal seryl residue catalyzed by a dedicated Ca$^{2+}$-independent PTPC protein kinase and PP2A (protein phosphatase type-2A) [1,2,56]. Phosphorylation at this site enhances allosteric activation by hexose-phosphates while reducing inhibition by L-malate and L-Asp. Manipulation of these control properties can cause drastic effects on carbon and nitrogen metabolism within the cell [17]. Recently, a number of other mechanisms for the post translational control of PEPC, including the formation of Class-
2 complexes, have emerged. Class-1 PEPC is also subject to inhibitory monoubiquitination at a conserved Lys residue during COS (castor oil seed; *Ricinus communis*) germination, causing an increased $K_m$(PEP) and enhanced sensitivity to allosteric effectors [63]. A chloroplast-targeted Class-1 PEPC isozyme exists in rice leaves, although the degree to which this phenomenon occurs in other plant species remains to be determined [80]. In developing castor endosperm BTPC and PTPC subunits associate into allosterically insensitive Class-2 PEPCs. Lastly, BTPC subunits from developing castor endosperm are phosphorylated at multiple sites *in vivo* [72]. Only one of these sites has been identified (Ser$_{425}$), and it appears to cause inhibition of the BTPC subunits by increasing their $K_m$(PEP) and sensitivity to feedback inhibition [73].

The aim of the current study was to continue to elucidate the control properties of castor endosperm BTPC by identifying and characterizing its remaining phosphorylation sites. High sensitivity fourier transform mass spectrometry (FT-MS) was used to identify Thr$_4$ and Ser$_{879}$ as additional phosphorylation sites. Phosphosite-specific antibodies were then raised against these sites to confirm their existence and monitor their *in vivo* phosphorylation status. Lastly, phosphomimetic mutants of the Thr$_4$ and Ser$_{451}$ phosphorylation sites were created to analyze the kinetic effects of phosphorylation at these sites. The combined results suggest that pSer$_{451}$ is an additional regulatory phosphorylation site of COS BTPC, whereas pThr$_4$ forms part of a phosphothreonine-dependent protein interaction domain known as a forkhead-associated (FHA) domain.
2.3 Materials and Methods

Plant material
Castor bean plants (*Ricinus communis*; cv. Baker 296) were cultivated in a greenhouse at 24 °C and 70% humidity under natural light supplemented with 16 h of artificial light. Pods containing COS at various developmental stages were harvested at midday unless otherwise indicated. For depodding experiments, stems containing intact pods of developing COS were excised and placed in water in the dark at 24 °C. Developing endosperm and cotyledon tissues were rapidly dissected, frozen in liquid N₂, and stored at -80°C.

Co-immunopurification and protein phosphatase treatments
Co-IP of BTPC polypeptides from developing COS extracts using an anti-(COS PTPC) immunoaffinity column was conducted as previously described [72]. Enrichment of BTPC polypeptides was achieved using 100 mM glycine pH 2.8 to elute bound BTPC from the co-IP column. Incubation of co-IP samples with exogenous λ-phosphatase (New England BioLabs) was as previously described [48,72].

LC MS/MS analysis and phosphopeptide identification
Proteins were reduced with 10 mM dithiothreitol (DTT), alkylated with 55 mM iodoacetamide and dialyzed against 10 mM ammonium bicarbonate. Following tryptic digestion, the protein digest was dissolved in 0.2% formic acid (FA) for LC MS/MS analysis on either the Nano-Acquity ultra-performance liquid chromatography system (UPLC, Waters, Milford, MA) coupled to a 7-tesla hybrid linear ion-trap Fourier transform ion cyclotron resonance mass spectrometer (LTQ-FT ICR, Thermo Fisher) or
the Agilent 1100 capillary HPLC system coupled to a QStar XL quadrupole time-of-flight instrument (QqTOF, AB Sciex). The peptides were trapped and subsequently separated by a C18 analytical column of 1.7 µm BEH130 (100 µm x 100 mm, UPLC, Waters) or 3 µm PepMap 100 (75 µm x 150 mm, HPLC, LC packings) through a 90 min gradient from 5% to 30% of solvent B (acetonitrile containing 0.1% FA), then 85% at a flow rate of 0.5 µl/min. Automated data dependent acquisition was employed to obtained MS and MS/MS measurements at a mass range of m/z 300-2000. Dynamic exclusion was enabled for a period of 180 s.

Phosphopeptide identification was performed using an in-house Mascot Server (version 2.3.0, Matrix Science), and the data were searched against the National Center for Biotechnology Information (NCBI) database for *viridiplantae* (green plants). The parameter settings allowed trypsin digestion for maximum 2 missed cleavage sites, and a fixed peptide modification of cysteine carbamidomethylation. Deamidation of asparagine and glutamine, methionine oxidation, phosphorylation of serine, threonine and tyrosine were considered as variable modifications. Mass tolerances were set up to 10 ppm for the FT MS ions and 1 Da for ion trap MS/MS fragment ions, 100ppm for the QqTOF MS and 0.1 Da for MS/MS measurements. Phosphorylation sites were validated by manual inspection of MS/MS spectra with predicted fragments.

*Site-directed mutagenesis and heterologous expression of recombinant PEPCs*

Full-length cDNAs for AtPPC3 (PTPC isozyme from *Arabidopsis thaliana*) and *RcPPC4* (BTPC isozyme from castor plant) were cloned into a pET28b His-tag vector (Novagen) and transformed into *E. coli* (BL21-CodonPlus (DE3)-RIL) (Stratagene) and heterologously expressed as previously described [71,73]. All AtPPC3 constructs used
encoded inactive (R644A) mutants as per [71]. The Quickchange II Site-Directed mutagenesis kit (Stratagene) was used to generate the desired mutant AtPPC3 construct (R644A) and RcPPC4 constructs (S451D, T4D and S451D+S425D) as per previously described [71]. Oligonucleotide primer pairs used to introduce the mutations were as follows, forward 5’-GAATCCAAGATAGGAAGATCTGATTTCCAGAAAACCTTCTA-3’ and reverse 5’-CTAGAAGTTTCTGGAAATCAGATCTTCTCTATCTTTGGATT-3’ and for T4D forward 5’-GCCATATGACGGACGACACAGATGATACGC-3’ and reverse 5’-GCGATATCATCTGTGTCGTCCGTCATATGGC-3’; underlined is an introduced EcoRV restriction site. The double mutant (S425D+S451D) was created by further mutagenesis of the S425D plasmid [71] with the S451D primers. The amplified vectors were transformed into E. coli using electroporation and positive clones selected on LB plates containing 50 µg/ml kanamycin were further screened for the desired mutation by restriction digestion and sequencing. The PEPC coding portion in each plasmid from the confirmed clones was sequence verified.

Purification of recombinant Class-2 PEPCs

All purifications were conducted as previously described [71,73]. Briefly, E. coli cells containing the heterologously expressed RcPPC4 or the inactive AtPPC3 were lysed by passage through a French press and then mixed together. The resulting Class-2 PEPCs were purified using PrepEase™ His-Tagged High Yield Purification Ni²⁺-affinity resin (USB Corp.) followed by Superdex-200 and Superpose-6 gel filtration FPLC (GE Biosciences). Pooled peak fractions were concentrated with an Amicon Ultra-15 centrifugal filter unit (100-kDa cut-off), frozen in liquid N₂, and stored at -80 °C. The
PEPC activity of the preparations were stable for at least 2 months when stored under these conditions.

**Preparation of phosphosite specific antibodies against pThr\textsuperscript{4}, pSer\textsuperscript{451}, and pSer\textsuperscript{879} of COS BTPC**

Synthetic phospho- and dephospho-peptides were made corresponding to the regions around the putative Thr\textsuperscript{4}, Ser\textsuperscript{451} and putative Ser\textsuperscript{879} phosphorylation sites of COS BTPC with an additional cysteine at the N- terminus (Ser\textsuperscript{451} and Ser\textsuperscript{879}) or C-terminus (Thr\textsuperscript{4}) by the Sheldon Biotechnology Centre, McGill University, Montreal QC, Canada (Fig. 2.3). Purified phosphopeptide (1 mg) was coupled to maleimide-activated keyhole limpet haemocyanin (Pierce Chemicals) according to the manufacturer’s protocols. C-terminus coupling with an additional cysteine was attempted for a pThr\textsuperscript{4} containing phosphopeptide but was non immunogenic so a second coupling strategy was attempted. A new pThr\textsuperscript{4} peptide containing a pair of lysine residues at its C-terminus (Fig. 2.3) was coupled to both bovine serum albumin (BSA)(Pierce) and keyhole limpet haemocyanin with 2.5% (v/v) glutaraldehyde. pThr\textsuperscript{4} peptide was dissolved at 10 mg/ml in PBS at pH 7.4. The dissolved peptide (250 µl) was then mixed with either 1000 µl of 10 mg/ml each of KLH and BSA and 25 µl of 2.5 % (v/v) glutaraldehyde. A 0.5 h time point was taken for both the KLH and BSA conjugates (400 µl) and mixed with 50 µl of 1 M Tris (pH 7.5) to quench excess glutaraldehyde. The remaining peptide-glutaraldehyde mixture was left at room temperature for several hours until the reaction turned yellow and then mixed with 100 µl of 1M Tris pH 7.5. All four mixtures (BSA coupled (0.5 h and end point) and KLH coupled (0.5 h and end point) were mixed together. For both coupling strategies the conjugates were desalted into phosphate buffered saline via Sephadex G-25 gel
filtration FPLC (GE Healthcare), filter sterilized, and emulsified with Titermax Gold adjuvant (CytRx Corp.). Following collection of pre-immune serum, 500 µg of phosphopeptide conjugate was injected subcutaneously into a 2-kg New Zealand rabbit. A 250 µg booster injection was administered 30 d later. Seven days after the final injection, blood was collected in Vacutainer tubes (Becton Dickinson) by cardiac puncture, and the immune serum frozen in liquid N₂ and stored at -80 °C. For immunoblotting, anti-pSer⁴⁵¹ and anti-pSer⁸⁷⁹ immunoglobulin G was affinity-purified as previously described [48] using 500 µg of the corresponding synthetic phosphopeptide bound to nitrocellulose.

**Electrophoresis and immunoblotting**

SDS- and P₁-affinity-PAGE using a Bio-Rad Protean III minigel system were conducted as previously described [10]. For immunoblotting, minigels were electroblotted onto poly(vinylidene difluoride (PVDF) membranes and probed using antibodies described in the relevant figure legends. Antigenic polypeptides were visualized using an alkaline phosphatase-conjugated secondary antibody and chromogenic detection [81]. Rabbit anti-BTPC [anti-(COS BTPC)-IgG] was raised against homogeneous recombinant RePPC4 as described previously [73]. All immunoblot results were replicated a minimum of three times with representative results shown in the various figures.

**Enzyme and protein assays and kinetic studies**

PEPC activity was assayed at 25 °C by following NADH oxidation at 340 nm using a kinetics microplate reader (Molecular Devices) and the following optimized 200-µl assay mixture: 50 mM Hepes/KOH (pH 8.0) containing 10% (v/v) glycerol, 10 mM PEP, 5 mM KHCO₃, 10 mM MgCl₂, 2 mM dithiothreitol, 0.15 mM NADH and 5 units•ml⁻¹ of porcine
muscle L-malate dehydrogenase (Roche). One unit of PEPC is defined as the amount of PEPC resulting in the production of 1 µmol of oxaloacetate•min⁻¹. PEP saturation kinetic data for Class-2 PEPCs were fitted to a single active site model using nonlinear regression analysis software and apparent $I_{50}$ values (inhibitor concentration producing 50% inhibition of PEPC activity) were calculated using a nonlinear least-square regression computer program [82]. All kinetic parameters represent means of at least four independent experiments and are reproducible to within ±15% S.E.M. of the mean value. Metabolite stock solutions were made equimolar with MgCl₂ and adjusted to pH 7.0. Protein concentrations were determined by the Coomassie Blue G-250 dye-binding using bovine $\gamma$-globulin as the protein standard [10].

Statistics

Data were analyzed using the Student’s $t$-test, and deemed significant if $p < 0.02$

2.4 Results and Discussion

**BTPC phosphosite mapping**

Previous work has conclusively shown that a 118-kDa BTPC subunit is phosphorylated at multiple sites during COS development [72]. Both LC MS/MS and immunoblotting using anti-pSer₄²⁵ specific antibodies identified Ser₄²⁵ as an *in vivo* COS BTPC phosphorylation site [71,72]. Here, increased resolution and sensitivity afforded by the FT-ICR and QqTOF MS analysis of co-IP’d COS BTPC (93.4% sequence coverage) has independently verified the Ser₄²⁵ phosphorylation site while identifying two additional sites, corresponding to Thr⁴ and Ser⁴⁵¹ (Fig. 2.1). Two abundant phosphopeptides were identified by FT-ICR-MS in which the intensity of peptide
corresponding to residues 447-454 at m/z 501.7430 (RT 14.61 min) was approximately three fold higher than that of the peptide corresponding to residues 413-427 at m/z 736.3142 (RT 11.41 min) (Fig. 2.1A). Fragmentation of the doubly charged ion of m/z 501.74 displayed an apparent loss of phosphoric acid (-49 m/z) resulting in a major fragment ion at 452.99. Analysis of the N-terminal b_n and C-terminal y_n ion series presented a 69 Da mass difference between two high-intensity fragments of y_3 and y_4-18. This corresponds to an abnormal dehydroalanine residue at Ser^{451} which results from the loss of a P_i group from serine (Fig. 2.1B). The MS/MS spectrum of the precursor ion at m/z 736.31 unambiguously identified this phosphorylation site as the previously established pSer^{425} (Fig. 2.1C) [71,73]. Similar LC MS/MS was performed by QqTOF mass spectrometry, and identified a large phosphorylated peptide corresponding to residues 2-34 at m/z 970.95 (RT 30.11 min, Fig. 2.1D). Manual inspection of the data set indicated this low-abundance peptide had a charge state of 4+ (Fig. 2.1E). The collision induced dissociation of this quadruple charged ion generated a set of C-terminal y_n fragments (n=1-12) in the MS/MS spectrum and identified a spacing of 83 Da at b_2 indicating an abnormal dehydroaminobutyric acid residue caused by dephosphorylation of Thr^4 (Fig. 2.1F).

Thr^4 appears to exist in an FHA binding domain and also correspond to an acidic protein kinase recognition motif (Fig. 2.2). FHA domains are present in a wide variety of proteins and have gained considerable prominence as phospho-Thr dependent binding modules. Two prominent FHA binding motifs described so far are pTXXD and pTXXI/L/V [83]. The former motif occurs at the N-terminus of BTPC. By contrast, Ser^{451} corresponds to a basophilic kinase motif having an arginine residue located at the -2
position (Fig. 2.2). Most basophilic kinases prefer substrates with basic residues in close proximity to the phosphorylated residue commonly at either the -2 or -3 position [84]. The location of Ser\textsuperscript{451} is also of interest because secondary structure analysis using the Phyre server [85] predicted that this region of COS BTPC exists in a largely coiled, unstructured, and highly flexible conformation known as an intrinsically disordered region [71]. Disordered region containing proteins are ubiquitous to all organisms. Disordered regions typically exist as a flexible linker that freely twist and rotate through space to mediate protein:protein interactions [86]. PTMs such as proteolysis and phosphorylation at residues within disordered regions are common due to their high surface accessibility [86]. In this case, the COS BTPC disordered region also contains the Ser\textsuperscript{425} phosphorylation site, and the known BTPC proteolysis site at Lys\textsuperscript{446} [7,71]. Both Thr\textsuperscript{4} and Ser\textsuperscript{451} are conserved in almost all other orthologous plant BTPC protein sequences deduced to date. The apparent exceptions are a substitution and deletion of Thr\textsuperscript{4} in *Glycine max* and *Mimulus guttatus* respectively, although these could represent errors in sequencing (Fig. 2.2).

*Phospho-site specific antibodies confirm the phosphorylation of Ser\textsuperscript{451} in vivo*

Phospho-site specific antibodies have proven to be essential tools for confirming and studying the specific *in vivo* phosphorylation of proteins, especially those containing multiple phosphorylation sites [71]. To further analyze the phosphorylation status of COS BTPC, a series of phosphosite-specific antibodies were made corresponding to identified and putative BTPC phosphorylation sites. Antibodies were successfully raised against synthetic phosphopeptides corresponding to the sequences around pSer\textsuperscript{451} and putatively phosphorylated Ser\textsuperscript{879} (Fig. 2.3). The Ser\textsuperscript{879} site was included as it was
previously suggested to be a potential phosphorylation site due to its presence in a well characterized plant SNF1-related protein kinase 1 (SnRK1) phosphorylation motif that appears to be conserved in other plant BTPC orthologs [72]. Each of the phospho-site specific antibodies was specific for the phosphorylated version of the corresponding synthetic peptide. The cross-reaction between the antibodies and the phosphopeptides was abolished when the antibody was pre-incubated with corresponding blocking phosphopeptide, whereas the addition of corresponding non-phosphorylated blocking peptide did not quench the cross-reaction (Fig. 2.3). The use of these blocking peptides alongside the inclusion of non-phosphorylated BTPC control lanes served to verify the specificity of these phosphosite-specific antibodies in subsequent immunoblots. Unfortunately, several different peptides corresponding to the Thr⁴ phosphorylation site were non-immunogenic as they failed to cross react with the corresponding phosphopeptide (Fig. 2.3A and 2.3B).

To verify and/or establish the presence of *in vivo* phosphorylation sites, co-IP’d native BTPC from stage VII developing COS endosperm was probed with each of the phosphosite-specific antibodies. Immunoblots with anti-pSer⁴⁵¹ specifically detected the 118 kDa BTPC polypeptide (p118) and this signal was eliminated by λ-phosphatase pre-treatment of the sample or by incubation with blocking phosphopeptide (Fig. 2.4A). This result independently confirms the FT-ICR MS data that COS BTPC is phosphorylated *in vivo* at Ser⁴⁵¹. In contrast, anti-pSer⁸⁷⁹ failed to detect corresponding BTPC polypeptides on SDS-PAGE immunoblots. Although, this does not rule out the possibility that this site may be phosphorylated in other tissues and/or stress conditions, it indicates it is not *in vivo* phosphorylated in the BTPC of developing COS endosperm. FT-ICR MS data
indicates that Thr\(^4\) of COS BTPC is an *in vivo* N-terminal phosphorylation site. This is consistent with the phosphorylation prediction software NetPhos (http://www.cbs.dtu.dk/services/NetPhos/) which predicts Thr\(^4\) to have a high probability of phosphorylation (0.833) (eg. relative to Thr\(^5\) (0.244)) (Appendix A).

*Monitoring Ser\(^{451}\) phosphorylation status throughout COS development and following COS depodding*

Anti-pSer\(^{451}\) immunoblots were used to monitor changes in Ser\(^{451}\) phosphorylation status of BTPC during COS development and in response to the removal of photosynthate supply. These results are interpreted in comparison with those previously obtained using phosphosite-specific antibodies towards the single regulatory N-terminal Ser\(^{11}\) phosphorylation site of the PTPC subunit of Class-1 PEPC from developing COS [39,48] as well as the established Ser\(^{425}\) phosphorylation site of COS BTPC [71]. An affinity-purified antibody raised against recombinant COS BTPC (anti-BTPC) detects BTPC polypeptides independent of their phosphorylation status and allowed for normalization of total BTPC on immunoblots [71,73]. Parallel anti-pSer\(^{451}\) and anti-BTPC immunoblots of co-IP’d samples from four stages of COS endosperm revealed a distinct developmental pattern in which phosphorylation at Ser\(^{451}\) decreased from stage III-V, increased again at stage VII, and then rapidly decreased by stage IX (Fig. 2.4B and Appendix B). In COS endosperm, developmental stage III marks rapid cell expansion, stage V–VII represent the major phases of oil and protein accumulation, and at stage IX the seed is almost mature, has lost vascular connection with the parent plant, and has begun to desiccate [25,87,88]. The developmental profile of PTPC Ser\(^{11}\) phosphorylation was somewhat similar to that of BTPC Ser\(^{451}\), with phosphorylation
peaking at stage VII, the maximal stage of oil production, and declining by stage IX [39,48], the decrease in phosphorylation at Ser\(^{451}\) from stage III-V, however, is not seen in PTPC Ser\(^{11}\) phosphorylation. By contrast, the Ser\(^{425}\) phosphorylation status of BTPC continuously increased throughout development becoming maximal in stage IX developing COS [71]. A depodding treatment, which eliminates photosynthate import, had no apparent effect on Ser\(^{451}\) phosphorylation after 4 days (Fig. 2.4C). This contrasts with Ser\(^{425}\) phosphorylation of COS BTPC which 4 days after depodding displayed a significant increase [71]. In contrast, the regulatory phosphorylation at Ser\(^{11}\) is dependent upon the presence of imported photosynthate such that depodding or prolonged darkness caused the complete dephosphorylation and subsequent monoubiquitination of Class-1 PTPC after 48 h (concomitant with disappearance of PPCK activity) [39,48]. Therefore, like Ser\(^{425}\), phosphorylation of BTPC at Ser\(^{451}\) appears to be regulated independently of corresponding COS PTPC. However, differences in their developmental profile and also in the nature of their phosphorylation site motifs suggest that in vivo Ser\(^{451}\) and Ser\(^{425}\) phosphorylation status of BTPC are likely controlled by different kinases/phosphatases.

**Characterization of phosphomimetic mutants suggests that Ser\(^{451}\) phosphorylation inhibits BTPC within a Class-2 PEPC complex**

Numerous lines of evidence indicate that green algal and vascular plant BTPC subunits only exist in vivo as part of the heteromeric Class-2 PEPC complex [12,58,67,74]. For example heterologously expressed COS BTPC aggregate and precipitate upon purification on their own but spontaneously rearrange to form stable Class-2 PEPC complexes when mixed with a Class-1 PEPC [73]. Within Class-2 PEPC, BTPC functions as both a catalytic and regulatory subunit [71,73]. Kinetic analysis of Class-2
PEPC using catalytically inactive mutant subunits showed that the PTPC subunits constitute allosterically sensitive, low \( V_{\text{max}} \) and low \( K_m(\text{PEP}) \) catalytic sites, whereas the BTPC subunits constitute allosterically insensitive, high \( V_{\text{max}} \) and high \( K_m(\text{PEP}) \) catalytic sites [71,73]. A phosphomimetic mutant of COS BTPC (RcPPC4_S425D) was mixed with a catalytically inactive Arabidopsis PTPC subunit (AtPPC3_R644A) and kinetics of the resultant Class-2 PEPC were compared with kinetics of a parallel recombinant Class-2 PEPC containing wild-type COSBTPC [71]. This strategy avoided the complicating effect of having simultaneously active PTPC and BTPC subunits and was clearly able to show that the S425D BTPC mutation was inhibitory, causing an increase in both its \( K_m(\text{PEP}) \) and sensitivity to feedback inhibition by malate and Asp [71]. The same strategy was used here to determine the effect of phosphomimetic mutations at both Thr\(^{4}\) and Ser\(^{451}\). Site-directed mutagenesis was performed to create the mutant subunits RcPPC4_T4D and RcPPC4_S541D. A third double BTPC mutant was also created which contained both the S425D and S451D mutations. E. coli lysates containing the mutant BTPCs were combined with the inactive PTPC subunit AtPPC3_R644A to form mutant Class-2 PEPCs. Each lysates containing Class-2 PEPC was then purified to homogeneity by nickel affinity and gel filtration FPLC as previously described (Fig. 2.5 and Appendix A) [71,73]. The mutations did not appear to affect binding between the PTPC and BTPC subunits as the complexes all eluted off the final gel filtration column as stable hetero-octamers of ~900 kDa.

Kinetic properties of the three phosphomimetic mutants were compared to the wild type BTPC and previously described RcPPC4_S425D BTPC subunits [71]. The PEP substrate saturation kinetics at both optimal and suboptimal pH, (8 and 7.3 respectively),
are shown in Table 2.1. The S451D mutation caused an approximately 2.5-3 fold increase in the $K_m(PEP)$ compared to wild type at both pH levels. This is very similar to the results obtained with the S425D mutation [71]. The double S425D+S451D mutant appeared to be less inhibited than either of the single mutations. By contrast, T4D mutation did not affect BTPC’s $K_m(PEP)$ value, but elicited a relatively minor 1.4-fold increase in this parameter at pH 7.3. It does not appear that any of the mutations had a significant influences on the enzyme’s $V_{max}$. The sensitivity to allosteric effectors was also analyzed (Fig. 2.6). Each mutant was tested against various PEPC allosteric effectors (2 mM each) at pH 7.0 and subsaturating (1 mM) PEP. The effectors included the PTPC inhibitors glutamate, malate, aspartate and the PTPC activators glucose-1-phosphate, fructose-6-phosphate, glucose-6-phosphate and glycerol-3-phosphate. Malate and aspartate were the only two metabolites that appeared to have a significant effect on the activity of the BTPC subunits (Fig. 2.6). The S451D mutation caused an approximately 2-fold decrease in the $I_{50}$ (malate) and $I_{50}$ (Asp) values under these conditions. This result was similar to the effect of the S425D mutation [71], whereas the double S425D+S451D mutant showed a less pronounced increase in sensitivity to feedback inhibition than either single mutation. By contrast the T4D mutation had no significant effect on the sensitivity to feedback inhibition by Asp or malate.

The effect of pH on the activity of the S451D and T4D BTPC mutants was determined at saturating (15 mM) and subsaturating PEP (2 mM). No differences in pH dependence of the two phosphomimetic mutants were noted at either PEP concentration (Fig. 2.7). Altogether, the S451D mutation appears to be inhibitory in nature, causing an increase in the enzyme’s $K_m(PEP)$ and sensitivity to feedback inhibition by malate and
Asp. This provides strong evidence that the introduction of the negative charges associated with a phosphate group to the Ser$^{451}$ residue (i.e. *in vivo* phosphorylation) would have a similar inhibitory impact. Ultimately, this conclusion needs to be experimentally verified by analysis of the addition or removal of a phosphate group from Ser$^{451}$ of COS BTPC. As native COS BTPC cannot be purified intact due to proteolysis [7], work is underway to identify either an endogenous or exogenous kinase that is capable of site-specific phosphorylation at this residue.

Remarkably, the inhibitory effects of the S451D mutation appear to be quite similar to the previously described S425D mutation [71]. The fact that there are two regulatory phosphorylation sites within close proximity clearly implicates the intrinsically disordered region as a site of regulatory control for BTPC. An unexpected finding was that the BTPC double mutant S425D+S451D was less inhibited than either or the single mutants. However, as no structural data is available for BTPC's disordered region, it is difficult to predict what interaction between these two residues may occur to possibly account for this attenuation of inhibition. Based on earlier findings using P$_i$-affinity PAGE [72], it is clear that BTPC from developing COS endosperm exists in a high state of multi-site phosphorylation. Combined with their developmental patterns and response to photosynthate limitation, it appears that there would be considerable overlap between the occurrences of the Ser$^{425}$ and Ser$^{451}$ phosphorylation sites. Clearly, more research is needed to provide a rational for multisite phosphorylation in this region as a new mechanism of PEPC enzyme control. In this regard, it will be of great interest to identify the corresponding kinases and phosphatases that control phosphorylation at these sites as well as to determine the nature of the upstream signaling pathway.
2.5 Conclusion

Within the developing COS endosperm there exist two distinct subunits and isoforms of PEPC. Class-1 PEPC is controlled by phosphorylation of its PTPC subunits at Ser\textsuperscript{11} to be in tune with the presence of imported photosynthate from the leaves and the developmental stage of the seed. This strongly suggests a role for Class-1 PEPC in regulating carbon metabolism in support of the accumulation of storage compounds in the developing endosperm. The Class-2 PEPC has kinetic properties that would allow it to function as a metabolic overflow mechanism, sustaining flux from PEP to malate when PEP levels were high but Class-1 PEPCs may be largely inactivated by feedback inhibition. In this capacity, BTPC and Class-2 PEPC may allow an increased rate of biosynthesis in the developing seeds. However, verification of this hypothesis requires: (i) further examples of the existence of Class-2 PEPC in similar metabolic environments outside the developing COS endosperm and (ii) the elucidation of mechanisms of control for BTPC that are in line with this proposed physiological role. The current study furthers previous research which proposed multisite phosphorylation as a post-translational mechanism for the control of COS BTPC \textit{in vivo} [71,72]. Both Ser\textsuperscript{425} and Ser\textsuperscript{451} have been shown to be \textit{in vivo} phosphorylation sites whose corresponding phosphomimetic mutations cause inhibition of the BTPC subunits. It is not yet clear how phosphorylation at these sites contributes to the regulation of carbon metabolism \textit{in vivo}, and the discovery of environmental stimuli or cellular conditions that modulate phosphorylation at these sites would thus be a major advancement. Nevertheless, the additional post-translational control mechanisms would allow endosperm PEPC activity to respond more dynamically and rapidly to meet the needs of the cell. Conversely, pThr\textsuperscript{4} does not appear
to play a regulatory role in COS BTPC as suggested by kinetic analysis of the T4D phosphomimetic mutant. Thr$^4$ does, however, exist in a FHA binding motif pTXXD known to mediate protein-protein interactions through pThr. Additional work is needed to confirm the role of pThr$^4$ in BTPC and its potential role mediating protein-protein interactions in developing COS.
Figure 2.1: **COS BTPC MS/MS analysis and phosphorylation site mapping**

Ultra Performance Liquid Chromatography (UPLC) LTQ-FT MS/MS identification of the *in vivo* phosphorylation of bacterial-type phosphoenolpyruvate carboxylase. (A) The extracted ion chromatograms (XICs) of the phosphopeptide ions at m/z 736.3142 and m/z 501.7430. (B) MS/MS spectrum of the doubly-charged ion at m/z 736.31. (C) MS/MS spectrum of the doubly-charged ion at m/z 501.74. RT refers to LC retention time. HPLC QqTOF identified a large phosphorylated peptide at site Thr^4. (D) The extracted chromatogram of the phosphopeptide ion at m/z 970.95 indicating position of the Pi groups. (E) MS spectrum of the ion shows a charge state of 4+. (F) MS/MS spectrum of the quadruply-charged ion at m/z 970.95. RT refers to LC retention time.
Figure 2.2: Partial alignment of COS BTPC Thr\(^4\) and Ser\(^{451}\) domains with other vascular plant BTPCs. Available full length plant BTPC amino acid sequences were aligned using the TCooffee program (available online at http://www.tcoffee.org/). Ser\(^{425}\), Ser\(^{451}\) and Thr\(^4\) are indicated by the arrows. \(*\) : and . indicate decreasing degrees of conservation. The protein sequences were identified using NCBI’s BLAST program and the NCBI (http://www.ncbi.nlm.nih.gov/) or Phytozome (www.phytozome.org) databases.
Figure 2.3: **Specificity of phosphosite-specific antibodies.** (A-C) The synthetic peptide sequences used in the creation phosphosite-specific antibodies are shown with the corresponding COS BTPC residue numbers. Each peptide contains an additional N- or C-terminal lysine or cysteine residue utilized for coupling to the Keyhole limpet hemocyanin (KLH) and BSA carrier proteins. Phosphorylated and dephosphorylated peptides were probed the corresponding phosphosite-specific antibodies pre-incubated with 10 μg/ml of the dephosphopeptide, phosphopeptide or no peptide.
Figure 2.4: \textit{In vivo} phosphorylation status of Ser$^{451}$ in developing COS endosperm

\textbf{(A)} Co-immunopurified BTPC from stage VII developing endosperm was incubated with (+) and without (-) λ-phosphatase (λ-P’tase). Samples were subjected to SDS-PAGE followed by immunoblotting with anti-BTPC or anti-pSer$^{451}$ in the presence of 10 µg/mL of dephosphopeptide or corresponding phosphopeptide (B). Co-IP COS extracts were loaded based on equal BTPC content, subjected to SDS-PAGE, then immunoblotted with anti-pSer$^{451}$ and anti-BTPC. The negative control (deP BTPC) is purified recombinant (dephosphorylated) castor-BTPC expressed in \textit{E. coli}. Biological replicates are shown in Appendix B. \textbf{(C)} Co-IP’d extracts from Stage VII developing endosperm harvested at mid-day was used as a standard and compared to stage VII endosperm from intact developing COS that had been depodded for four days.
Figure 2.5: **Purification of recombinant Class-2 PEPC mutants** Final preparations of three Class-2 PEPC mutants were subjected to SDS/PAGE followed by Coomassie Brilliant Blue 250 (CBB-R250) staining and immunoblotting with anti-BTPC or anti-PTPC. The lanes were loaded in the following order (PTPC/BTPC): lane 1, AtPPC3_R644A/RcPPC4_T4D; lane 2, AtPPC3_R644A/RcPPC4_S451D; lane 3, AtPPC3_R644A/RcPPC4_S425D-S451D.
Figure 2.6: Sensitivity of recombinant Class-2 PEPC mutants to effectors. (A and B) PEPC activity was determined at pH 7.0 with subsaturating PEP (2 mM) in the presence of increasing concentrations of L-malate and L-aspartate. Shown are the \( I_{50} \) (malate) (A) or \( I_{50} \) (aspartate) (B) values for several subunit combinations of Class-2 PEPC. The PTPC subunit was an inactive mutant (AtPPC3_R644A). The BTPC subunit was either wild-type (wt)(RcPPC4), or active (RcPPC4_T4D, RcPPC4_S451D, RcPPC4_S425D (data for R644A/S425D are from [71]) and RcPPC4_S425D+S451D) site-directed mutants. All values represent the means ± S.E.M. of at least four separate determinations. Asterisks denote values are significantly different from R644A/wt (p < 0.02).
Figure 2.7: **pH activity profile of recombinant Class-2 PEPC mutants.** PEPC activity for R644A / wt, R644A / T4D and R644A / S451D was determined with (A) saturating PEP (15 mM) or (B) subsaturating PEP (2 mM) at various pH values using a mixture of 50 mM bis-Tris-propane and 50 mM MES as the buffer. All values represent the mean of at least three separate determinations and are reproducible to within ± 10% S.E.M. of the mean value.
Table 2.1: **PEP saturation kinetics of wild-type and phosphomimetic mutants of heterologously expressed Class-2 PEPC**

All values represent the mean of four separate determinations and are reproducible to within ±15% S.E.M. of the mean value.

<table>
<thead>
<tr>
<th>Class-2 PEPC Isoform</th>
<th>pH 7.3</th>
<th></th>
<th>pH 8.0</th>
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<tr>
<td></td>
<td>$V_{\text{max}}$</td>
<td>$K_m$(PEP)</td>
<td>$V_{\text{max}}$</td>
<td>$K_m$(PEP)</td>
</tr>
<tr>
<td>AtPPC3 / RcPPC4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R644A / wild type</td>
<td>9.4</td>
<td>0.76</td>
<td>11</td>
<td>0.74</td>
</tr>
<tr>
<td>R644A / S451D</td>
<td>12.5</td>
<td>2.4</td>
<td>11.7</td>
<td>2.1</td>
</tr>
<tr>
<td>R644A / T4D</td>
<td>11.7</td>
<td>1.1</td>
<td>13.8</td>
<td>0.76</td>
</tr>
<tr>
<td>R644A / S451D-S425D</td>
<td>10.0</td>
<td>1.6</td>
<td>11.2</td>
<td>0.95</td>
</tr>
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</table>
Chapter 3. General Discussion

Work presented in this thesis has furthered our understanding of multisite \textit{in vivo} phosphorylation of Class-2 PEPC’s BTPC subunits in developing COS. Initial purification of two distinct classes of PEPC from developing COS not only determined the presence of the Class-2 PEPC, but led to kinetic evaluation deeming the novel Class-2 PEPC heteromeric complex to have a lower affinity for PEP and be less sensitive to allosteric effectors [10]. Subsequent studies on BTPC phospho-sites provided a solid foundation for characterizing the remaining PTMs of COS BTPC.

With the ability to employ protease inhibitors to largely suppress the proteolytic problems first faced when purifying native COS BTPC, co-IP phosphoproteomic studies determined that BTPC was phosphorylated at multiple sites \textit{in vivo} [72]. This represents the first reported case of vascular plant BTPC phosphorylation. LTQ-FT MS/MS of co-IP’d COS BTPC was then used to identify its specific phospho-sites to Thr\textsuperscript{4}, Ser\textsuperscript{425} and Ser\textsuperscript{451}. Work by Brendan O’Leary on Ser\textsuperscript{425} provided a useful foundation for studying BTPC phospho-sites [71]. His kinetic studies of S425D phosphomimetic mutants indicated that was found that Ser\textsuperscript{425} acted as an inhibitory regulator of BTPC catalytic activity when phosphorylated.

This thesis characterized the BTPC phospho-sites Ser\textsuperscript{451} and Thr\textsuperscript{4}. Ser\textsuperscript{879} was ruled out as \textit{in vivo} phosphorylation sites due to (i) the inability of anti-pSer\textsuperscript{879} to cross-react with native co’IP’d BTPC, despite being specific for its phospho-peptide, and (ii) failure of FT-ICR MS to identify Ser\textsuperscript{879} as a phospho-site. Ser\textsuperscript{451} was confirmed as an \textit{in vivo} phospho-site and was found to be phosphorylated in developing COS endosperm in a pattern quite differently than the Ser\textsuperscript{11} of COS PTPC and Ser\textsuperscript{425} of BTPC. Ser\textsuperscript{451}
showed a marked decrease in phosphorylation at stage V followed by an increase at stage VII and another decrease at stage IX (Fig. 2.4B). This pattern along with the fact that depodding does not change phosphorylation status suggests Ser\textsuperscript{451} is controlled by a mechanism yet again different from PTPC and now Ser\textsuperscript{425} in BTPC. In addition, phosphomimetic S451D, demonstrated similar kinetic characteristics to S425D, indicating that Ser\textsuperscript{451} phosphorylation functions as a regulatory inhibitor in BTPC. Interestingly, when a double mutant (S425D+S451D) was created sensitivity to allosteric inhibitors and affinity for PEP was intermediate to the wild type and the single mutants. This may be due to some interaction between the two phospho-sites and/or the fact that Asp is not identical to phospho-Ser but further studies are needed to understand this effect.

Unfortunately, attempts with several synthetic Thr\textsuperscript{4} phosphopeptide did not produce any antibodies. However, a phosphomimetic T4D mutant demonstrated that this phosphorylation site is not regulatory. Interestingly, pThr\textsuperscript{4} exists in a FHA binding motif. The sequence surrounding Thr\textsuperscript{4} coincides with the one of the two prominent FHA binding motifs described thus far, pTXXD [83]. This indicates that phosphorylation at this site may play a role in mediating some sort of protein:protein interaction. FHA binding domains are typically found in signal transduction pathways and have been recently found in plants in the KAPP and DDL proteins, involved in receptor kinases and miRNA/siRNA biogenesis, respectively [89,90]. If BTPC pThr\textsuperscript{4} mediates \textit{in vivo} binding of BTPC to an FHA domain containing protein it would be the first record of FHA binding proteins involved in a plant metabolic pathway.
With more known about this novel Class-2 PEPC questions arise regarding its function in COS endosperm. Why does COS endosperm contain two distinct oligomeric isoforms of PEPC? Evaluation of the kinetic properties of recombinant Class-2 PEPC show that the catalytic contribution of its BTPC subunits allows the complex to remain active in the presence of high levels of malate. The estimated malate concentration in developing COS is ~5 mM [24], at this concentration the PTPC containing Class-1 PEPC (whose \( I_{50} \) malate is ~0.15 mM) would be completely inhibited. By contrast, BTPC has an \( I_{50} \) malate of ~8 mM (Fig. 2.6). BTPC may therefore be acting in response to cytosolic malate concentrations. However, BTPC does have a high \( K_m(PEP) \) of around 0.7 mM, so may require relatively high concentrations of PEP to become noticeably active. Class-2 PEPC is then potentially acting as a metabolic overflow mechanism to allow continued flux from PEP to organic acids during periods of rapid biosynthesis when elevated malate concentrations may inhibit Class-1 PEPC. The benefit of having such a mechanism to keep malate concentrations high in the cell may be to increase sink strength and support higher rates of fatty acid and amino acid biosynthesis in developing COS.

Understanding these pathways and more specifically Class-1 and -2 PEPCs may lead to potential sites of control of carbon partitioning in developing seed. This could lead to rational metabolic engineering efforts aimed at increasing crop yield. It may be a promising area to engineer as BTPC is expressed in tissues of high biosynthetic activity such as the developing seed, which could lead to potentially increasing sink storage. It has been previously shown that modifications to PEPC that reduce feedback inhibition are more effective than simple overexpression [18,44,91,92]. BTPC is already
desensitized to feedback inhibition, making it an attractive candidate for PEPC metabolic engineering in vascular plants.

Another promising area would be to further address the PTMs of BTPC, by creating Ser to Ala mutants that cannot be phosphorylated at the regulatory sites (Ser$^{425}$ and Ser$^{451}$). This may provide more information as to how the two phosphorylation sites are interacting with each other and could lead to furthering the information presented with the double phosphomimetic mutant (S425D+S451D). Finally, searching for an FHA-domain containing binding partner of the pThr$^{4}$ site would give insight into a possible BTPC protein interactome. This could be approached by eluting clarified COS extracts through a pThr$^{4}$ peptide affinity column to search for endogenous protein interactors. Parallel elution through a control dephosphorylated Thr$^{4}$ peptide column would be necessary to confirm pThr$^{4}$ dependence of any putative interactors. In addition, despite the fact that BTPC’s Ser$^{879}$ is not phosphorylated in developing COS endosperm future work needs to consider its possible phosphorylation in BTPC of other castor tissues or plant species.

Finally, with three phosphorylation sites identified in COS BTPC it will be crucial to investigate the kinases and phosphatases (and related signaling pathways) responsible for regulating site-specific BTPC phosphorylation. Each BTPC phosphorylation site also appears to exist in very different kinase recognition motifs (acidic (Thr$^{4}$), proline directed (Ser$^{425}$) and basophilic (Ser$^{451}$)) suggesting that each site may in fact be regulated by different kinases and phosphatases.
References


mass isoforms of this enzyme in the unicellular green alga *Selenastrum minutum*. J. Biol. Chem. 276, 12588-12601


69 Rivoal, J., Dunford, R., Plaxton, W. C. and Turpin, D. H. (1996) Purification and properties of four phosphoenolpyruvate carboxylase isoforms from the green alga Selenastrum minutum: Evidence that association of the 102-kDa catalytic subunit with unrelated polypeptides may modify the physical and kinetic properties of the enzyme. Arch. Biochem. Biophys. 332, 47-57


Appendix A

NetPhos phospho-site prediction score and phosphomimetic mutant purifications

Table A1  NetPhos phospho-site prediction scores for Thr$^4$, Thr$^5$, Ser$^{451}$, Ser$^{425}$ and Ser$^{879}$ of COS BTPC

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<th>Position</th>
<th>Context</th>
<th>Prediction Score</th>
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<tr>
<td>Thr$^4$</td>
<td>-MTD(pT)TDDI</td>
<td>0.833</td>
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<tr>
<td>Thr$^5$</td>
<td>MTD(pT)DDIA</td>
<td>0.244</td>
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<tr>
<td>Ser$^{425}$</td>
<td>NSSG(pS)PRAS</td>
<td>0.996</td>
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<td>KIGR(pS)SFQK</td>
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<td>Ser$^{451}$</td>
<td>IGRS(pS)FQKL</td>
<td>0.834</td>
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<tr>
<td>Ser$^{879}$</td>
<td>TRRKS(pS)TGI</td>
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Table A2  Purification of recombinant Class-2 PEPC from combined extracts originating from 5 g of AtPPC3_R644A- and 10 g of RePPC4_T4D-expressing E. coli

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<th>Protein (mg)</th>
<th>Specific Activity (units/mg of protein)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
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61
Table A3  Purification of recombinant Class-2 PEPC from combined extracts originating from 9 g of AtPPC3_R644A- and 12 g of RcPPC4_S451D-expressing *E. coli*

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<th>Specific Activity (units/mg of protein)</th>
<th>Purification (fold)</th>
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<tr>
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</tbody>
</table>

Table A4  Purification of recombinant Class-2 PEPC from combined extracts originating from 3 g of AtPPC3_R644A- and 10 g of RcPPC4_S425D+S451D-expressing *E. coli*

<table>
<thead>
<tr>
<th>Step</th>
<th>Activity (units)</th>
<th>Protein (mg)</th>
<th>Specific Activity (units/mg of protein)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combined extracts</td>
<td>126</td>
<td>1375</td>
<td>0.09</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ni$^{2+}$-affinity FPLC</td>
<td>115</td>
<td>28.6</td>
<td>4</td>
<td>44</td>
<td>91</td>
</tr>
<tr>
<td>Superdex-200 FPLC</td>
<td>57.7</td>
<td>4.6</td>
<td>12.4</td>
<td>138</td>
<td>46</td>
</tr>
</tbody>
</table>
Biological Replicates of *in vivo* phosphorylation status of Ser\(^{451}\) in developing COS endosperm

**Figure B1:** Biological replicates of *in vivo* phosphorylation status of Ser\(^{451}\) in developing COS endosperm. Co-IP COS extracts were loaded based on equal BTPC content, subjected to SDS-PAGE, then immunoblotted with anti-pSer\(^{451}\) and anti-BTPC as described in Fig. 2.4. Stages III, V, VII, and IX correspond to the heart-shaped embryo, mid-cotyledon, full cotyledon, and maturation stages of COS developing respectively [71].