Bioorganic & Medicinal Chemistry Letters, Vol.3, No.7, pp. 1415-1420, 1993
Printed in Great Britain

0960-894X/93 $6.00 + .00

RESPONSE OF AROMATIC PATHWAY ENZYMES OF PLANT SUSPENSION CELLS TO PHOSPHATE LIMITATION

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(*Received* 1 *September* 1992; *accepted* 25 *October* 1992)

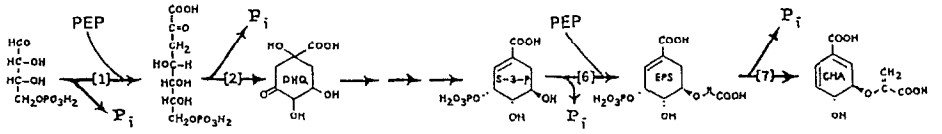
Abstract: The differential expression of aromatic pathway enzymes in suspension cultures of *Brassica nigra* and *Nicotiana silvestris* in response to phosphate availability suggests that carbon flow into the aromatic pathway and P_i release is maintained in the subcellular location of the cytosol but not in plastids during phosphate limitation.

A basic response of plants to phosphate limitation involves the more efficient acquisition and utilization of P_i from the environment, as well as the mobilization of cellular P_i reserves. Investigations of P_i limitation in plant suspension cultures, as well as in whole plants, have generally reported a repertoire of metabolic and physiological adaptations such as increased activities and secretion of phosphatases, increased P_i uptake, and decreased cellular concentrations of adenylates and P_i. A recent study[1] of the effect of P_i starvation on the glycolytic pathways in *Brassica nigra* cell cultures has revealed that the dramatic reductions in levels of P_i, ADP, and ATP were accompanied by 10- to 20-fold increases in the specific activities of several nucleoside phosphate or P_i-independent enzymes which were able to catalyze parallel reactions to adenylate-dependent or P_i-dependent glycolytic reactions. These inducible glycolytic "bypasses" were proposed to provide competence for continued hexose-P conversion to pyruvate despite the very low intracellular concentrations of P_i and adenylate which would otherwise impose drastic limitations upon glycolysis. Two of these reactions [PP_i:fructose-6-phosphate 1-phosphotransferase (PFP) and phosphoenolpyruvate (PEP) phosphatase] may perform an additional role as a "P_i-recycling" system, converting esterified phosphate moieties into P_i which could then be rapidly reassimilated into cellular metabolism.

The biochemical pathway of aromatic biosynthesis is important in nature as the source of several vitamin-like compounds and of the three aromatic amino acids (*L*-tyrosine, *L*-

phenylalanine, and L-tryptophan) that are required for protein synthesis.[2] In higher plants the pathway of aromatic biosynthesis has a vastly greater metabolic impact than in other organisms because it provides connecting pathways of secondary metabolism with starting substrate molecules.[3] Such secondary metabolites include a wide range of compounds including indoleacetic acid, quinate, chlorogenate, cyanogenic glycosides, alkaloids, flavanoids, coumarins, and lignins. The output of products derived from the aromatic pathway is massive since up to 60% or more of the ultimate plant mass (dry weight) may be represented by molecules that once traversed segments of the pathway[3]. In addition to its fractionally great contribution to the amount of cellular carbon flow, the aromatic amino acid pathway is energy intensive, utilizing 2 molecules of PEP and 1 ATP for every molecule of chorismate synthesized by the common trunk of the pathway. What is known of the spatial organization of the interfacing enzymes of carbohydrate metabolism, aromatic biosynthesis, and secondary metabolism in both the chloroplast and cytosol microenvironments of higher-plant cells has been reviewed recently.[4]

The response of enzymes of aromatic amino acid biosynthesis, as well as of enzymes in connecting metabolic networks, to environmental signals such as light and wounding are of great contemporary interest (reviewed in ref. 4). However, nothing is known about possible effects of P_i limitation upon enzymes of aromatic amino acid biosynthesis. Of particular interest in this context is that the initial two steps of aromatic amino acid biosynthesis catalyze reactions which release P_i. The sixth and seventh steps, culminating with chorismate formation, also release P_i. Thus, the synthesis of chorismate generates four molecules of P_i at the expense of 1 erythrose-4-P, 1 ATP, and 2 PEP molecules.



Aside from certain vacuolar and extracellular enzymes, metabolic response to P_i limitation is thus far only known to involve cytosolic enzymes.[2,5,6] Of particular interest in this context is that the initial catalytic step of aromatic biosynthesis is mediated in plants by cytosolic and chloroplast-localized isoenzymes[6] of 3-deoxy-*D*-*arabino*-heptulosonate 7-phosphate (DAHP) synthase, an enzyme that condenses erythrose-4-phosphate and PEP. With these DAHP synthase isoenzymes as an initial point of focus, we have initiated investigation of the effects of P_i limitation on the aromatic pathway.

Considerable information concerning the various responses of *Brassica nigra* suspension cells to P_i limitation has been reported.[1,5] Since the regimen of P_i starvation has been well established for *B. nigra*, we initially determined activities for aromatic-pathway enzymes in 7-day cultures[1] which had been deprived of P_i. The P_i and adenylate levels are quite low in these cultures, while PFP and PEP phosphatase activities are high. An attractive feature of the aromatic amino acid pathway in plants is that the DAHP synthase isoenzymes possess highly distinctive properties[7] which permit their selective assay in crude extracts in order

to gauge pathway dynamics within the different subcellular compartments.

As shown in Table 1, the DAHP synthase isoenzyme designated DS-Co (located in the cytosol) exhibited a specific activity that was 4-fold greater in the P_i-deficient culture. In contrast, the plastid-localized DS-Mn isoenzyme yielded a specific activity in the P_i-deficient culture that was only 7% of the P_i-sufficient culture.

The total cellular specific activity for 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase was about 20% greater in the P_i-deficient culture. Similarly, two enzymes of secondary aromatic metabolism (phenylalanine ammonia-lyase, the first enzyme of the phenylpropanoid pathway segment, and chalcone synthase of the flavonoid pathway segment) showed greater than a 40% elevation in specific activities. These data suggest that cytosolic carbon flow through a large metabolic network may be enhanced by P_i limitation in cultures of *B. nigra*.

Table 1. Effect of phosphate limitation on aromatic pathway enzyme activities of *Brassica nigra* suspension cultures

	Specific activities[a]				
Culture	DS-Co	DS-Mn	EPSP synthase	Phenylalanine ammonia-lyase	Chalcone synthase
P_i-sufficient	12.7	16.5	17.0	0.43	1.8
P_i-deficient	50.5	1.2	20.6	0.61	2.6

[a]Specific activities: DS-Co, nmol 4,5-dihydroxy-2-oxovalerate produced $min^{-1}mg^{-1}$; DS-Mn, nmol DAHP produced $min^{-1}mg^{-1}$; EPSP synthase, nmol PEP utilized $min^{-1}mg^{-1}$; phenylalanine ammonia-lyase, pkat cinnamate produced mg^{-1}; chalcone synthase, pkat phenylalanine produced mg^{-1}. Specific activity values are the mean of two determinations.

N. silvestris in cell culture is one of the best characterized suspension culture systems available in terms of growth analysis[8] and the tracking of aromatic-pathway enzyme expression in relationship to growth[9]. Cells which have been maintained indefinitely in exponential-phase growth (denoted EE cells) were examined for their growth response to P_i deprivation, as well as for growth response of P_i-limited cultures to added P_i. Figure 1 illustrates the typical growth rate (doubling time = 36-38 h) obtained with a starting concentration of 1.25 mM P_i.

In response to P_i deficiency a biphasic pattern of growth ensued over a 12-day interval. An immediate shift to a slower (2-fold) but still exponential growth rate persisted for 4 days, followed by an abrupt shift to a still greater doubling time of about 9 days. Restoration of P_i was carried out after an elapsed time of 4 days, exactly at the transition point of the biphasic curve. One day after P_i addition, the growth rate increased

progressively for 3 days until the maximal growth rate was again achieved.

Enzyme assays were carried out for DS-Co, DS-Mn, acid phosphatase and alkaline phosphatase using culture flasks of *N. silvestris* from the same experiment diagrammed in Fig. 1. The results are shown in Table 2.

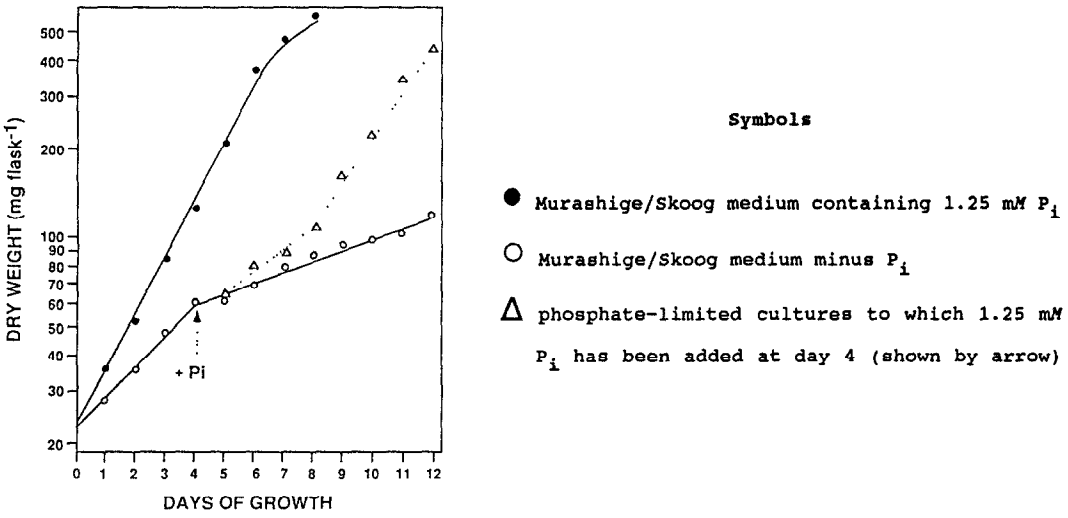


Figure 1 Growth curves obtained from suspension cultures of *N. silvestris*. Dry cell weight (mg flask^{-1}) is plotted on a logarithmic scale as a function of days following subculture of EE cells into media supplemented as indicated by the symbols on the right of Fig. 1 above.

Table 2. Time-course of DAHP synthase and phosphatase activities[a] from phosphate-limited *Nicotiana silvestris* suspension cultures

Culture Conditions	Days in Culture	DS-Co SA	DS-Co Units/ g dry wt.	DS-Mn SA	DS-Mn Units/ g dry wt	Ratio of DS-Co to DS-Mn	Acid Phosphatase SA	Acid Phosphatase Units/ g dry wt	Alkaline Phosphatase SA	Alkaline Phosphatase Units/ g dry wt	Soluble Protein (mg) per g dry wt
EE Cells	0	80.4	14.5	30.6	5.5	2.6	2123	382.2	80	14.3	180
P_i-deprivation	3	77.8	7.8	15.6	1.6	5.0	5080	508.5	323	32.3	100
	4	142.3	14.3	22.0	2.2	6.5	5271	497.4	353	35.5	99
	5	126.9	11.8	17.2	1.6	7.4	5400	494.0	363	33.5	92
	7	117.0	9.4	16.3	1.3	7.2	5562	443.3	446	36.0	81
	10	82.7	6.4	12.3	0.95	6.7	5596	347.1	252	19.5	78
P_i-restoration	6	129.1	17.2	37.8	5.0	3.4	2286	305.5	123	16.4	134
	7	79.3	10.7	34.8	4.7	2.3	1728	232.9	104	14.1	135
	10	53.4	9.2	26.0	4.4	2.1	1391	240.6	33	5.7	173

[a]Changing activities of DS-Co and DS-Mn are given as specific activities (SA) (as indicated in Table 1) and as units (μmol product min^{-1}) per g dry weight. Changing activities of acid and alkaline phosphatases are expressed as nmol *p*-nitrophenylphosphate utilized min^{-1}mg^{-1} protein (specific activity) or as units (μmol *p*-nitrophenylphosphate utilized min^{-1}) per g dry weight. Activity values given were within 5% of those obtained in a preliminary experiment in which fewer samples were taken.

The specific activities of DS-Co and DS-Mn given in Table 2 varied in opposite directions following phosphate starvation. Since soluble protein content decreases with phosphate starvation, it appears that the changing ratio of DS-Co to DS-Mn largely reflects selective maintenance of existing levels of DS-Co in contrast to the turnover of DS-Mn. This is consistent with previous findings[10] that DS-Mn levels vary in positive correlation with growth rate. Addition of P_i to P_i-starved cultures resulted in a rapid recovery of DS-Mn and a slow diminution of DS-Co levels. The latter could be accounted for by growth dilution of existing enzyme.

A distinct burst of Ds-Co enzyme synthesis appears to occur just before onset of the slowed phase of growth on day 4 of P_i deprivation. Between days 3 and 4 the total units per flask of DS-Co activity increased from 326 to 740. The specific activity increased almost 2.3 fold under conditions where total soluble protein decreased about 1%. More detailed studies using closely spaced samples taken just before and after the time of the first growth transition will be needed to confirm this. Both alkaline phosphatase and acid phosphatase activities increased and decreased in response to P_i limitation and to restoration of P_i, respectively. However, the response of phosphatase appears to correlate with the initial phase of slow growth, whereas the response of DS-Co appears to correlate with the second phase of slow growth.

Perspective. Phosphate limitation is a common nutritional state encountered by higher plants. During growth conditions of P_i excess, various phosphorylated metabolites are formed which can be considered to be a potential source of P_i in subsequent conditions of P_i limitation. The enzymes, PFP, PEP phosphatase, and PEP carboxylase, have previously been cited[1,5,6] as components of a P_i-recycling system which can release esterfied P_i. Four enzymes in the common pathway of aromatic amino acid biosynthesis also release P_i during catalysis. The first (DAHP synthase) and sixth (EPSP synthase) steps involve the utilization of PEP and the release of P_i as reaction products. The second pathway enzyme (dehydroquinate synthase) releases P_i in the conversion of DAHP to dehydroquinate while the seventh (chorismate synthase) releases P_i in the conversion of EPSP to chorismate. Systems for mobilization of P_i from intracellular phosphorylated compounds thus far seem to be mainly cytosolic, and this is consistent with: (i) the increased ratio of cytosolic isoenzyme (DS-Co) to plastid isoenzyme (DS-Mn) in response to P_i deprivation, (ii) the increased specific activities of secondary-pathway enzymes such as PAL and chalcone synthase, and (iii) the common observation that flavonoids and phenolics accumulate following P_i limitation. Cytosolic isoenzymes for biosynthetic steps other than DAHP synthase and chorismate mutase have not yet been demonstrated. Thus, for enzymes such as dehydroquinate synthase, EPSP synthase, and chorismate synthase, the measurement of overall activities in crude extracts might be misleading if plastid-localized and cytosolic isoenzymes respond in opposite fashion to P_i limitation, as seen with DS-Co and DS-Mn. Shikimate, quinate, and their various derivatives, well known for their accumulation in tissues of whole plants, may prove to be

products of a P_i-recycling mechanism during P_i limitation.

A well-defined experimental system is in place to examine the exact timing of altered levels of aromatic-pathway enzymes which release P_i in response to P_i limitation. The two distinct phases of growth triggered by deficiency of P_i may be significant indicators of two classes of physiological response which may correspond to discrete enzymological adjustments.

Acknowledgement. This research was supported by grant DCB 8916265 from the National Science Foundation (to G.H.), contract DE-FG05-86ER13581 from the U.S. Department of Energy (to R.A.J.) and by the Natural Sciences and Engineering Research Council of Canada (to W.C.P.). This is Florida Agricultural Experiment Station Journal Series No. R-02666.

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