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Phosphofructokinase Activities in Photosynthetic Organisms[1]

THE OCCURRENCE OF PYROPHOSPHATE-DEPENDENT 6-PHOSPHOFRUCTOKINASE IN PLANTS AND ALGAE

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NANCY WIELAND CARNAL[2] AND CLANTON C. BLACK
Department of Botany and Department of Biochemistry, University of Georgia, Athens, Georgia 30601

ABSTRACT

A pyrophosphate-dependent phosphofructokinase (PPi-PFK) activity is detectable in extracts of a wide variety of primitive and advanced plants, the Charalean algae, and in the photosynthetic bacterium, *Rhodospirillum rubrum*. Angiosperms with extractable PPi-PFK activities 4- to 70-fold higher than the respective ATP-PFK activities tend to be succulent and to exhibit CAM. Even though PPi-PFK activity is not detected in crude extracts of some well known CAM plants, *e.g.* plants in the Crassulaceae, gel filtration of the extract and/or inclusion of the PPi-PFK activator, fructose 2,6-bisphosphate, in the assay reveals that a PPi-PFK activity is present in these species. Fructose 2,6-bisphosphate likewise activates PPi-PFK activities in extracts of C_3 and C_4 plants. C_3 and C_4 plant PPi-PFK activities are roughly equivalent to ATP-PFK activities in the same species. PPi-PFK activity is also detected in some bryophytes, lower vascular plants, ferns, and gymnosperms. The Charophytes, advanced algae presumed to be similar to species ancestral to vascular plants, exhibit at least 4-fold higher PPi-PFK than ATP-PFK activities. *R. rubrum* also exhibits a much higher PPi-PFK activity than ATP-PFK activity. These data indicate that PPi-PFK may serve as an alternate enzyme to ATP-PFK in glycolysis in a wide range of photosynthetic organisms.

Phosphofructokinase catalyzes the first unique reaction in glycolysis and is a major regulatory point of glycolytic carbon flow (21). The interpretation that an ATP-dependent PFK[3] catalyzes a rate-determining step in glycolysis in plant stems from the following observations: (a) ATP-PFK catalyzes an essentially irreversible reaction which under physiological conditions is found to be far from equilibrium; (b) extractable ATP-PFK activities are the lowest or among the lowest displayed by enzymes catalyzing reactions in the glycolytic sequence; (c) PFK activity changes in concert with induced changes in the rate of glycolysis; and (d) plant ATP-PFK, like the mammalian and bacterial counterparts, show complex kinetic behaviors and are modulated by an array of positive and negative effectors (4, 21).

In 1979, we reported the discovery of an additional PFK activity in pineapple leaves that is specific for PPi as the energy source and phosphate donor for the phosphorylation of Fru-6-P to Fru-1,6-bisP (2) A PPi-PFK has previously been identified as one of five PPi-specific phosphotransferase reactions in bacteria and in the enteric amoeba, *Entamoeba histolytica* (23). Recently, two other research groups have confirmed the presence of PPi-PFK in plants (3, 15).

We have proposed that PPi-PFK is involved in carbohydrate metabolism in pineapple since the PPi-PFK activity in pineapple leaf homogenates is about 15 times the ATP-PFK activity (2). Because of the potential importance of an alternate PPi-dependent PFK in the metabolism of plants, we conducted a survey of the plant kingdom for the occurrence of PPi-PFK. We recently reported that PPi-PFK is widely distributed among photosynthetic organisms (1). In the present report, we document the presence of PPi-PFK in these organisms by presenting PPi-PFK and ATP-PFK activities for a variety of plant species, for several green algae, and for the photosynthetic bacterium, *Rhodospirillum rubrum*.

MATERIALS AND METHODS

Biological Materials. Plants utilized in this survey, unless otherwise stated, were obtained from greenhouses maintained by the Botany or Horticulture Departments, University of Georgia, Athens. Leaf samples were collected, wrapped in moist toweling, placed on ice, and transported to the laboratory. Samples from CAM plants were collected in the afternoon to ensure low acid concentrations in the tissue. Spinach leaves were obtained from a local market. Pea leaves were harvested from 2-week-old plants grown in the laboratory under fluorescent lights.

Selected liverwort, moss, and *Lycopodium* species were collected from field sites near Ithaca, NY and mailed to us. These specimens were unpacked and placed in humid chambers (>90% RH, 22°C) at low light intensity for 2 d before using the material. Algal specimens were obtained from pure cultures maintained by the Botany Department, University of Georgia. *Chara* and *Nitella* specimens were kindly provided by Dr. Roger Spanswick, Cornell University, Ithaca, NY and Dr. Barry Palevitz, Botany Department, University of Georgia. Freeze-dried samples of *Rhodospirillum rubrum* were a gift from Dr. Donald Keister, C. F. Kettering Laboratory, Yellow Springs, OH.

Extraction Procedures. Leaf samples were washed in deionized H_2O and blotted dry. Midribs were excised when appropriate. Each sample was sliced into approximately 1 × 5 mm pieces, placed in a chilled mortar, and ground in about 3 volumes of cold (~4°C) extraction medium A: 100 mM Hepes-NaOH, pH 8.0, 150 mM potassium acetate, 30 mM β-mercaptoethanol, 5 mM $MgCl_2$, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, and 1% (w/v) PVP-40. The resulting leaf homogenate was filtered through eight layers of cheesecloth and then placed on ice.

Algal specimens were collected from liquid culture by centrifugation at 890*g*. The pelleted material was washed one or two times with extraction medium A. The washed specimens were resuspended in extraction medium A, sonicated on ice for 30 s,

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[3] Abbreviations: PFK, phosphofructokinase; PEP, phosphoenolpyruvate; Fru-6-P, fructose 6-phosphate; Fru-1,6-bisP, fructose 1,6-bisphosphate.

and further disrupted by one pass through a French pressure cell at 13,000 p.s.i. Charophytes were removed from the culture medium, washed extensively with deionized H_2O, blotted, and ground to a powder in liquid N_2. Extraction buffer A was added to the powder and grinding was continued. The resulting extract was filtered through eight layers of cheesecloth and then placed on ice. Because unicellular green algae were observed in some of the Charophyte culture media, we prepared a pellet (40,000*g* for 30 min) from a 150-ml sample of each culture medium. PFK activity was not detected in the pellet or in the supernatant. Thus, we feel confident that the PFK activities reported are those of the Charophyte species examined.

R. rubrum samples were ground in a chilled mortar in extraction medium A. The homogenate was filtered through eight layers of cheesecloth and placed on ice.

Extract Preparation for Detection of Fru-2,6-bisP Effects on PPi-PFK Activity. Crude extracts of selected species were prepared as stated above, then immediately centrifuged at 22,000*g* for 10 min. A 0.5–0.75-ml aliquot of the resulting supernatant was filtered through a 12.5 × 1.0 cm column of Sephadex G-25 which had been equilibrated in 50 mM Hepes-NaOH, pH 8.0. Fractions eluting after the void volume which were free of small mol wt inclusions (as indicated by the absence of Cl^- and β-mercaptoethanol) were combined. The total combined volume was between 1.0 and 1.5 ml. These desalted extracts were utilized for determining the effects of Fru-2,6-bisP or $(NH_4)_2SO_4$ on PPi-PFK activity.

PFK Assays. ATP-PFK and PPi-PFK were assayed at 30°C in either a 0.5 or 1.0 ml reaction mixture containing 100 mM Hepes-NaOH, pH 8.0, 2.5 mM $MgCl_2$, 0.08 mM NADH, 10 mM Fru-6-P, 6 units/ml aldolase, 1 unit/ml triose-P isomerase, 6 units/ml α-glycerol-P dehydrogenase, plant extract, and either 1 mM ATP or 1 mM PPi. Reaction mixtures used to assay PFK in crude plant extracts contained in addition to the above, about 24 mM $(NH_4)_2SO_4$ and 0.01% BSA which carried over from the coupling enzyme mixture. For assays of PPi-PFK in Sephadex G-25-filtered plant extracts, $(NH_4)_2SO_4$ was removed from a stock coupling enzyme preparation which contained no BSA by filtration on G-25. PFK reactions were initiated with either ATP or PPi. Reaction progress was monitored at 340 nm with a Gilford spectrophotometer (model 240). Reaction rates were corrected for endogenous rates of NADH oxidation. A short lag period preceded a linear reduction period in assays of PFK activity in crude plant extracts. We later discovered this lag period could be virtually eliminated by either removing $(NH_4)_2SO_4$ from the coupling enzyme preparation or by including Fru-2,6-bisP in the reaction mixture. PFK activities in the present paper are given as nmol Fru-1,6-bisP produced/min·mg protein or Chl during the linear reaction period.

Internal Standard to Assay for PPi-PFK Inhibition. In our early research with various plant crude extracts with no apparent PPi-PFK activity, we examined the possibility that the enzyme was inhibited by inclusions in the crude homogenates. An aliquot of a partially purified pineapple PPi-PFK preparation (free of ATP-PFK activity) was incubated for 5 min with the crude extract in question in the reaction mixture described above. The reaction was then initiated with PPi and the per cent inhibition or stimulation of the pineapple PPi-PFK was determined. These data are given in Tables I, II, and III.

Determination of Fru-2,6-bisP Effect on the Detection of PPi-PFK Activity. PPi-PFK activity was determined for Sephadex G-25-filtered extracts of selected species in the presence and absence of Fru-2,6-bisP. Full activation of PPi-PFK with respect to Fru-2,6-bisP was confirmed for each species by examining PPi-PFK activity at 0.5, 1.0, and 2.0 μM Fru-2,6-bisP. Each plant extract was preincubated in the reaction mix with Fru-2,6-bisP for about 2 min prior to initiation of the reaction with PPi. The preincubation was adopted as standard procedure because in some cases we obtained lower PPi-PFK activities when Fru-2,6-bisP was added to a reaction mix in which the PPi-PFK reaction had already been initiated than when the activator was added prior to reaction initiation.

Chl and Protein Determinations. Chl concentrations were determined by the method of Wintermans and DeMots (22). Protein concentrations were measured as previously described (2), using ovalbumin as the protein standard.

General Comments. Little attempt was made to optimize assay conditions for ATP-PFK or PPi-PFK for each species. Instead, we assayed all samples under conditions that were near optimal for PFK activities in pineapple leaf preparations. We initially did not attempt to remove inhibitors from crude extracts by dialysis or Sephadex G-25 filtration because pineapple leaf ATP-PFK is not stable to these treatments under any set of conditions we have tried.

We also considered the possibility that pyrophosphatases present in the crude extract might prevent PPi-PFK detection or effectively reduce PPi-PFK activity by competing for PPi. Since pyrophosphatase activities in pineapple leaf homogenates are completely inhibited at pH 8.0 by 10 mM NaF while PPi-PFK activity is unaffected, we included 10 mM NaF in the extraction and assay media for a second leaf sample of about 25 plants. Increased PPi-PFK activities were noted in only a few cases; hence, NaF inclusions were not continued.

RESULTS AND DISCUSSION

ATP-PFK and PPi-PFK activities for leaf tissue of over 100 angiosperm species are presented in Tables I and II, and for photosynthetic cells or tissues of nonangiosperm species in Table III. ATP-PFK activities for the majority of species range between 1 and 30 nmol·min^{-1}·mg protein^{-1}, and thus are similar to ATP-PFK activities for leaf tissues of various angiosperm species reported in the literature (Table IV). The present survey for PPi-PFK activity centered upon succulent angiosperms when it became apparent that PPi-PFK activities were frequently higher than ATP-PFK activities for these plants. Four- to 70-fold higher PPi-PFK than ATP-PFK activities are found for succulent plants classified in two dicotyledonous families and three monocotyledonous families; however, relatively little or no PPi-PFK activity is detected in crude extracts of succulent, CAM plants in other families (Table I). Likewise, PPi-PFK activities either are not detected in crude extracts of C_3 and C_4 plants or when detected, are nearly equivalent to or lower than ATP-PFK activities (Table II). Following the discovery of PPi-PFK activation by Fru-2,6-bisP (15), we reexamined selected species for PPi-PFK activity in the presence of the purported activator. With a few exceptions (Table V), PPi-PFK activity then is detected in extracts of the CAM, C_3, and C_4 plants in which we had not previously detected PPi-PFK, albeit PPi-PFK activities in these species still generally are equivalent to or lower than ATP-PFK activities (Tables I–III and V). Fru-2,6-bisP inclusion in PPi-PFK assays for species which initially displayed high PPi-PFK activities relative to ATP-PFK activities further enhances these PPi-PFK activites in all species examined except *R. rubrum* and *Thuidium* (Table V).

PFK Activities in Species Classified in Families with CAM Members. PFK activities for representatives of 12 of the 18 angiosperm families in which CAM has been reported (19, 20) are presented in Table I. For each of the families omitted from the survey, three or fewer species have been suggested to utilize CAM (19). PPi-PFK activity is detected in all species examined in the dicot families, Cactaceae and Asclepiadaceae. Eight of the 10 cacti examined display PPi-PFK activities at least 4 times higher than the respective ATP-PFK activities. Both *Mammillaria* and *Opuntia* PPi-PFK activities are activated approximately 2-fold when Fru-2,6-bisP is included in the assay mixture either under conditions utilized to generate the data in Table I or when small mol wt

Table I. Phosphofructokinase Activities of Species Classified in Families with CAM Members.

Family	Species	PP_i-PFK Activity $\mu mol \cdot min^{-1}$ mg $prot^{-1}$ ($mgChl^{-1}$)	ATP-PFK Activity $\mu mol \cdot min^{-1}$ mg $prot^{-1}$ ($mgChl^{-1}$)	Ratio PP_i-PFK: ATP-PFK	PP_i-PFK Standard ΔZ
		DICOTS			
Aizoaceae	*Pleiospilos sp.*	N.D.	101 (318)	---	-41.1
Asclepiadaceae	*Hoya carnosa*	204 (8151)	5 (201)	40.6	---
	Stapelia gigantea	3 (52)	58 (922)	0.1	-35.1
	(+NaF)	16 (248)	--- (---)		
Asteraceae	*Kleinia repens*	5 (102)	14 (293)	0.3	-13.8
	Senecio Doyleanus	N.D.	19 (428)	---	-12.5
Cactaceae	*Cephalocereus senilis*	26 (---)	7 (---)	3.7	---
	Cereus sp.	--- (3473)	--- (414)	---	---
	Cryptocereus anthonyanus	5 (414)	22 (1779)	0.2	- 6.3
	Epiphyllum hybrid	63 (4716)	4 (319)	14.8	---
	Hatiora salicornioides	22 (2182)	N.D.	---	---
	Lemaireocereus pruinosus	45 (2344)	11 (561)	4.2	---
	Mammillaria sp.	197 (6516)	13 (418)	15.6	---
	Opuntia sp.	101 (8031)	7 (565)	14.2	---
	Pereskia aculeata godseffiana (leaves)	23 (579)	19 (474)	1.2	+10.7
	Zygocactus sp.	163 (3600)	4 (91)	39.6	---
Crassulaceae	*Crassula perfossa*	N.D.	N.D.	---	-100.0
	C. argentea	N.D.	N.D.	---	-100.0
	Echeveria glauca	N.D.	28 (153)	---	- 58.1
	Kalanchoe daigremontiana	N.D.	30 (308)	---	- 16.6
	Sedum fedtschenkoi	N.D.	36 (83)	---	- 8.8
	Sedum telephium	N.D.	22 (---)	---	- 29.8
Euphorbiaceae	*Codiaeum variegatum pictum*	N.D.	6 (534)	---	- 17.1
	Euphorbia splendens	N.D.	N.D.	---	-100.0
	Euphorbia sp.	N.D.	12 (631)	---	- 54.0
	Pedilanthus tithymaloides	4 (252)	29 (2048)	0.1	- 30.1
Piperaceae	*Peperomia nivalis*	--- (N.D.)	--- (87)	---	- 1.1
	P. obtusifolia	5 (130)	N.D.	---	---
Portulacaceae	*Portulacaria afra*	N.D.	N.D.	---	- 27.3
		MONOCOTS			
Agavaceae	*Agave americana marginata*	87 (1608)	10 (189)	8.5	---
	A. attenuata	38 (1691)	8 (355)	4.8	---
	A. reginae variegata	184 (5193)	10 (298)	17.4	---
	Yucca sp.	144 (3446)	14 (340)	10.1	---
	Yucca sp.	N.D.	21 (404)	---	- 39.5
Bromeliaceae (Pitcairnioideae)	*Dyckia brevifolia*	--- (N.D.)	--- (1601)	---	---
	Dyckia ensholeroides	--- (N.D.)	--- (959)	---	---
	Dyckia reourvata	--- (N.D.)	--- (595)	---	---
	Pitcairnia verokleana	N.D.	27 (192)	---	- 8.1
	Puya mirabilis	N.D.	75 (888)	---	- 9.7
Bromeliaceae (Bromelioideae)	*Aechmea bracteata*	140 (7354)	4 (215)	34.2	---
	A. caudata variegata	102 (1985)	N.D.	---	---
	A. fasciata	94 (1880)	25 (494)	3.8	---
	A. hyb. 'Bert'	66 (1971)	18 (538)	3.7	---
	A. penduliflora	154 (2356)	2 (32)	73.6	---
	A. ramosa	38 (1631)	4 (155)	10.5	---
	A. 'Royal Wine'	34 (788)	5 (116)	6.8	---
	Ananas comosus	576 (18719)	30 (988)	18.9	---
	A. comosus variegatus	174 (12533)	18 (1298)	9.6	---
	Billbergia fantasia	34 (901)	3 (89)	10.1	---
	B. horrida tigrina	51 (1342)	15 (403)	3.3	---
	B. nutans	129 (2804)	21 (468)	6.0	---
	B. pyramidalis concolor	611 (14200)	15 (359)	39.6	---
	Cryptanthus beuckeri	274 (3811)	7 (101)	37.7	---
	C. bivittatus minor	186 (3675)	29 (579)	6.3	---
	C. bromelioides tricolor	25 (3571)	6 (853)	4.2	---
	C. lacerdae	42 (1863)	N.D.	---	---
	Cryptanthus sp.	154 (3616)	13 (310)	11.7	---
	C. zonatus zebrinus	16 (956)	N.D.	---	---
	Orthophytum saxicola	N.D.	8 (392)	---	+ 1.7
	Neoregelia carolinae tricolor	152 (3983)	4 (99)	40.2	---
	N. spectabilis	118 (3653)	6 (194)	18.8	---
	Nidularium innocentii	4 (190)	3 (143)	1.3	-26.4
Bromeliaceae (Tillandsioideae)	*Catopsis nutans*	N.D.	25 (280)	---	+ 3.1
	Tillandsia circinnata	11 (372)	17 (597)	0.6	-14.2
	Tillandsia ayanea	N.D.	33 (196)	---	+ 1.1
	T. fasciculata	6 (116)	38 (724)	0.2	- 7.7
	T. fasciculata clavispica	3 (131)	30 (1513)	0.1	-20.0
	T. flexuosa	22 (252)	143 (1654)	0.2	-13.5
	T. lomantha	N.D.	98 (905)	---	-17.9
	T. leiboldiana	--- (N.D.)	--- (117)	---	---
	T. myosura	N.D.	117 (1196)	---	+47.0
	T. pruinosa	8 (69)	60 (552)	0.1	-13.5
	T. recurvata	--- (N.D.)	--- (790)	---	0.0
	Tillandsia sp.	N.D.	33 (1268)	---	-28.8
	T. tricolor	--- (N.D.)	--- (300)	---	---
	T. usneoides	N.D.	33 (816)	---	-21.8
	Vriesea sp.	N.D.	6 (25)	---	-15.1
Liliaceae	*Allium sp.*	N.D.	N.D.	---	- 1.8
	Aloe ciliaris	10 (142)	35 (522)	0.3	-29.1
	Beaucarnea recurvata	--- (433)	--- (348)	1.2	---
	Bowiea volubilis	--- (N.D.)	--- (327)	---	-20.0
	Clivia miniata	27 (284)	13 (140)	2.0	---
	Dracaena sanderiana	8 (130)	8 (131)	1.0	-21.8
	Gasteria liliputana	181 (3771)	34 (710)	5.3	---
	G. trigona	247 (5343)	58 (1261)	4.2	---
	Hippeastrum vittatum	11 (140)	17 (226)	0.6	-23.2
	Scilla violacea	--- (N.D.)	--- (328)	---	-23.6
	Sansevieria subspicata	102 (1434)	24 (345)	4.2	---
	S. trifasciata	191 (3071)	33 (525)	5.8	---
	S. trifasciata Hahnii	277 (3980)	30 (427)	9.3	---
	Tulipa sp.	N.D.	4 (50)	---	-17.5
Orchidaceae	*Cattleya sp.*	20 (469)	12 (281)	1.7	---
	Dendrobium nobile	6 (142)	17 (379)	0.4	-13.4
	Vanda Miss Agnes Joachim	--- (652)	--- (1666)	0.4	---
	Vanilla sp.	--- (313)	--- (120)	2.6	+12.7

N.D.=Not Detectable

Table II. Phosphofructokinase Activities of Species Classified in Families Without CAM Members.

Family	Species	PP_i-PFK Activity $\mu mol \cdot min^{-1}$ mg $prot^{-1}$ ($mgChl^{-1}$)	ATP-PFK Activity $\mu mol \cdot min^{-1}$ mg $prot^{-1}$ ($mgChl^{-1}$)	Ratio PP_i-PFK: ATP-PFK	PP_i-PFK Standard ΔZ
		DICOTS			
Acanthaceae	*Beloperone guttata*	N.D.	N.D.	---	- 32.4
Anacardiaceae	*Mangifera indica*	N.D.	N.D.	---	-100.0
Araceae	*Scindapsus pictus argyraeus*	N.D.	N.D.	---	- 16.4
Chenopodiaceae	*Spinacea oleracea*	5 (139)	8 (262)	0.5	---
Cruciferae	*Brassica rapa*	N.D.	2 (71)	---	- 38.3
Gesneriaceae	*Aeschynanthus speciosus*	--- (N.D.)	--- (177)	---	- 9.8
Lauraceae	*Persea americana*	N.D.	N.D.	---	- 12.3
Leguminosae	*Pisum sativum*	N.D.	7 (154)	---	- 3.6
Marantaceae	*Calathea insignis*	N.D.	N.D.	---	- 3.8
Moraceae	*Ficus elastica*	N.D.	N.D.	---	18.2
Myrsinaceae	*Ardisia crispa*	N.D.	N.D.	---	- 91.3
Myrtaceae	*Eugenia uniflora*	14 (120)	N.D.	---	- 37.1
Polygonaceae	*Homalocladium platycladum*	--- (N.D.)	--- (221)	---	+ 4.0
Pontederiaceae	*Eichhornia crassipes*	N.D.	N.D.	---	- 10.5
Saxifragaceae	*Saxifraga sarmentosa tricolor*	N.D.	N.D.	---	+ 12.7
Urticaceae	*Pilea nummulariifolia*	N.D.	N.D.	---	+ 2.6
		MONOCOTS			
Cyperaceae	*Cyperus ligularis*	10 (254)	10 (243)	1.0	+16.5
Gramineae	*Avena sativa*	--- (72)	--- (202)	0.4	-21.9
	Chloris gayana	1 (21)	3 (63)	0.3	-40.1
	Digitaria sanguinalis	N.D.	3 (44)	---	- 2.8
	Eleusine indica	--- (N.D.)	--- (322)	---	-19.2
	Panicum maximum	N.D.	3 (79)	---	-44.7
	Poa pratensis	2 (32)	9 (140)	0.2	---
	Triticum aestivum	--- (N.D.)	--- (828)	---	-19.2
	Zea mays	N.D.	20 (803)	---	-17.0
Iridaceae	*Neomarica gracilis*	41 (1127)	22 (624)	1.8	+17.8
Musaceae	*Musa nana*	N.D.	N.D.	---	- 1.4
Palmae	*Caryota mitis*	N.D.	19 (236)	---	+38.0
	Chamaedorea elegans	16 (331)	10 (183)	1.8	0.0
	C. elegans bella	20 (464)	8 (182)	2.5	+10.7

N.D.=Not Detectable

inclusions are removed from the extracts and coupling enzyme preparations (Table V). *Pereskia aculeata godseffiana*, one of the few non-CAM cacti (14), and *Cryptocereus anthonyanus* exhibit PPi-PFK activities respectively equivalent to or lower than ATP-PFK activities. Neither plant was examined for Fru-2,6-bisP activation of PPi-PFK. The relatively low PPi-PFK activity expressed in *C. anthonyanus* crude extracts, on first approximation, does not appear to be due to inhibition by inclusions in the extract since partially purified pineapple PPi-PFK is not significantly inhibited when incubated with this extract (Table I); however, it must be realized that the PPi-PFK from different plant sources may be differentially sensitive to extract inclusions.

Both *Stapelia gigantea* and *Hoya carnosa* (Asclepiadaceae) utilize the CAM pathway of CO_2 assimilation (19). PPi-PFK activity in *H. carnosa* is 40 times greater than the ATP-PFK activity, whereas, PPi-PFK activity in *S. gigantea* crude extracts is less than that of the ATP-PFK activity (Table I). Inclusion of NaF with the later extract results in a 5-fold increase in detectable PPi-PFK activity (Table I). Likewise, PPi-PFK activity is increased approximately 2.5-fold when fully activating concentrations of Fru-2,6-bisP are present (Table V). Fru-2,6-bisP (1 μM) included with the crude extract results in a 7-fold increase in PPi-PFK activity, *i.e.* to the same level as the activity expressed in the assay with desalted extract (data not shown). None of the treatments, however, increased PPi-PFK activity in *S. gigantea* extracts to the level of ATP-PFK activity.

Little or no PPi-PFK activity is detected in crude extracts of succulent species in the Aizoaceae, Asteraceae, Crassulaceae, Euphorbiaceae, Portulacaceae, or Piperaceae (Table I). Failure to detect PPi-PFK in these extracts is in part due to inhibition of the enzyme by inclusions in the crude extracts. Partially purified pineapple PPi-PFK is inhibited between 20 and 100% when incubated with extracts of species in the latter families. Secondly, using desalted extracts, PPi-PFK activity is expressed in *Portulacaria afra* and *Kalanchoë daigremontiana* even without inclusion of the activator Fru-2,6-bisP (data not shown). Fru-2,6-bisP further enhanced the activity of PPi-PFK in the latter species both in desalted and crude extracts (Table V). PPi-PFK activity is noted

Table III. Phosphofructokinase Activities of Selected Bacteria, Algae, Bryophyte, Lower Vascular Plants, Ferns, and Gymnosperms.

Species	PP_i-PFK Activity nmol·min^{-1} mg prot^{-1}(mgChl^{-1})	ATP-PFK Activity nmol·min^{-1} mg prot^{-1}(mgChl^{-1})	Ratio PP_i-PFK: ATP-PFK	PP_i-PFK Standard Δ%
BACTERIA				
Rhodospirillum rubrum	36 (---)	N.D. (---)	---	---
R. rubrum (G-9)	48 (---)	5 (---	9.6	---
ALGAE				
Chlamydomonas reinhardii	--- (N.D.)	--- (16)	---	+31.1
Eudorina elegans	--- (N.D.)	--- (356)	---	- 3.1
Gonium sp.	--- (N.D.)	--- (N.D.)	---	+34.2
Volvox sp.	--- (N.D.)	--- (N.D.)	---	-40.4
Oedogonium cardiacum	--- (N.D.)	--- (386)	---	---
Chara corallina	46 (548)	3 (33)	16.6	---
Chara corallina	37 (339)	N.D.	---	---
Nitella translucens	73 (822)	N.D.	---	---
N. axillaris	113 (906)	N.D.	---	---
BRYOPHYTES				
Bazzania trilobata	N.D.	4 (79)	---	-23.4
Dicranum viride	N.D.	N.D.	---	+ 5.5
Leucobryum sp.	4 (114)	7 (207)	0.6	- 7.8
Mnium affine	N.D.	11 (310)	---	+16.7
M. cuspidatum	N.D.	13 (403)	---	-10.6
Sphagnum sp.	6 (232)	7 (268)	0.9	- 8.3
Thuidium sp.	6 (262)	25 (1049)	0.2	-15.7
SEEDLESS VASCULAR PLANTS				
Psilotum sp.	13 (555)	4 (193)	2.9	---
Lycopodium complanatum	4 (128)	1 (24)	5.3	-32.3
L. lucidulum	4 (269)	5 (322)	0.8	- 8.5
L. obscurum	N.D.	4 (118)	---	-41.3
Selaginella sp.	6 (109)	N.D.	---	- 5.5
Equisetum arvense	2 (44)	4 (89)	0.5	-25.4
(+NaF)	9 (222)	--- (---)		
Pyrrosia adnascens	3 (31)	3 (26)	1.2	-18.8
P. longifolia	N.D.	17 (198)	---	---
Polypodium sp.	N.D.	N.D.	---	-100.0
Marsilea sp.	N.D.	N.D.	---	-28.4
GYMNOSPERMS				
Cycas revoluta	1 (42)	3 (101)	0.4	-30.8
Ginkgo biloba	N.D.	N.D.	---	-23.6
Podocarpus macrophylla	N.D.	N.D.	---	-35.4

N.D. = Not Detectable

Table IV. Reported Activities of ATP-Phosphofructokinase in Crude Homogenates of Plant Leaves.

Species	CO_2 Fixation Pathway	ATP-PFK Activity	Reference
		nmol·min^{-1}·mg^{-1}	
Atriplex hastata	C_3	320 Chl	16
Pisum sativum	C_3	1-12 protein[b]	6
Spinacea oleracea	C_3	3 protein[a]	7
		4.2 protein[a]	8
Triticum aestivum	C_3	16-28 protein[b]	5
		1140 Chl	16
Atriplex spongiosa	C_4	300 Chl	16
Zea mays	C_4	0.3-0.7 fresh wt	9
		690 Chl	16
Bryophyllum bloesfeldiana	CAM	1-7 dry wt	13
Bryophyllum pinnatum	CAM	1330 Chl	16
Kalanchoe daigremontiona	CAM	800 Chl	16

[a]Values calculated from data given in reference.

[b]Crude preparation included an initial centrifiguration step, therefore, value is a maximum value.

in *Sedum fedtschenkoi* and a *Pleiospilos* sp. (Aizoaceae) only in the presence of Fru-2,6-bisP (Table V). The PPi-PFK activities for the Crassulacean species and for *Pleiospilos* at the highest activation levels achieved in this survey are still lower than ATP-PFK activities expressed in crude extracts of these species

Table V. Influence of Fructose-2,6-bisphosphate on PPi-Phosphofructokinase Activity in Selected Photosynthetic Organisms.

Species		PP_i-PFK Activity nmol·min^{-1}·mg protein^{-1}	
		Control	Plus Fru-2,6-P_2[a]
BACTERIA, ALGAE, AND LOWER PLANTS			
Rhodospirillum rubrum[c]		108	98
Chara corallina		211	230
Thuidium sp.		3.7	3.5
Lycopodium lucidulum		7	11
ANGIOSPERMS			
Helianthus annuus	(C_3)[b]	N.D.[d]	8
Pisum sativum	(C_3)	N.D.	10
Spinacea oleracea	(C_3)	26	39
Triticum aestivum	(C_3)	5	8
Digitaria sanguinalis	(C_4)	N.D.	9.6
Eleusine indica	(C_4)	N.D.	6.5
Zea mays	(C_4)	N.D.	2.5
Agave attenuata[c]	(CAM)	8	9
Ananas comosus	(CAM)	1500	1787
Kalanchoe daigremontiana	(CAM)	3.3	11
Mammillaria sp.	(CAM)	230	429
Opuntia sp.[c]	(CAM)	61	116
Pleiospilos sp.	(CAM)	N.D.	12
Portulacaria afra[c]	(C_3/CAM)	14	18
Sedum fedtschenkoi	(CAM)	N.D.	2.5
Stapelia gigantea	(CAM)	13	30
Tillandsia cyanea	---	0.7	2.6
Tillandsia usneoides	(CAM)	N.D.	N.D.

[a]Fru-2,6-P_2 was determined to be saturating at 1 μM for all species.

[b]CO_2 fixation pathway utilized.

[c]Extract not treated by Sephedex G25 filtration.

[d]N.D. = Not Detectable.

(Table I).

Succulent monocots in three families, *i.e.* the Agavaceae, Bromeliaceae, and Liliaceae, also exhibit PPi-PFK activities at least 4 times greater than respective ATP-PFK activities (Table I). PPi-PFK activities are noted among CAM Orchidacean species, but these activities are roughly equivalent to or lower than ATP-PFK activities in these species (Table I).

All plants assayed in the Liliaceae and Agavaceae that are known to exhibit CAM (*Agave, Aloe, Gasteria, Sansevieria, Yucca* [19]) except *Aloe ciliaris* exhibit PPi-PFK activities at least 4 times greater than ATP-PFK activities in the same extracts (Table I). *Agave attenuata* shows a slight enhancement of PPi-PFK activity when Fru-2,6-bisP is included in the assay (Table V). The PPi-PFK activities in *A. ciliaris* and the *Yucca* species in which we did not detect a PPi-PFK activity may be different than the levels indicated because purified pineapple PPi-PFK is inhibited 30 and 40%, respectively, in the presence of these crude extracts (Table I).

PPi-PFK activity in extracts of C_3 species in the Liliaceae display either no PPi-PFK activity (*Tulipa, Scilla, Allium*) or PPi-PFK activities roughly equivalent to or less than ATP-PFK activities (*Beaucarnea, Clivia, Dracaena, Hippeastrum*). Pineapple PPi-PFK is inhibited about 20% when incubated with extracts of these species; thus, it is possible that PPi-PFK activities in these species are higher than our data indicate.

A PPi-PFK is the predominant PFK activity in 21 or the 23 species assayed in the Bromelioideae subfamily of the Bromeliaceae, whereas, ATP-PFK is the predominant PFK activity among members of the Pitcairnoideae and Tillandsioideae subfamilies (Table I). All species in the Bromelioideae which have been examined fix CO_2 via CAM (10, 11, 19), whereas only one genus in the Pitcairnoideae, *Dyckia,* is reported to have characteristics consistent with CAM. Some Tillandsioid species fix CO_2 via CAM

while others utilize only the C_3 mode of CO_2 fixation (11).

All but two species in the Bromelioid subfamily of the Bromeliaceae show a 4- to 70-fold higher PPi-PFK than ATP-PFK activity (Table I). PPi-PFK is not detectable in *Orthophytum saxicola* and the PPi-PFK and ATP-PFK activities in *Nidularium innocentii* are roughly equivalent. Pineapple PPi-PFK is not inhibited when incubated with *O. saxicola* extract. However, *N. innocentii* extract inhibits the pineapple enzyme by 26%; hence, PPi-PFK activity in the latter species may be higher than reported here. CAM is reported as the means of CO_2 fixation in all species which have been examined in this subfamily (11), including *N. innocentii* (10) and an *Orthophytum* sp. (19). *Ananas comosus* exhibits the highest PPi-PFK specific activity of any species studied and it exhibits about 20% activation when Fru-2,6-bisP is included in the assay with near saturating concentrations of Fru-6-P and PPi (Table V).

PPi-PFK activity is not detected in crude extracts of selected species in the Pitcairnoideae nor in 10 of the 15 Tillandsioid species (Table I). When PPi-PFK activity is detected among the Tillandsioid bromeliads, its activity is less than that of the ATP-PFK activity. Inhibition of partially purified pineapple PPi-PFK by extracts of Pitcairnoid and Tillandsioid bromeliads is generally less than 20% and is probably insufficient to mask PPi-PFK detection, presuming similar sensitivities of the PPi-PFK from these sources to crude extract inclusions. The effects of Fru-2,6-bisP on the detection of PPi-PFK for representative *Tillandsia* species are shown in Table V. The fact that a 3.5-fold stimulation of the small PPi-PFK activity detected in desalted *T. cyanea* extracts is noted may indicate that relatively low PPi-PFK activities are present in other Tillandsioid bromeliads as well. However, PPi-PFK is not detected in *T. usneoides* even when Fru-2,6-bisP is included in the assay. In general, activation of all species in Table V is less than 3-fold under the substrate conditions utilized in our standard PPi-PFK assay. Thus, even if all *Tillandsia* species show the maximal activation by Fru-2,6-bisP which we observe for PPi-PFK for other species, the PPi-PFK activities in the *Tillandsia* species would still be lower than the respective ATP-PFK activities.

PFK Activities in Families without CAM Members. Neither an ATP-PFK nor PPi-PFK activity is readily detectable among crude extracts of many C_3 dicot species we assayed (Table II). ATP-PFK activities similar to ATP-PFK activities reported in the literature (Table IV) are detected in pea, spinach, and turnip extracts (Table II) and in *Codiaeum variegatum pictum*, a C_3 Euphorb (Table I). Neither PFK activity, however, is noted in the C_3 Euphorb, *Euphorbia splendens*. In contrast to dicot C_3 species, ATP-PFK activities are readily detectable in extracts of C_3 and C_4 monocot species (Table II).

A PPi-PFK activity is reproducibly detected in spinach leaf crude extracts. Spinach PPi-PFK under substrate conditions utilized in the standard assay is activated by about 50% when Fru-2,6-bisP is included in the assay mix. Kelly and Latzko (8) report that PPi will not serve as a substrate for PFK in spinach chloroplast preparations; however, they do not state whether PPi was tested as a substrate for less purified preparations. Cseke *et al.* (3), however, have recently detected PPi-PFK in spinach and have shown that spinach PPi-PFK activity is stimulated by Fru-2,6-bisP under a variety of substrate and pH conditions.

PPi-PFK activities equal to or lower than ATP-PFK activities are noted in crude preparations of the C_4 species *Chloris gayana* and *Cyperus ligularis* (Table II) and in several C_3 monocots, including the C_3 species in the Liliaceae (Table I) discussed earlier.

Failure to detect PPi-PFK in other C_3 and C_4 plants may in part be due to inhibition by extract inclusions and to $(NH_4)_2SO_4$ in the initial assays, and in part to improper activation of the enzyme. Inhibition of pineapple PPi-PFK when incubated with extracts of C_3 and C_4 species is generally less than 20%; however, extracts of several grass species, turnip, and mango inhibit the pineapple enzyme between 40 and 100% (Table II). The data in Table V show that $(NH_4)_2SO_4$ removal from assay components and desalting the extracts of C_3 and C_4 species, except for spinach and wheat, generally does not result in the detection of a PPi-PFK activity. However, for all C_3 and C_4 species examined, PPi-PFK activity is detected when Fru-2,6-bisP is included in the assay mixture (Table V).

PFK Occurrence in Other Photosynthetic Organisms. PPi-PFK and ATP-PFK activities for *R. rubrum*, various algae, lower plants, ferns, and gymnosperms are presented in Table III. PPi-PFK activity is at least 10 times the ATP-PFK activity in the wild type and G-9 strain of *R. rubrum*. PPi-PFK has been partially purified and characterized from *R. rubrum* (12) and PPi-PFK activities have been detected in a number of other bacterial species (*e.g.* 2, 23). We did not detect Fru-2,6-bisP stimulation of activity in *R. rubrum* (Table V) either at 10 mM Fru-6-P or at nonsaturating Fru-6-P concentrations.

A PPi-PFK activity is at least 17 times that of the ATP-PFK activity is noted in *Chara corallina* and PPi-PFK activities are the only PFK activities we observed in crude extracts of *Nitella* species (Table III). Fru-2,6-bisP does not appear to stimulate significantly *C. corallina* PPi-PFK at either saturating or subsaturating Fru-6-P concentrations. ATP-PFK activities but not PPi-PFK activities are detected in crude extracts of other green algae. Pineapple PPi-PFK is not significantly inhibited by incubation with algal extracts, except with *Volvox*. Therefore, if pineapple PPi-PFK can serve as a model for algal PPi-PFK, then the enzyme does not appear to be inhibited.

Among the bryophytes examined, ATP-PFK is the predominant PFK activity; however, a PPi-PFK of lower activity is noted in a few species (Table III). We did not note Fru-2,6-bisP activation of PPi-PFK activity in *Thuidium* (Table V). The pineapple PPi-PFK control generally is inhibited by less than 15% when incubated with bryophyte extracts (Table III).

PPi-PFK activities equivalent to or somewhat higher than ATP-PFK activities are noted in extracts of lower vascular plants. Extracts of several *Lycopodium* species and of *Equisetum* cause considerable inhibition of pineapple PPi-PFK; hence, PPi-PFK values for these plants may be higher than reported. *Lycopodium* PPi-PFK activity is activated by about 65% when Fru-2,6-bisP is added to the assay mix (Table V). Inclusion of 10 mM NaF in the extraction and assay media for *Equisetum* increased the detectable PPi-PFK activity to levels higher than the ATP-PFK activity (Table III). Fern and gymnosperm extracts inhibit the standard pineapple PPi-PFK considerably and generally neither PFK activity could be detected. Species of *Pyrrosia*, ferns reported to utilize CAM (19), show either equivalent PPi- and ATP-dependent PFK activities or only an ATP-PFK activity (Table III).

CONCLUSIONS

In conclusion, PPi-PFK has been shown to be present in *R. rubrum*, in algae (Charophytes) similar to those which presumably gave rise to vascular plants (18), and among both lower and higher plant species. Among the angiosperms, PPi-PFK is detected in C_3, C_4, and CAM species. Extractable PPi-PFK activities in C_3 and C_4 plants are roughly equivalent to or lower than respective ATP-PFK activities, whereas extractable activites 4- to 70-fold higher than respective ATP-PFK activities are noted in CAM plants in five angiosperm families.

Even though a comparative discussion of PFK activities among plants based upon detectable activities in crude extracts is fraught with uncertainty due to undefined inclusions which may activate or inhibit the enzymes in question, we cautiously present the following generalizations.

(a) PFK specific activities in CAM species tend to be higher than those in C_3 and C_4 species. ATP-PFK values in leaf extracts

of C_3, C_4, and many CAM species, including those with PPi-PFK:ATP-PFK activity ratios greater than 4, are generally between 1 and 30 nmol/min·mg protein (Tables I and II). The ATP-PFK activities we obtained are comparable to values reported or calculated from data available in the literature (Table IV). Some CAM species with little or no detectable PPi-PFK, *e.g.* Tillandsioid bromeliads, *Pleiospilos* (Aizoaceae), *S. gigantea* (Asclepiadaceae), however, exhibit higher extractable ATP-PFK specific activities, *e.g.* between 30 and 150 nmol/min·mg protein. Athough ATP-PFK values for succulent plants with PPi-PFK:ATP-PFK activity ratios greater than 4 do not differ from those of C_3 or C_4 plants, the total extractable PFK activity obviously is higher, falling between 20 and 200 nmol/min·mg protein, with a few bromeliads exhibiting values up to 600 nmol/min·mg protein. Thus, higher total PFK activities may be associated with CAM plants irrespective of the relative contributions of ATP-PFK and PPi-PFK.

(b) PPi-PFK is ubiquitously detected among CAM species with the possible exception of some bromeliads, *e.g. Tillandsia usneoides* (Table V), and its presence likely is involved in the regulation of glycolysis associated with CAM. Both Pierre and Queiroz (13) and Sutton (16, 17) present data which indicate ATP-PFK as a regulatory point for the conversion of storage carbohydrate to the malate precurser, PEP, during the acidification phase of CAM. Already, three activators of PPi-PFK (15) and over 10 modulators of ATP-PFK from a variety of non-CAM plant tissues (21) have been described. Among CAM plants, regulatory properties of ATP-PFK from *K. daigremontiana* have been examined (17); the enzyme is 100 times less sensitive to inhibition by PEP than ATP-PFK from C_3 plants and is inhibited by malate. The properties of the *Kalanchoë* ATP-PFK are consistent with the functioning of the enzyme during nighttime acidification (17). The high activities of PPi-PFK in some CAM plants make it likely that the enzyme is involved in the diurnal carbohydrate turnover characteristic of CAM (1), but clearly, data outlining the kinetics of PFK in different plants as well as substrate and effector concentrations in the locale of the PFKs are needed before we can assess the relative roles of ATP-PFK and PPi-PFK in CAM.

(c) PPi-PFK also is likely to be important in glycolysis in non-CAM plants. PPi-PFK is detected in C_3 and C_4 plants (Tables II and V) with activities generally equivalent to ATP-PFK activities. Cseke *et al.* (3) indicate that PPi-PFK is not readily detectable in spinach leaves unless the activator Fru-2,6-bisP is present. Although we detected substantial PPi-PFK in spinach with only a small stimulation by Fru-2,6-bisP under our assay conditions (Table V), detection of PPi-PFK in other C_3 and C_4 plants was dependent upon the presence of Fru-2,6-bisP.

Collectively, the results of this survey demonstrate that a PPi-PFK activity occurs in most photosynthetic organisms along with an ATP-PFK activity. Clearly much work remains to be done to elucidate the roles of the two PFK activities in plant metabolism, but the presence of the PPi-PFK in the cytoplasm (1, 3) and ATP-PFK in both the chloroplast and the cytoplasm (8) of plant cells clearly supports the possibility that two glycolytic pathways operate in green cells.

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