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MECANISMO CINETICO Y REGULACION ALOSTERICA DE LA ENZIMA FOSFOENOLPIRUVATO CARBOXILASA DE HOJA DE MAIZ

T E S I S

QUE PARA OBTENER EL GRADO DE

D O C T O R E N C I E N C I A S

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ÍNDICE

ABREVIATURAS

3-PGA	3-fosfoglicerato
Ala	alanina
C_3	plantas que fijan CO2 exclusivamente vía Ciclo de Calvin
C_4	plantas que poseen el ciclo de los ácidos dicarboxílicos (C_4) además del Ciclo de Calvin
CAM	metabolismo ácido de las crasuláceas
DTT	ditiotreitol
EDTA	ácido etilendiaminotetracético
fMg^{2+}	magnesio libre
fPEP	fosfoenolpiruvato libre
Glc6P	glucosa-6-fosfato
Gly	glicina
HEPES	ácido N-(2-hidroxietil)piperacina-N`-(2-etanosulfónico)
HPLC	cromatografía líquida de alta presión
MgPEP	complejo entre magnesio y fosfoenolpiruvato
NAD^+	dinucleótido de nicotinamida y adenina oxidado
NADH	dinucleótido de nicotinamida y adenina reducido
$NADP^+$	dinucleótido de nicotinamida y adenina-3-fosfato oxidado
NADPH	dinucleótido de nicotinamida y adenina-3-fosfato reducido
OAA	oxaloacetato
Pi	fosfato
PEG	polietilénglicol
PEP	fosfoenolpiruvato
PEPC	fosfoenolpiruvato carboxilasa
PIR	piruvato
PLP	piridoxal-5`-fosfato
SDS-PAGE	electroforesis desnaturalizante en geles de poliacrilamida

RESUMEN

La enzima fosfoenolpiruvato carboxilasa (PEPC) cataliza la formación de oxalacetato y Pi utilizando fosfoenolpiruvato (PEP) y bicarbonato en presencia de magnesio. Esta reacción es el primer paso de la vía de asimilación de CO_2 atmosférico en plantas C_4, como es el maíz. La PEPC esta sugeta a una compleja regulación alostérica que involucra cooperatividad homotrópica, efectos heterotrópicos positivos (por azúcares fosfato y aminoácidos neutros) y negativos (por ácidos dicarboxílicos). Además, la PEPC esta sugeta a regulación por modificación covalente, que modula su respuesta a los efectores alostéricos. La enzima de plantas C_4 esta fosforilada durante el día y no fosforilada durante la noche. Con el fin de obtener un mejor entendimiento del mecanismo de la regulación alostérica de la actividad de la PEPC de C_4, medimos la unión de ligandos del sitio activo y del sitio alostérico para glucosa-6-fosfato (Glc6P) a la forma no fosforilada de la PEPC de hojas de maíz. Nuestros resultados indican que el mecanismo de la regulación alostérica de la PEPC no se basa en cambios de su estado oligomérico. Para estudiar los efectos de los iones PEP y Mg^{2+} sobre la actividad de la PEPC en sus formas no fosforilada y fosforilada, realizamos determinaciones de actividad en estado estacionario, a concentraciones saturantes de bicarbonato utilizando las formas libres de PEP (fPEP) y magnesio (fMg^{2+}), en un intervalo de concentracion cercana a la fisiológica. A pH 7.3, los resultados obtenidos en la ausencia de activadores con ambas formas de la enzima, son consistentes con la unión exclusiva del complejo MgPEP al sitio activo y de fPEP a un sitio alostérico activador. A pH 8.3 y en presencia de concentracion saturante de activadores, las especies libres también se unen al sitio activo de la enzima libre, pero con constantes de disociación de por lo menos 35 veces mayores a las estimadas para el complejo MgPEP. La saturación de la enzima con Glc6P elimina la activación por fPEP, lo que es consistente con un sitio común de unión; mientras que la saturación con glicina (Gly) aumenta la afinidad del sitio alostérico por fPEP. Bajo todas la condiciones evaluadas, nuestros datos sugieren que fPEPno es capas de unirse al sitio alostérico de la enzima libre. Además, Para evaluar la cinética de la enzima en condiciones cercanas a las fisiológicas, realizamos estudios cinéticos utilizando magnesio libre 0.4 mM y bicarbonato 0.1 mM, y encontramos que ambas formas de la enzima (no fosforilada y fosforilada) muestran un alto grado de cooperatividad en la unión de PEP, una mucho menor afinidad por este substrato y por los activadores, y una mayor afinidad por malato que la que muestra a altas concentraciones de magnesio libre y bicarbonato. La inhibición por malato, fue contrarestada por aminoácidos neutros, pero no por azúcares fosfato. Concentraciones fisiológicas de Ala activan significativamente a la PEPC, sugiriendo un papel fundamental para la regulación de la actividad de PEPC de hoja de maíz por este aminoácido. Nuestros resultados, también muestran que la actividad máxima presente *in vivo* puede ser menor del 50% de la medible *in vitro* en condiciones óptimas. Por lo que, el alto nivel de proteína PEPC presente en el citosol del mesófilo de plantas C_4 (10-15% de la proteína soluble total), pueden ser una adaptación para mantener la velocidad de asimilación de CO_2 del proceso fotosíntetico de de plantas C_4, considerando las limitantes impuestas por las propiedades cinéticas de la PEPC y de las condiciones medioambientales.

ABSTRACT

Phosphoenolpyruvate carboxylase (PEPC) catalyzes the essentially irreversible formation of oxaloacetate and Pi from phosphoenolpyruvate (PEP) and bicarbonate in the presence of magnesium. This reaction is the first step in the assimilation pathway of atmospheric CO_2 in C_4 plants, such as maize. Its importance in the photosynthetic metabolism is underscored by the abundance of the PEPC protein which accounts for 10-15% of the total soluble protein of mesophyll cells. PEPC is subjected to complex allosteric regulation involving homotropic cooperativity and heterotropic effects by phosphorylated sugars and neutral amino acids (activators) and by dicarboxylic acids (inhibitors). PEPC is also subjected to covalent modification, which modulates its response to the allosteric effectors. The enzyme of C_4 plants is phosphorylated during the day and nonphosphorylated during the night. In an effort to further the understanding of the mechanism of allosteric regulation of the activity of C_4-PEPC, we measured the binding of ligands to the active and glucose-6-phosphate allosteric sites of the nonphosphorylated form of maize-leaf PEPC, our results shown that the mechanism of PEPC allosteric regulation is not based on PEPC oligomerization changes. To study the effects of PEP and Mg^{2+} ions on the activity of the non-phosphorylated and phosphorylated forms of PEPC from *Zea mays* leaves, steady-state measurements have been carried out at saturating bicarbonate using the free forms of PEP (fPEP) and Mg^{2+} (fMg^{2+}), both in a near-physiological concentration range. At pH 7.3, in the absence of activators, the initial velocity data obtained with both forms of the enzyme are consistent with the exclusive binding of the complex MgPEP to the active site and of fPEP to an activating allosteric site. At pH 8.3. and in the presence of saturating concentrations of activators, the free species also combined with the active site in the free enzyme, but with dissociation constants at least 35-times larger than that estimated for MgPEP. Saturation of the enzyme with Glc6P abolished the activation by fPEP, consistent with a common binding site, while saturation with Gly increased the affinity of the allosteric site for fPEP. Under all the conditions tested, our data suggest that fPEP is not able to combine with the allosteric site in the free enzyme. In addition, to evaluate the kinetics of the enzyme at near physiological conditions, we have performed kinetic studies at 0.4 mM free magnesium and 0.1 mM bicarbonate, and found that both the nonphosphorylated and phosphorylated enzymes exhibited a high degree of cooperativity in the binding of PEP, a much lower affinity for this substrate and for activators, and a greater affinity for malate than at high concentrations of these ions. Inhibition by malate was overcome by neutral amino acids but not by sugar phosphates. Physiological concentrations of Ala caused significant activation, suggesting a pivotal role for this amino acid in regulating maize-leaf PEPC activity. Our results also showed that the maximum enzyme activity attainable *in vivo* would be less than 50% of that attainable *in vitro* under optimum conditions. Therefore, the high levels of phospho*enol*pyruvate carboxylase protein in the cytosol of C_4-mesophyll cells might be an adaptation for sustaining the steady-state rate of flux through the photosynthetic CO_2 assimilation pathway, despite the limitations imposed by the PEPC kinetic properties and the conditions of its environment.

1. INTRODUCCIÓN

Aproximadamente de un 90 a un 95% de la materia seca de un cultivo se deriva del CO_2 atmosférico asimilado durante la fotosíntesis [Zelitch, 1979]. Por ello, la comprensión de los procesos bioquímicos responsables de la asimilación de este compuesto es del máximo interés, no sólo científico sino también económico.

El maíz pertenece al grupo de plantas con fotosíntesis C_4, que se caracteriza por un alto nivel de eficiencia en su crecimiento bajo condiciones extremas de luz, altas temperaturas y niveles elevados de oxígeno. Las bases moleculares de dichas características estriban en la existencia de la ruta metabólica de los ácidos dicarboxílicos (ciclo C_4) que minimiza la pérdida de energía debida a la fotorrespiración [Leegood & Osmond, 1990].

La enzima fosfoenolpiruvato carboxilasa (PEPC, E.C. 4.1.1.31) juega un papel clave en la asimilación fotosintética de CO_2 atmosférico en las plantas C_4, pues cataliza la primera reacción de fijación del CO_2 en el metabolismo C_4 [Hatch, 1978]. Debido a ello, la PEPC es objeto de numerosas investigaciones con el fin de llegar a entender su funcionamiento y regulación *in vivo*. Sin embargo, el mecanismo cinético y la regulación alostérica de esta enzima no han sido completamente elucidados hasta la fecha. Por lo anterior, es de gran interés llevar a cabo un estudio a profundidad de estos dos importantes aspectos funcionales de la PEPC de hoja de maíz.

2. ANTECEDENTES

Los antecedentes están divididos en tres apartados principales: el metabolismo C_4 presente en las hojas de maíz, la enzima PEPC de plantas C_4 y su regulación, con especial atención en la enzima de maíz.

2.1. El metabolismo C_4 (tipo enzima $NADP^+$-málico)

Existen tres tipos del metabolismo C_4 dependiendo de la enzima que cataliza la descarboxilación del malato en la vaina vascular. Así existen plantas que usan a la enzima $NADP^+$-málico, otras a la enzima NAD^+-málico y otras a la enzima fosfoenolpiruvato carboxicinasa (PEPCK) [Leegood & Osmond, 1990]. A continuación se presenta un resumen del metabolismo C_4 que presentan las plantas de maíz (tipo enzima $NADP^+$-málico) y cuyo esquema se muestra en la figura 2.1. [página 3].

Dos diferentes clases de células cooperan durante la fotosíntesis en las plantas C_4, las llamadas células del mesófilo y las células de la vaina vascular. Las células de la vaina vascular presentan cloroplastos sin grana y poseen la enzima málica-$NADP^+$ y las enzimas del Ciclo de Calvin. Estos cloroplastos son deficientes en fotosistema II y son incapaces de realizar el transporte de electrones no cíclico que produce O_2 y NADPH, aunque pueden producir ATP mediante el transporte de electrones cíclico. En contraste los cloroplastos de las células del mesófilo realizan tanto el transporte de electrones no cíclico como el cíclico (produciendo O_2, NADPH y ATP) y no contienen ni la enzima descarboxilante ni las enzimas del Ciclo de Calvin. Otra diferencia importante entre los dos tipos de células de las hojas de plantas con metabolismo C_4, es que mientras la acumulación de almidón está restringida a los cloroplastos de las células de la vaina vascular, la síntesis de sacarosa se lleva a cabo principalmente en el citosol de las células del mesófilo [Leegood & Osmond, 1990].

Esta especialización celular permite suprimir la liberación de O_2 en las células de la vaina vascular manteniendo altas concentraciones de CO_2 en éstas células, favoreciendo la carboxilación de ribulosa 1,5-bifosfato; que requiere de la rápida transferencia del carbono fijado y la energía generados en ambos tipos de células.

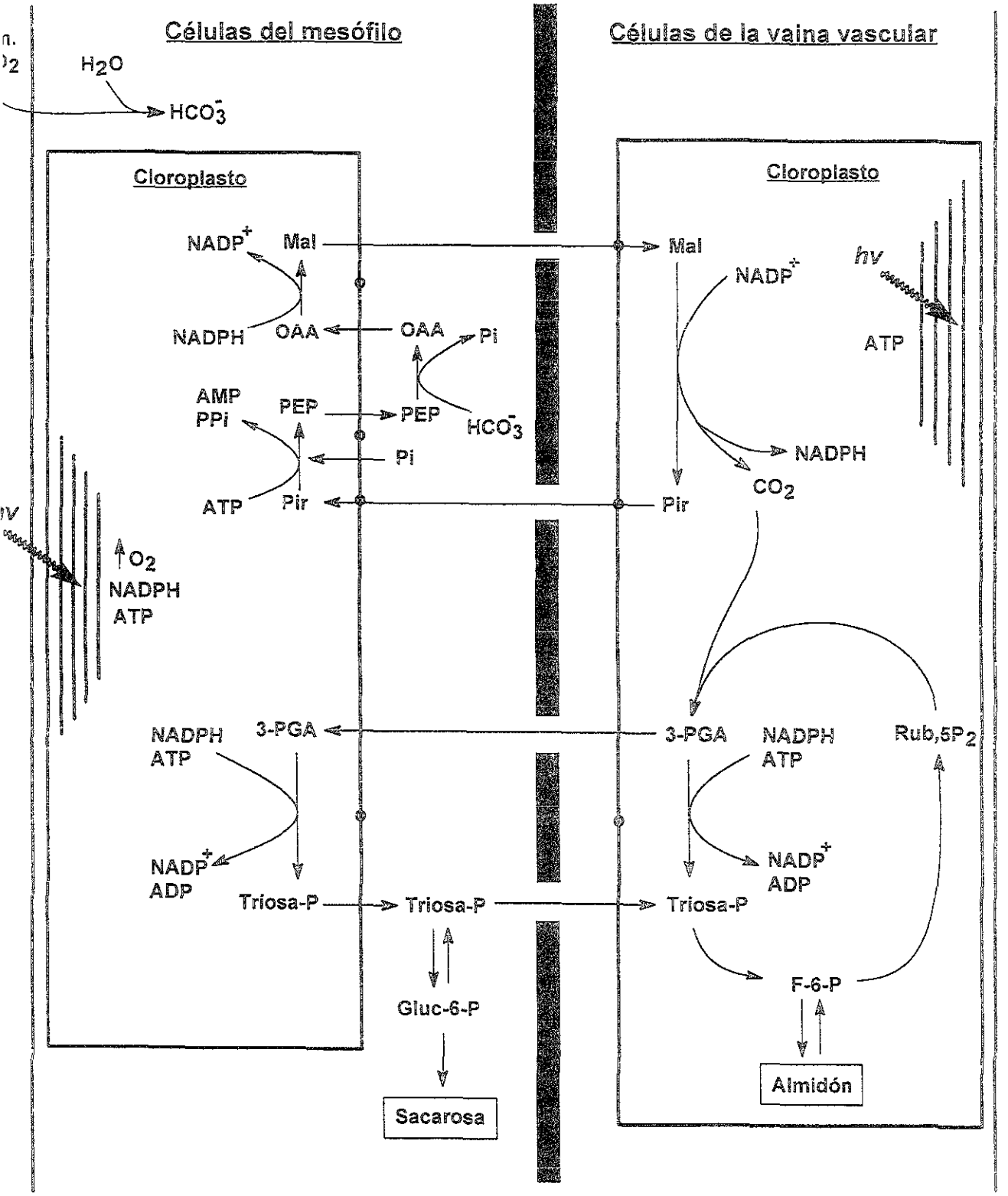


Figura 2.1. Metabolismo C_4 (tipo enzima $NADP^+$-málico) presente en las hojas de maíz [adaptado de Leegood & Osmond, 1990].

Dos intercambios de metabolitos entre los dos tipos de células permiten el proceso. En primer lugar, un intercambio de malato y de piruvato permite proporcionar NADPH y CO_2 al Ciclo de Calvin que se realiza en las células de la vaina vascular: El malato se mueve desde las células del mesófilo a los cloroplastos de las células de la vaina vascular donde es convertido en CO_2, NADPH y piruvato por la enzima málica-$NADP^+$. El piruvato regresa al mesófilo donde es transformado en malato por una secuencia de reacciones que involucran su conversión a PEP por la piruvato fosfato dicinasa (PPDK) en los cloroplastos, la carboxilación del PEP por la PEPC en el citosol y la reducción del oxalacetato a malato por la $NADP^+$-malato deshidrogenasa en el cloroplasto. El CO_2 liberado en las células de la vaina vascular es re-fijado por la ribulosa bisfosfato carboxilasa oxigenasa (RUBISCO) produciendo dos moléculas de 3-fosfoglicerato (3-PGA). Sólo una de estas moléculas puede ser reducida utilizando el NADPH transportado por el intercambio malato/piruvato, por lo que se requiere un segundo intercambio entre la vaina vascular y el mesófilo: el 3-PGA no reducido en la vaina vascular se mueve a los cloroplastos del mesófilo donde es reducido para formar triosas fosfato. Al menos dos terceras partes de estas triosas fosfato retornan al Ciclo de Calvin en los cloroplastos de las células de la vaina vascular, mientras que la restante tercera parte se utiliza para la síntesis de sacarosa en el mesófilo [Leegood & Osmond, 1990].

Así, la concentración de metabolitos del Ciclo de Calvin (triosas fosfato y 3-PGA) y del ciclo C_4 (malato y PEP) aumenta como consecuencia de la inducción de la fotosíntesis por luz [Leegood, 1985; Stit & Heldt, 1985a]. Estos cambios de concentración son importantes ya que el movimiento de metabolitos entre los dos tipos de células presentes en las plantas C_4, se dá por diferencias de concentración. El mayor gradiente de concentración entre los dos tipos de células fotosintéticas (células del mesófilo y de la vaina vascular) es el de malato, por lo cual una alta concentración de malato está presente en las células del mesófilo [Leegood & Osmond, 1990] y que se ha estimado puede ser del orden de 10 a 20 mM [Stitt & Heldt, 1985].

Como puede verse [Figura 2.1., página 3], la PEPC realiza la fijación inicial del CO_2 atmosférico; por lo cual, la regulación de esta enzima permite la integración del ciclo C_4 con el Ciclo de Calvin y con otras rutas biosintéticas. Dada la elevada concentración de malato, metabolito que inhibe la enzima PEPC como se describe en la sección 2.3.1, (de 20 a 40 mM) y la baja concentración de PEP (aproximadamente 3 mM), presente en las células del mesófilo en los períodos de luz, la PEPC se encontraría totalmente inhibida en los períodos de luz [Leegood & Osmond, 1990]. Sin embargo, la actividad de la PEPC en la luz se ha explicado como consecuencia de la modulación positiva de la enzima por fosforilación y por regulación metabólica, como se detalla en la sección 2.3.

2.2. Fosfoenolpiruvato carboxilasa de plantas C_4

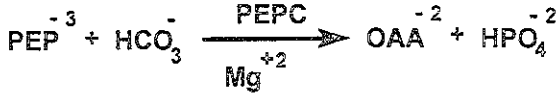
Las PEPC de plantas se han clasificado en cuatro grupos [Ting & Osmond, 1973c]: 1) PEPC de hojas de plantas C_3 con una baja $Km_{(PEP)}$ y una baja actividad específica, 2) PEPC de hojas de plantas C_4 con una alta $Km_{(PEP)}$ y una actividad específica elevada, 3) PEPC de hojas de plantas CAM con una baja $Km_{(PEP)}$ y una elevada actividad específica y 4) PEPC de tipo no autotrófico ó de tejidos no verdes con una $Km_{(PEP)}$ y actividad específica baja. Además, las diferentes isoformas de PEPC de plantas superiores se pueden agrupar en dos grandes categorías atendiendo a sus funciones [Toh *et al.*, 1994]: 1) PEPC de hojas de plantas C_3 y de tejidos no fotosintéticos de todo tipo de plantas, que está involucrada en funciones anapleróticas como es el reabastecimiento de oxalacetato al ciclo del ácido cítrico para la síntesis de aminoácidos y porfirinas, y 2) PEPC de tejido fotosintético de plantas C_4 y CAM, que está asociada a la fijación inicial en el proceso fotosintético de estas plantas [Hatch, 1987].

A la fecha existen varias revisiones bibliográficas sobre la enzima PEPC de plantas [O'Leary, 1982; O'Leary, 1983; Andreo *et al.*, 1987; Deroche & Carrayol, 1988; Jiao & Chollet, 1991; Rajagopalan *et al.*, 1994; Chollet *et al.*, 1996]. A continuación se resume la información existente acerca de la enzima de hoja de plantas C_4 que es

pertinente para enmarcar teóricamente el presente trabajo, con algunas referencias a las otras isoformas cuando es necesario.

2.2.1. Reacción catalizada

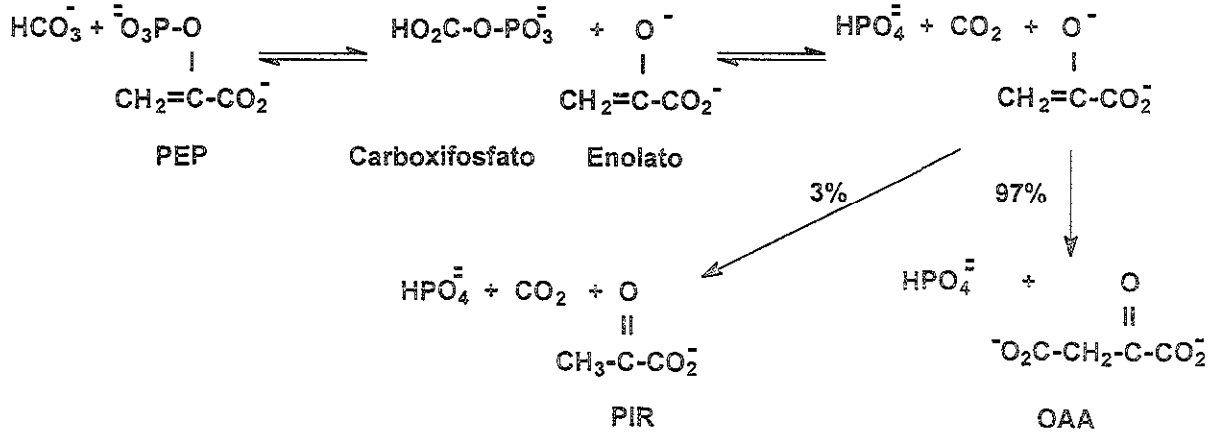
PEPC cataliza la β-carboxilación, irreversible, de PEP para producir oxalacetato y fosfato inorgánico [Bandurski *et al.*, 1953; Bandurski & Greiner, 1953].



Esta reacción es altamente exergónica (ΔG^{o} = -7.2 Kcal/mol) y requiere un catión divalente que fisiológicamente parece ser el Mg^{2+} [Bandurski, 1955; Tchen & Vennesland, 1955], aunque *in vitro* la enzima puede usar Mn^{2+} ó Co^{2+} [Miziorko *et al.*, 1974; O'Leary, 1981; Nguyen *et al.*, 1988]. A diferencia de otras reacciones de carboxilación catalizadas enzimáticamente donde el substrato es bicarbonato, la reacción de PEPC no es dependiente de biotina [Cooper & Wood, 1971; Mukerji, 1977].

2.2.2. Mecanismo químico

La reacción catalizada por la PEPC involucra la formación de la forma enólica de piruvato, la cual es estabilizada por el ión magnesio [Ausenhus & O`Leary, 1992]. El mecanismo químico de catálisis propuesto para esta enzima se ilustra a continuación:



El primer paso de la reacción consiste en la transferencia del fosfato del PEP al bicarbonato para formar carboxifosfato y el ión enolato. Después, el carboxifosfato se descompone y forma CO_2 y Pi. El CO_2 es el agente nucleofílico que ataca al enolato del piruvato generando el oxalacetato. Los dos primeros pasos de la reacción son reversibles. Además, se sabe que la reacción no es 100% eficiente, ya que a pH 7.8 y en presencia de magnesio, aproximadamente el 3% del CO_2 proveniente del carboxifosfato se escapa de la enzima y el intermediario enólico forma piruvato espontáneamente [Chollet *et al.*, 1996].

2.2.3. Características moleculares

La PEPC de hoja de maíz y de otras plantas C4 es un homotetrámero [Ting & Osmond, 1973b; Uedan & Sugiyama, 1976]. El monómero de la enzima de maíz tiene una masa molecular de 109408 Da según su cDNA [Matsuoka & Minami, 1989], que es similar a la masa molecular aparente (109 kDa) de la enzima purificada en presencia del inhibidor de proteasas quimostatina [McNauhton *et al.*, 1989].

Recientemente, se ha reportado la estructura cristalina del complejo del inhibidor aspartato con la PEPC de *Escherichia coli* [Kai *et al.*, 1999]. Las cuatro subunidades del tetrámero están arregladas en forma de "dímero de dímeros". Cada subunidad posee un barril α/β, que está formado por 8 hojas plegadas β rodeadas por 40 hélices α.

Estudios de mutagénesis dirigida han indicado que los residuos His-138, His-579, Arg-587, Arg-581 y Arg-396 son esenciales para la actividad de la PEPC y que el residuo Lys-546 está involucrado en la unión del substrato bicarbonato, por lo cual se sugiere que el sitio activo se ubica cerca del extremo carboxilo del barril-β. El sitio de unión de aspartato se localiza a 20 Å del sitio activo; cuatro residuos (Lys-773, Arg-832, Arg-587 y Asn-881) están involucrados en la unión del inhibidor. Como la Arg-587 es catalíticamente esencial y se encuentra en una asa rica en glicina altamente conservada en la escala evolutiva, se ha sugerido que aspartato causa inhibición por separación de este asa del sitio activo [Kai *et al.*, 1999].

En adición al sitio activo y al sitio alostérico de unión de ácidos dicarboxílicos como aspartato y malato, la enzima de hojas de maíz tiene otros dos sitios alostéricos, uno al que se unen hexosas y triosas fosfato [Tovar-Méndez *et al.*, 1997] y otro al que se unen aminoácidos neutros [Bandarian *et al.*, 1992]. En base a resultados obtenidos en estudios de modificación química se ha sugerido que el primero de estos dos sitios alostéricos, mejor conocido como sitio alostérico para Glc6P, posee residuos de histidina [Taghizadeh *et al.*, 1991], de cisteína [Wedding *et al.*, 1989], de lisina [Wu & Wedding, 1994; Tovar-Méndez *et al.*, 1997] y de ácido aspártico ó glutámico [Maralihalli & Bhagwat, 1993] involucrados en la unión de sus ligandos. Por otra parte, no existe a la fecha ninguna información sobre la estructura del sitio alostérico para aminoácidos neutros.

2.2.4. Propiedades cinéticas

Esta sección se ha dividido en cinco apartados con el fin de resaltar las diferentes características cinéticas de la enzima.

a) Saturación por substratos: La enzima de hojas de plantas C_4 muestra cooperatividad homotrópica positiva en la cinética de saturación por PEP a pH 7 o cercanos a 7 [Uedan & Sugiyama, 1976; Hayakawa *et al.*, 1981; Rodríguez-Sotres & Muñoz-Clares, 1986], y no muestra cooperatividad a valores de pH alrededor de 8 [Uedan & Sugiyama, 1976; Mukerji, 1977; O`Leary *et al.*, 1981; Doncaster & Leegood, 1987].

La saturación de la PEPC de hoja de maíz por magnesio parece no presentar cooperatividad [Uedan & Sugiyama, 1976; Nguyen *et al.*, 1988], aunque también se ha encontrado cooperatividad negativa en la saturación por este metal de la enzima de hojas de maíz [Mukerji, 1977] y de *Crassula argentea* [Nguyen *et al.*, 1988].

La saturación de la enzima de hojas de plantas C_4 por bicarbonato ha sido reportada como no cooperativa [Uedan & Sugiyama, 1976; Bauwe, 1986; Jancet *al.*, 1992; Dong *et al.*, 1997], aunque un reporte reciente dice que si lo es [Parvathi*et al.*, 1998].

b) Mecanismo cinético: En la literatura hay dos propuestas diferentes para el mecanismo de unión de magnesio y de PEP al sitio activo de la PEPC de hoja de maíz.

1.- Estudios cinéticos realizados con la forma no fosforilada de la enzima, en los que se consideraron las especies libres y el complejo de magnesio y PEP (MgPEP), indican que el complejo MgPEP es el verdadero substrato [Mukerji, 1977; Wedding *et al.*, 1989; Rodríguez-Sotres & Muñoz-Clares, 1990] y que PEP libre (fPEP) [Rodríguez-Sotres & Muñoz-Clares, 1990] o magnesio libre (fMg^{2+}) [Mukerji, 1977] se comportan como activadores de la enzima de hoja de maíz.

2.- A partir de estudios cinéticos realizados con la enzima no fosforilada y usando concentraciones totales de PEP y magnesio, se ha concluido que la unión de magnesio a la enzima precede la unión de PEP y de bicarbonato, y que la unión de estos es al azar, pero que el orden preferido es la unión de PEP seguida de la unión de bicarbonato [Janc *et al.*, 1992].

c) Activación por PEP libre: La activación de la enzima de hoja de maíz por el 2-dihidroxifosfinoilmetil-2-propenoato ($MeCH_2PEP$), un análogo de PEP que tiene el carboxilo metilado y un fosfonato en lugar de fosfato, ha sugerido que la PEPC posee dos sitios a los que el PEP se une: el sitio activo y un sitio regulatorio, y que el $MeCH_2PEP$ se une preferencialmente al sitio regulatorio [Jenkins *et al.*, 1986]. Igual conclusión se alcanzó con el estudio de la cinética de activación de la enzima de maíz por otro análogo de PEP, el fenil fosfato [Rodriguez-Sotres & Muñoz-Clares, 1990]. Adicionalmente, estudios de unión sugieren que PEP se une tanto al sitio activo como al sitio alostérico para el activador Glc6P [Rustin *et al.*, 1988; Rustin *et al.*, 1991]. A este respecto, se ha sugerido que aunque el sitio activo y este sitio alostérico parecen compartir numerosas características estructurales ya que unen los mismos ligandos, difieren en la forma del ligando que unen: mientras que el sitio activo une al complejo Mg-ligando, el sitio alostérico para Glc6P une la forma libre del ligando [Mújica-Jiménez *et al.*, 1998]. Esta diferencia es crucial para que los activadores, que se encuentran mayoritariamente en forma no acomplejada con el

magnesio *in vivo*, no inhiban a la enzima por competencia con el substrato por el sitio activo [Mújica-Jiménez *et al.*, 1998].

2.3. REGULACIÓN DE LA PEPC DE PLANTAS C_4

La actividad de la enzima es regulada por ciertos metabolitos del medio intracelular y por modificación covalente reversible (fosforilación). A continuación se describe dicha regulación, información que se resume en la tabla 2.1. [página 16].

2.3.1. Regulación por metabolitos

Se han descrito tres tipos de metabolitos efectores de la actividad de PEPC. Estos metabolitos tienen efecto más pronunciado a valores de pH neutros.

a) Acidos dicarboxílicos como malato y aspartato son inhibidores de todas las PEPC estudiadas hasta el momento. En el caso de la enzima de maíz, el inhibidor de mayor relevancia fisiológica es malato [Huber & Edwards, 1975; Leegood & Osmond, 1990]. Malato se comporta como un inhibidor competitivo en la saturación de la enzima por PEP a pH neutro [González *et al.*, 1984] y aumenta la intensidad de la cooperatividad presente en la saturación de la enzima por PEP [Wu & Wedding, 1985].

b) Azúcares fosforilados como Glc6P activan la enzima de hojas de plantas C_4 [Coombs *et al.*, 1973; Huber & Edwards, 1975; Uedan & Sugiyama, 1976; Marâres & Leblová, 1980; Stiborová & Leblová, 1985] y CAM [Rusting *et al.*, 1988; Wedding *et al.*, 1989]. Su principal efecto es disminuir la *Km* para el substrato PEP y aumentar ligeramente la *Vmax* [Coombs *et al.*, 1973; Huber & Edwards, 1975; Uedan & Sugiyama, 1976; Marâres & Leblová, 1980; Stiborová & Leblová, 1985]. Además, la Glc6P elimina la cooperatividad de la cinética de saturación por el substrato PEP [Coombs *et al.*, 1973; Uedan & Sugiyama, 1976]. Se ha reportado que a valores de pH de 7 o menores, la saturación por Glc6P es cooperativa, pero a valores de pH 8 o mayores no lo es [Stiborová & Leblová, 1985]. Otro factor que afecta la afinidad de la PEPC por Glc6P es el estado de saturación del sitio activo. Así, la enzima con el sitio activo saturado presenta una constante de activación aparente para Glc6P menor que en presencia de concentraciones de substrato subsaturantes [Duff *et al.*, 1995].

Triosas fosfato como la DHAP y el 3-PGA [Doncaster & Leegood, 1987; Selioniti *et al.*, 1985; Coombs *et al.*, 1973; Wong & Davies, 1973] y hexosas fosfato como manosa-6-fosfato y galactosa-6-fosfato [Bandarian*et al.*, 1992; Doncaster & Leegood, 1987] activan a la enzima PEPC al unirse al mismo sitio que Glc6P [Tovar-Méndez *et al.*, 1997].

c) Aminoácidos neutros activan exclusivamente a la PEPC de hoja de plantas C_4 monocotiledóneas [Nishikido & Takanashi, 1973]. Gly aumenta ligeramente la *Vmax* y disminuye en mucha mayor proporción la *Km* para el substrato PEP [Nishikido & Takanashi, 1973; Uedan & Sugiyama, 1976; Stiborová & Leblová, 1985]. Gly también reduce la *Km* para magnesio, pero no tiene un efecto significativo sobre la *Km* para bicarbonato [Gillinta & Grover, 1995]. La enzima puede ser activada también por Ala [Garson & Gray, 1991., Bandarian*et al.*, 1992] y por Ser [Bandarian *et al.*, 1992], sin embargo, se ha encontrado que posee mayor afinidad por Gly que por los otros aminoácidos neutros [Bandarian *et al.*, 1992].

2.3.2 Regulación por fosforilación

La fosforilación de la enzima disminuye la $S_{0.5}$ para PEP, aunque la diferencia entre la forma no fosforilada y la fosforilada es pequeña (dos veces) [Duff*et al.*, 1995]. Algunos reportes indican que la fosforilación de la PEPC aumenta la *Vmax* [Duff *et al.*, 1995]; aunque otros reportes sugieren que la fosforilación no conlleva a ningún aumento en este parámetro cinético [Karabourniotis *et al.*, 1983; Huber *et al.*, 1986; Doncaster & Leegood, 1987; Nimmo *et al.*, 1987].

Por otra parte, la fosforilación de la PEPC produce una fuerte disminución (de hasta 10 veces) en la afinidad aparente de la PEPC por el inhibidor malato [Huber *et al.*, 1986; Doncaster & Leegood, 1987; Rodríguez-Sotres*et al.*, 1987; Echevarria *et al.*, 1994; Duff *et al.*, 1995]. Además, se ha reportado que la fosforilación de PEPC aumenta la afinidad aparente de la enzima por Glc6P [Duff*et al.*, 1995] y por Gly [Bakrim *et al.*, 1998],

Se ha planteado un modelo para la regulación de la enzima por fosforilación-defosforilación [Jiao & Chollet, 1991; Chollet *et al.*, 1996] el cual propone que la

forma no fosforilada es menos activa, más sensible a inhibición por malato y menos sensible a activación por Glc6P, que la forma fosforilada.

En todas las PEPC esta regulación se lleva a cabo por fosforilación y desfosforilación del grupo hidroxilo de una Ser en el extremo amino terminal de la proteína, que es la Ser-15 en el caso de la PEPC de hoja de maíz [Jiao & Chollet, 1989] y Ser-8 en el caso de la enzima de sorgo [Lepiniec *et al.*, 1994]. La fosforilación también se ha documentado en la enzima tipo CAM [Nimmo*et al.*, 1986; Nimmo *et al.*, 1987] y tipo C_3 [Zhang *et al.*, 1995; Duff & Chollet, 1995].

En hojas de plantas C_4 la fosforilación de la PEPC se induce por luz, de manera que la forma fosforilada está presente durante el día [Jiao *et al.*, 1991; Jiao & Chollet, 1991; Duff & Chollet, 1995]. La fosforilación y desfosforilación de PEPC son catalizadas respectivamente por la PEPC-cinasa [Carter *et al.*, 1991; Jiao *et al.*, 1991; Jiao & Chollet, 1991] y una proteína fosfatasa tipo A2 [Carter*et al.*, 1990; McNaughton *et al.*, 1991].

El mecanismo por el cual se regula la PEPC-cinasa en hojas de plantas C_4 aún no se conoce en detalle, aunque se ha propuesto que el 3-PGA (originado en el Ciclo de Calvin en las células de la vaina vascular) al entrar al citosol de las células del mesófilo es parcialmente protonado a su forma dianiónica, y que luego entra al cloroplasto. Dicha protonación incrementa el pH del citosol en las células del mesófilo. Este cambio de pH, asociado con un subsecuente aumento en la concentración citosólica del ión calcio (que se presume proviene de la vacuola) puede causar un incremento en la síntesis de la PEPC-cinasa y así, aumentar la actividad de la PEPC-cinasa; que finalmente, se traduce en la fosforilación de la PEPC [Giglioli-Guivarc`h *et al.*, 1996].

2.3.3. La modulación de la PEPC durante los períodos de luz

Como se mencionó anteriormente, la actividad de la PEPC bajo las condiciones prevalecientes en los períodos de luz (bajas concentraciones de PEP y altas concentraciones de malato) se ha explicado como consecuencia de la modulación positiva de la enzima por fosforilación y por regulación metabólica

Se considera que la activación por triosas fosfato y hexosas fosfato es un medio de contrarrestar la inhibición inducida por malato. Se ha sugerido que esta activación determina la respuesta de la PEPC al suplemento de 3-PGA durante la fotosíntesis, desde el Ciclo de Calvin a las células del mesófilo [Leegood & Osmond, 1990]. A este respecto, se ha estimado que en las células del mesofilo durante períodos de luz puede tener una concentración de 2-3 mM de Glc6P y de hasta 10 mM de triosas fosfato [Stitt & Heldt, 1985b].

También, se ha sugerido que la activación por aminoácidos neutros como Gly puede ser un medio fisiológico para contrarrestar la inhibición por malato [Bandarian *et al.*, 1992; Gillinta & Grover, 1995; Gao & Woo, 1996]. A este respecto, Gly puede actuar como una señal del metabolismo de fotorrespiración de las células de la vaina vascular [Bandarian *et al.*, 1992; Gillinta & Grover, 1995], de manera que la activación de la PEPC por Gly sería un medio para minimizar la fotorrespiración en las células de la vaina vascular. Sin embargo, la concentración estimada de Gly en las células del mesófilo durante los periodos de luz en muy baja (2-3 mM) [Weiner & Heldt, 1992].

Finalmente, se considera a la fosforilación de la enzima como un proceso fundamental ya que al disminuir su sensibilidad frente a la inhibición por malato, favorece la actividad durante los períodos de luz [Leegood & Osmond, 1990; Chollet *et al.*, 1996]. Sin embargo, se ha sugerido que la fosforilación por si no es suficiente para permitir la actividad de la enzima durante los períodos de luz [Bakrim *et al.*, 1993; Gao & Woo, 1996].

Se ha reportado que Glc6P y Gly contrarrestan la inhibición por malato en forma sinérgica [Gillinta & Grover, 1995], aunque Gly es más efectivo que Glc6P en contrarrestar dicha inhibición [Gao & Woo, 1996]. En adición, Gly aumenta la afinidad de la enzima por Glc6P [Gillinta & Grover, 1995] y viceversa [Mújica-Jiménez *et al.*, 1998]. La fosforilación de la PEPC también actúa de manera sinérgica con ambos tipos de activadores de la enzima contrarrestando la inhibición de la PEPC por malato [Gao & Woo, 1996; Bakrim *et al.*, 1998], por lo que se ha sugerido que la actividad de la PEPC y, así, la fijación atmosférica del CO_2 es el resultado del balance entre la

regulación positiva (activación por triosas fosfato, Glc6P y aminoácidos neutros como Gly), la regulación negativa (inhibición por malato) y la fosforilación, que refuerza el efecto de los efectores positivos [Bakrim *et al.*, 1998].

2.3.4. Mecanismo de regulación alostérica

Como se ha mencionado anteriormente, la PEPC de hoja de plantas C_4 es una enzima alostérica que a valores de pH cercanos a la neutralidad presenta efectos homotrópicos y heterotrópicos. El mecanismo subyacente a estos efectos no ha sido bien establecido. Sin embargo, se han propuesto los siguientes modelos:

a) Modelo de asociación-disociación oligomérica [Podestá & Andreo, 1989; Willeford *et al.*, 1990]. Este modelo plantea que la enzima PEPC de maíz puede ser regulada *in vivo* vía un equilibrio dímero-tetrámero, donde la forma tetramérica sería la forma totalmente activa y con la mayor afinidad por el substrato, siendo el dímero mucho menos activo o inactivo y el monómero totalmente inactivo. El modelo es apoyado por las siguientes observaciones: (1) la enzima diluida a pH 7 y a concentraciones menores de 0.1 mg/ml es una mezcla de dímeros y tetrámeros [Podestá & Andreo, 1989], (2) El substrato PEP desplaza el equilibrio hacia la forma tetramérica [Willeford *et al.*, 1990], lo cual explicaría la cooperatividad positiva observada en las cinéticas de saturación por PEP. (3) El activador Glc6P también favorece la forma tetramérica [Willeford & Wedding, 1992], lo que explicaría la ausencia de cooperatividad positiva y el aumento de la afinidad por PEP en la presencia de Glc6P. (4) El inhibidor malato, que favorece la disociación del tetrámero en dímero [Willeford *et al.*, 1990], tiene efectos contrarios a Glc6P, disminuye la afinidad por el substrato y aumenta la cooperatividad positiva por el substrato. (5) Glicerol al 20% (v/v) elimina la cooperatividad positiva de la enzima en la cinética de saturación por PEP, lo que concuerda con que la enzima diluida en la presencia de estas altas concentraciones de glicerol se encuentra principalmente en forma tetramérica [Podestá & Andreo, 1989].

b) Mecanismo concertado de transición alostérica. Jawali (1990) ha sugerido que en presencia de iones magnésio la enzima (formando un complejo con magnesio) se encuentra en una forma relajada (R) y que ésta se transforma en la forma tensa (T) cuando PEP se une a la enzima, en un proceso reversible. La forma R de la enzima tiene una menor afinidad por los substratos. El autor se basa en las siguientes observaciones hechas a pH 7: (1) la cinética de unión del ion magnesio a la enzima libre es hiperbólica y cambia a una cinética con cooperativad positiva en la presencia de 2-fosfoglicerato (un análogo de PEP) y (2) la cinética de estado estacionario para el ión magnesio tiene cooperatividad positiva y cambia a hiperbólica en la presencia de 2-fosfoglicerato.

Recientemente, Frank *et al.* (1999) proponen que en presencia de iones magnesio la PEPC existe en dos formas I y II, con diferentes afinidades por PEP. La forma I es la conformación con baja afinidad por PEP, y la forma II en la conformación con mayor afinidad por PEP. Ambas formas están en equilibrio y la unión de PEP a la forma II puede modificar el equilibrio favoreciendo la forma II. Estos autores se basan en que el cambio en la fluorescencia extrínseca de la enzima, medido por mezclado rápido, inducido por la unión de PEP en la presencia de iones magnesio presenta dos tiempos de relajación. Un tiempo de relajación rápido asociado al proceso de unión de PEP y un tiempo de relajación lento atribuido al proceso de isomerización entre las formas I y II.

Tabla 2.1. Resumen del efecto de metabolitos y de la fosforilación de la PEPC sobre las propiedades cinéticas regulación de la PEPC de plantas C_4.

Ácidos dicarboxílicos como malato, inhiben la enzima disminuyendo la afinidad por PEP [González *et* al., 1984] y aumentando la intensidad de la cooperatividad presente en la saturación de la enzima por PEP [Wu & Wedding, 1985].
Azúcares fosforilados como Glc6P, activan la enzima aumentando la afinidad por PEP, aumentan ligeramente la *Vmax* [Coombs *et al.,* 1973; Huber & Edwards, 1975; Uedan & Sugiyama, 1976] y eliminan la cooperatividad presente en la saturación de la enzima por PEP [Coombs *et al.,* 1973; Uedan & Sugiyama, 1976]. Además, los azúcares fosforilados disminuyen la inhibición de la enzima por malato [Leegood & Osmond, 1990], y aumentan la afinidad de la enzima por Gly [Mújica-Jiménez *et al.*, 1998].
Aminoácidos neutros como Gly, activan la enzima aumentando la afinidad por PEP, aumentan ligeramente la *Vmax* [Nishikido & Takanashi, 1973; Uedan & Sugiyama, 1976; Stiborová & Leblová, 1985] y también aumentan la afinidad de la enzima por magnesio [Gillinta & Grover, 1995]. Además, los aminoácidos neutros disminuyen la inhibición de la enzima por malato [Leegood & Osmond, 1990], y aumentan la afinidad de la enzima por Glc6P [Mújica-Jiménez *et al.*, 1998].
La fosforilación de la PEPC aumenta la afinidad de la enzima por PEP [Duff*et al.*, 1995] y disminuye la afinidad de los ácidos dicarboxílicos (malato) [Huber*et al.*, 1986; Doncaster & Leegood, 1987; Rodríguez-Sotres *et al.*, 1987; Echevarria *et al.*, 1994; Duff *et al.*, 1998]. La fosforilación aumenta la *Vmax* de la enzima [Duff *et* al., 1995] aunque hay reportes que no soportan este efecto [Karabourniotis *et al.*, 1983; Huber *et al.*, 1986; Doncaster & Leegood, 1987; Nimmo *et al.*, 1987]. Además la fosforilación aumenta la afinidad aparente de la enzima por Glc6P [Duff *et al.*, 1995] y por Gly [Bakrim *et al.*, 1998].

3. PLANTEAMIENTO DEL PROBLEMA

Gran parte de los estudios cinéticos sobre PEPC reportados hasta la fecha han sido realizados usando enzima purificada en condiciones en las que se proteoliza el extremo NH2-terminal [McNaugthon *et al.*, 1989; Ausenhus & O'Leary, 1992; Wang *et al.*, 1992], que incluye al sitio de fosforilación [Jiao & Chollet, 1989; Lepiniec *et al.*, 1994]. Dicha proteólisis disminuye la sensibilidad de PEPC frente a la inhibición por malato [Ausenhus & O'Leary, 1992, Wang *et al.*, 1992] y probablemente aumente la sensibilidad frente a activación por Glc6P y por Gly [Qun & Jiao-nai, 1988]. Por ello, los resultados obtenidos con PEPC sin el extremo NH2-terminal no necesariamente reflejan las características cinéticas y alostéricas de la PEPC nativa.

Además, aspectos importantes de la PEPC como el mecanismo cinético, la importancia del proceso de asociación-disociación en la regulación alostérica y el papel de los factores que modifican la actividad de la enzima a la concentración de substratos cercana a la fisiológica, no han sido bien establecidos. Por lo que nos propusimos en este trabajo hacer un estudio a profundidad de estos aspectos de la enzima PEPC de hoja de maíz.

HIPÓTESIS

Si las propiedades alostéricas de la PEPC nativa de hoja de maíz descritas en estudios cinéticos de estado estacionario no dependen exclusivamente del estado de agregación de la enzima, entonces, la forma tetramérica de la PEPC poseerá propiedades, tanto homotrópicas como heterotrópicas.

4. OBJETIVOS

Objetivo General

Establecer el mecanismo cinético y la regulación alostérica de la PEPC de hojas de maíz (*Zea mays*) en condiciones cercanas a las presentes *in vivo*.

Objetivos Particulares

1. Determinar las propiedades alostéricas homotrópicas y heterotrópicas de la forma tetramérica de la enzima. Para ello:

1.1. Establecer la relación concentración-estado oligomérico de la enzima.

1.2. Determinar la cinética de inactivación de la PEPC usando concentraciones de enzima similares a las usadas en los estudios cinéticos, en ausencia y presencia de malato

1.3. Caracterizar la unión de los ligandos del sitio activo a la forma tetrámerica de la enzima.

1.4. Caracterizar la unión de Glc6P a la forma tetramérica de la enzima.

1.5. Evaluar el efecto que otros ligandos de la enzima y cosolutos como glicerol y PEG tienen sobre la unión de ligandos del sitio activo y del sitio alostérico de Glc6P.

2. Establecer la(s) especie(s) de magnesio y de PEP que reconoce(n) el sitio activo de la PEPC e investigar el posible papel regulador de las formas libres. Para ello:

2.1. Determinar la cinética de la saturación de la enzima por fMg^{2+} y fPEP en un rango de concentración cercano al fisiológico (de 0.25 a 4 mM) y a concentración de bicarbonato saturante.

2.2. Determinar los efectos del pH (7.3 y 8.3) sobre la activación alostérica por Glc6P, por Gly y por la fosforilación de la enzima sobre la cinética de la saturación indicada en el objetivo 1.1.

3. Establecer las características cinéticas de la enzima en la presencia de las concentraciones de substratos y protones estimadas en el citosol de células del mesófilo de maíz. Para ello:

3.1. Determinar los parámetros cinéticos de la saturación de la enzima por fosfoenolpiruvato a pH 7.3, fMg^{2+} 0.4 mM y bicarbonato 0.1 mM.

3.2. Determinar los parámetros cinéticos de la saturación de la enzima por los efectores alostéricos Glc6P, Gly y malato, y evaluar sus interacciones, en las condiciones indicadas en el objetivo 3.1., a concentraciones fisiológicas de PEP.

3.3. Determinar el efecto de la fosforilación de la enzima sobre los puntos indicados en los objetivos 3.1. y 3.2.

3.4. Comparar las características cinéticas de la enzima determinadas a concentración fisiológica de fMg^{2+} y bicarbonato, con las características cinéticas de la enzima determinadas a altas concentraciones de fMg^{2+} y bicarbonato.

3.5. Estimar el grado de respuesta de la PEPC a los cambios en la concentración de substratos y de sus posibles reguladores alostéricos y al cambio en su grado de fosforilación que tienen lugar *in vivo* durante el ciclo noche-día.

5. MATERIAL Y MÉTODOS

5.1. Reactivos

PEP, NADH, málico deshidrogenasa de corazón de cerdo, PEG (peso molecular de 6000), DTT, Glc6P, fosfoglicolato, HEPES, malato y PLP se adquirieron de Sigma Chemical Co, el de EDTA de Merck, el $NaBH_3CN$ de Aldrich, y el glicerol de Mallinckrodt, Inc. Todos los otros reactivos empleados fueron de grado analítico y se adquirieron de los proveedores habituales.

5.2. PEPC

En el presente trabajo se utilizó PEPC no fosforilada (forma de obscuridad) extraída y purificada por el método descrito por Tovar-Méndez *et al.* (1997). La enzima así purificada tenía una pureza mayor al 99 % de acuerdo a un SDS-PAGE [Laemli, 1970] teñido con plata [Wray *et al.*, 1981]. Su actividad específica era de 33 unidades (µmoles/min) por mg de proteína determinada en el amortiguador de ensayo a pH 7.3, en presencia de PEP 5 mM y de Mg^{2+} 10 mM.

El I_{50} para malato de la enzima fue de 0.18 mM (determinado en HEPES 100 mM, pH 7.3, EDTA 0.1 mM, PEP 2.5 mM y $MgCl_2$ 5 mM) y su valor no disminuyó después de incubación exhaustiva con fosfatasa alcalina. Además, la incubación de la enzima con proteína cinasa A según Wang *et al.* (1992), incrementó el I_{50} para malato a 1.54 mM. En conjunto, estos datos nos indicaron que la enzima PEPC utilizada estaba completa (no proteolizada) y que no estaba fosforilada, de acuerdo con lo reportado por Wang *et al.* (1992) y por Duff *et al.* (1995).

La enzima en la preparación usada en todos los experimentos era tetramérica, lo que se determinó por cromatografía de exclusión molecular en Superosa 6/HR (conectada a un sistema de HPLC Waters), que es el último paso del proceso de purificación [Tovar-Méndez *et al.*, 1997].

La cantidad de proteína se determinó por el método de Bradford (1976).

5.3. Determinación de la actividad PEPC

La actividad PEPC se midió espectrofotométricamente mediante un ensayo acoplado, usando malato deshidrogenasa, y siguiendo la oxidación del NADH a 340

nm en un volumen final de 0.5 ml con un espectrofotómetro Beckman DU-7500 equipado con un programa cinético. La temperatura del medio de reacción se mantuvo a 30 ± 0.1 °C con un equipo de circulación de agua con temperatura controlada. El amortiguador de actividad utilizado en los experimentos mostrados en la sección de resultados 6.1. consistió en trietanolamina-HCl 100 mM (pH 7.3) conteniendo EDTA 1 mM, NADH 0.2 mM, $NaHCO_3$ 10 mM, 4 unidades de malato deshidrogenasa y las concentraciones de $MgCl_2$ y de PEP indicadas en cada caso. En los experimentos mostrados en los Artículos el amortiguador fue como se indica en la respectiva sección de cada artículo. El ensayo acoplado fue el siguiente:

$$PEP^{-3} + HCO_3^- \xrightarrow[Mg^{+2}]{PEPC} OAA^{-2} + HPO_4^{-2}$$

$$OAA^{-2} + NADH + H^+ \xrightarrow{MDH} Malato^{-2} + NAD^+$$

En los experimentos en los que se controló la concentración de especies libres y de los complejo Mg-ligando, la cantidad de magnesio total y de ligando total se calculó utilizando el programa de cómputo descrito por Rodríguez-Sotres (1990).

5.4. Determinación de la cinética de inactivación de la PEPC por dilución

La PEPC tetramérica pura con una concentración de 2.5 μM (equivalente a 1 mg de proteína/ml) fue diluída a una concentración del tetrámero de 50 nM (equivalente a 20 μg/ml) en amortiguador de actividad a pH 7.3 en ausencia de substratos. Estas muestras se incubaron a 30 °C y, a diferentes tiempos después de la dilución, la actividad se disparó con la adición de $MgCl_2$ y PEP, a una concentración final respectiva de 5 y 10mM

5.5. Relación entre la concentración y la actividad de la PEPC

La PEPC tetramérica pura (con una concentración de 1 mg de proteína/ml) se diluyó a diferentes concentraciones en amortiguador de actividad a pH 7.3 en ausencia de substratos y conteniendo DTT 5 mM. Después de 8 horas de incubación a 30 °C, se tomó una alícuota conteniendo 10 μg de proteína, para medir la actividad

específica de la enzima de cada una de las diluciones. La actividad se midió en amortiguador de actividad a pH 7.3 conteniendo $MgCl_2$ 5 mM y PEP 10 mM.

5.6. Determinación de la unión de ligandos del sitio activo y del sitio alostérico para Glc6P

La modificación química reversible, de la PEPC con PLP provoca su inactivación e insensibilización a activación por Glc6P [Tovar-Méndez *et al.*, 1997]. Para determinar la unión de ligandos del sitio activo y del sitio alostérico para Glc6P, nosotros utilizamos la protección ofrecida por ligandos del sitio activo frente a la inactivación y la protección ofrecida por Glc6P frente a insensibilización a activación por Glc6P y por PEP.

Reacción de la PEPC con PLP: Se utilizó siempre una solución de PLP recién preparada en amortiguador de modificación (trietanolamina-HCl 50 mM, pH 7.3, EDTA 1 mM y DTT 5 mM). La concentración de PLP en esta solución se determinó a 388 nm usando el valor 4900 M^{-1} cm^{-1} como coeficiente de extinción molar [Blackburn & Schachman, 1976]. La PEPC tetramérica disuelta en el amortiguador de modificación (sin ó con PEG 6 % (p/v) ó con glicerol 20 % (v/v)) se incubó con PLP a 22 °C en ausencia o presencia de los ligandos indicados en cada experimento. Las incubaciones se realizaron en tubos cubiertos con papel aluminio para protegerlas de la luz. Después de 2 hr de incubación, tiempo suficiente para alcanzar el equilibrio de la inactivación y de la insensibilización a activación por Glc6P de la enzima, por modificación con PLP [Tovar-Méndez *et al.*, 1997], se tomaron alícuotas de 5 µl y se determinó la actividad enzimática en amortiguador de actividad a pH 7.3, conteniendo $MgCl_2$ 5 mM y PEP 1 mM en presencia y en ausencia de Glc6P 10 mM. Nosotros definimos la sensibilidad a Glc6P como: $(v_a-v_o)/ v_o$, donde v_a y v_o son la actividad enzimática en presencia y en ausencia de Glc6P, respectivamente. La dilución de la alícuota en la cubeta de reacción (100 veces) evita la reacción posterior del PLP con la PEPC. Ninguno de los compuestos presentes en la incubación afectaron la estabilidad del PLP bajo nuestras condiciones experimentales.

Determinación de unión de ligandos: La unión del ligando se determinó considerando el efecto inducido por la presencia del ligando (expresado en porcentaje), en la inactivación o en la insensibilización a activación por Glc6P de la PEPC, sobre el equilibrio de la modificación de la PEPC con PLP. Los datos mostrados en la sección de resultados 6.1 se analizaron con las siguientes ecuaciones:

$$\Delta A_{eq} = \Delta A_{eq\,max}\,[L]^h / (L_{0.5}{}^h + [L]^h)$$

$$\Delta S_{eq} = \Delta S_{eq\,max}\,[L]^h / (L_{0.5}{}^h + [L]^h)$$

donde ΔA_{eq} y ΔS_{eq} son la actividad y la sensibilidad a activación por Glc6P de la PEPC respectivamente, en el equilibrio de la modificación de la PEPC con PLP, $\Delta A_{eq\ max}$ y $\Delta S_{eq\ max}$ son los respectivos valores máximos cuando la enzima esta saturada con el ligando, [L] es la concentración de ligando, $L_{0.5}$ es la concentración de ligando que dá la mitad del $\Delta A_{eq\,max}$ ó del $\Delta S_{eq\,max}$, y h es en número de Hill.

6. RESULTADOS

6.1. Caracterización de las propiedades alostéricas de la forma tetramérica de la PEPC de hoja de maíz

Esta sección incluye resultados no publicados, así como resultados publicados en una memoria en extenso de un congreso internacional.

6.1.1. Efecto de la dilución sobre la actividad de la PEPC

Se conoce que la enzima PEPC de hoja de maíz es un homotetrámero que se disocia cuando se diluye en ausencia de magnesio y de PEP [Selenioti *et al.*, 1987; Podestá & Andreo, 1989]. La disociación del tetrámero provoca la pérdida de la actividad enzimática, ya que el monómero es inactivo y el dímero es inactivo o parcialmente activo [Uedan & Sugiyama, 1976; Marês & Leblová, 1980; Wu & Wedding, 1985].

Nosotros encontramos que la inactivación de la PEPC tetramérica por dilución sigue una cinética de primer orden con un tiempo medio de 190 minutos, y una constante de inactivación de 0.00354 minutos^{-1}. En presencia de malato 20 mM, el tiempo medio de inactivación es de 30 minutos y la constante de inactivación es de 0.02314 minutos^{-1}. Estos resultados en conjunto nos indican que la disociación de la PEPC tetramérica bajo nuestras condiciones de ensayo de actividad en el tiempo de reacción (0.5 minutos) es despreciable, tanto en ausencia (menos del 1%) como en presencia de malato 20 mM (1.1%). Por lo que los resultados obtenidos en los estudios cinéticos de velocidad inicial son atribuibles a la forma tetramérica de la PEPC de hoja de maíz.

Los estudios de unión se realizan en tiempos mucho mayores de 0.5 minutos, por lo cual, los estudios de unión de ligandos de la PEPC requerían establecer la concentración mínima en que la enzima se encuentra principalmente como tetrámero. Para ello procedimos a determinar la relación entre la concentración y la actividad de la PEPC, después de incubar la enzima a diferentes concentraciones por 8 horas para permitir que se alcanzara el equilibrio de la disociación del tetrámero. Los resultados se muestran en la figura 6.1. [página 25]. Como se puede observar, la

actividad específica de la enzima permaneció constante a concentraciones iguales ó superiores a 0.625 μM del tetrámero (equivalente a 0.25 mg de proteína/ml), lo que sugiere que la PEPC se encuentra principalmente como tetrámero a concentraciones iguales o superiores a 0.625 μM. A partir de los datos de la figura 6.1. se calculó una Kd del equilibrio tetrámero-(2)dímero de 0.045 μM de tetrámero (equivalente a 0.018 mg de proteína/ml), considerando que, a pH 7, el tetrámero de la PEPC se disocia a dímero sin posterior disociación a monómero [Wagner *et al.*, 1987; Podestá & Andreo, 1989]. Así, la concentración en la que la PEPC es principalmente tetrámero, es tres ordenes de magnitud inferior a la concentración de PEPC que se estima existe *in vivo* en plantas C_4 (15 mg de PEPC/ml que correponde a 37.5 μM del tetrámero) [Jiao & Chollet, 1991].

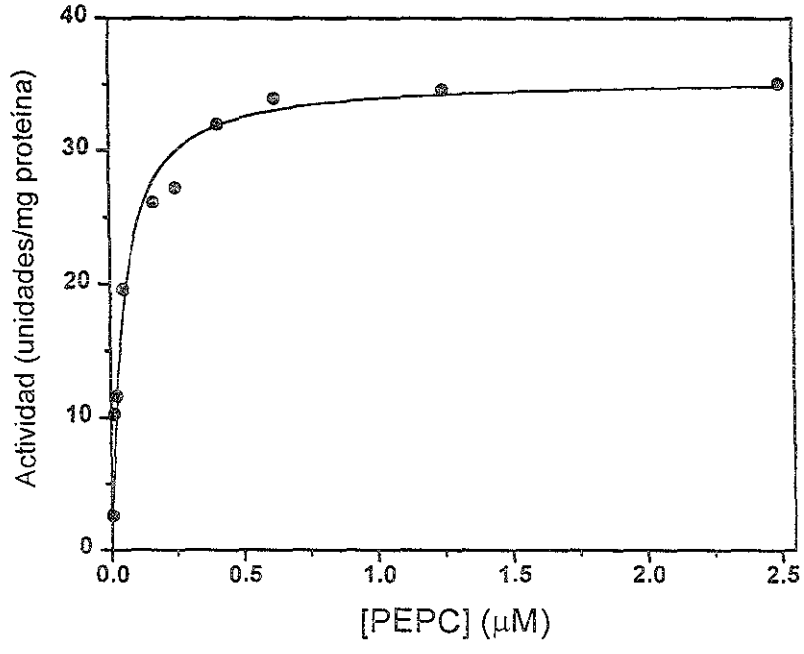


Figura 6.1. Relación entre la actividad y la concentración de la PEPC. La enzima tetramérica fue diluída e incubada por 8 horas a 30 °C y, posteriormente, se determinó la actividad en amotiguador de actividad (pH 7.3) conteniendo $MgCl_2$ 10 mM y PEP 10 mM, a cada una de las diluciones. La línea es el ajuste a una hipérbola rectangular.

Considerando lo anteriormente expuesto, los estudios de unión se realizaron con una concentración de 0.5 mg de PEPC/ml, para asegurar que la enzima se encontrara mayoritariamente como tetrámero.

6.1.2. Unión de ligandos del sitio activo y del sitio alostérico activador para Glc6P a la PEPC tetramérica

6.1.2.1 Proccedings of the Xth International Photosynthesis Congress:

Binding of ligands to the glucose-6-phosphate allosteric site in maize-leaf phosphoenolpyruvate carboxylase (In: P. Mathis (Ed.), Photosynthesis: from light to biosphere, Vol. V, Kluwer, Dordrecht, 1995, pp. 155-158).

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BINDING OF LIGANDS TO THE GLUCOSE-6-PHOSPHATE ALLOSTERIC SITE IN MAIZE-LEAF PHOSPHOENOLPYRUVATE CARBOXYLASE

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1. Introduction

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) from C4 plants is allosterically activated by glucose 6-phosphate (Glc6P) (1). In addition to these heterotropic effects, at pH values close to the neutrality the enzyme exhibits positive homotropic cooperativity, which is abolished by the activator Glc6P (2). At present, little is known about the full relationship between binding and observed steady-state kinetics. In an effort to further the understanding of the mechanisms of regulation of the activity of C4-PEPC, we have now measured the binding of homotropic and heterotropic effectors to the active and Glc6P allosteric sites of the nonphosphorylated form of maize-leaf PEPC.

It is known that treatment with pyridoxal 5'-phosphate (PLP), a specific reagent for lysine, inactivates the PEPC from maize leaves (3,4). Recently, lysyl residues have been implicated in the response to Glc6P of the *Crassula* (5,6) and of the maize-leaf enzymes (7,8). We took advantage of the protection provided by ligands against inactivation (3,4) and desensitization to Glc6P (7) by PLP to measure the binding to the active and allosteric sites, and to elucidate the factors that modulate this binding.

2. Materials and Methods

2.1. *PEPC Extraction and Purification*- The enzyme was extracted after a 4 h dark-period and purified as described elsewhere (C. Mújica-Jiménez and R.A. Muñoz-Clares, manuscript in preparation). The enzyme was more than 99% homogeneous by the criterion of SDS-PAGE and silver staining, and has a specific activity of 33 U/mg protein. The sensitivity to malate of this enzyme preparation was the expected for the nonphosphorylated form of the enzyme (9), and no decreases in Ki(malate) were observed after exhaustive incubation with alkaline phosphatase.

2.2. *PEPC Assay*- PEP carboxylase activity was measured spectrophotometrically at pH 7.3 and 30 °C in a coupled enzyme assay using malate and lactate deshidrogenase, as described (10). We defined the Glc6P sensitivity as the percentage of activation of the enzyme by 10 mM Glc6P when 1 mM total PEP and 5 mM total Mg^{2+} were used in the assay.

2.3. *Reaction of PEPC with PLP*- Incubation of the enzyme (0.5 mg/ml) with freshly

P. Mathis (ed.), Photosynthesis: from Light to Biosphere, Vol. V, 155–158.

prepared PLP was carried out at 22 °C in triethanolamine-ClH, pH 7.3, 1 mM EDTA, 5 mM DTT and 6% (w:v) polyethylene glycol 6000, in the absence or presence of the indicated ligands. Incubation mixtures were protected from light with metal foil. At specified intervals after the addition of PLP, 5 ml-aliquots of the incubation mixture were withdrawn and immediately assayed for catalytic activity and sensitivity to Glc6P. Recovery of the activity or sensitivity to Glc6P upon dilution in the assay medium was negligible in the time taken for the assays.

2.4. *Data Analysis.* Chemical-modification data were analyzed using commercial nonlinear-regression-analysis computer programs. Pseudo-first-order analysis of time courses of inactivation or desensitization were performed using the equation for a first-order reversible reaction. Protection data, i.e., remaining activities or sensitivities at equilibrium obtained in the presence of ligands, were fitted to the Hill or the rectangular hyperbola equations, after subtracting the remaining activity or sensitivity obtained in the absence of ligands for each experiment.

3. Results and Discussion

3.1. *Effect of PLP on Activity and Sensitivity to Glc6P of Maize-Leaf PEPC-* After incubation of the purified enzyme with PLP, there is a time dependent, reversible loss of activity or Glc6P activation. The two processes are not related, since desensitization is a much faster process than inactivation and it is still observed when inactivation is prevented by saturation of the active site.

3.2. *Protection of Maize-Leaf PEPC against Desensitization to Glc6P by PLP-* Loss of activation by Glc6P caused by incubation of the enzyme with PLP is prevented by Glc6P itself, being the initial rate of desensitization and the equilibrium point dependent on the concentration of Glc6P in the incubation medium. On the contrary, Glc6P accelerates the inactivation process. These results suggest that the modified lysyl residue involved in the response to Glc6P is at or near the Glc6P binding site, and that the conformational changes elicited by the binding of Glc6P expose the lysyl residue of the active site. Interestingly, the contrary is true in the case of the substrate Mg-PEP, which protects the enzyme from inactivation while accelerates the desensitization process. This is consistent with the increases in the affinity of the enzyme for the substrate in the presence of Glc6P (2) and the increases in the affinity of Glc6P at saturating concentrations of substrate (9) found in kinetic studies.

3.3. *Binding of Ligands to the Active and Glc6P Allosteric Sites of Maize-Leaf PEPC-* We took advantage of the protection against inactivation and desensitization offered by the substrate and by Glc6P, respectively, to study the binding of these compounds to the enzyme. Thus, by measuring the remaining activities and sensitivities at equilibrium after incubation of the enzyme with a fixed [PLP] at several different ligand concentrations, we determined: (i) dissociation constant values for PEP, Mg-PEP and Glc6P from their respective complexes with the enzyme, and (ii) the shape of the corresponding saturation curves. The results are summarized in Table I. As can be see, in the absence of Glc6P, binding of PEP and Mg-PEP to the active site is cooperative, while in the presence of 10 mM Glc6P, the binding of the substrate is hyperbolic and the dissociation constant, measured as $L_{0.5}$, decreases more than 60-times. On the other hand, the binding of Glc6P to the free enzyme is highly cooperative, while the binding is hyperbolic and the $L_{0.5}$ is 3-times lower when the active site is saturated by Mg-PEP. These results are in full agreement with those obtained in initial velocity studies (2,9).

The free species PEP and Mg^{2+} have different effects on the binding of Glc6P: while the former increases the affinity without modifying the cooperativity, the latter disminishes the cooperativity and increases the affinity in a similar extent. Two other allosteric effectors of the enzyme, the inhibitior malate and the activator glycine, have no significant effects on the binding of Glc6P to the allosteric site.

Table I. *Binding parameters of PEPC ligands measured by protection against inactivation and desensitization to Glc6P by PLP* [a].

Ligand	$L_{0.5}$ (mM)	*n*	Maximum Protection (%)
ACTIVE SITE			
PEP	5.33 ± 0.32	2.43 ± 0.33	100
Mg-PEP[b]	0.45 ± 0.03	2.21 ± 0.29	100
Mg-PEP, 10 mM Glc6P	0.007 ± 0.001	1[c]	100
Mg-PEP (20 % glycerol)[d]	0.089 ± 0.014	1[c]	100
Mg-PEP (without cosolute)[d]	0.51 ± 0.03	1.63 ± 0.13	100
ALLOSTERIC SITE			
Glc6P	4.71 ± 0.16	3.99 ± 0.38	70
Glc6P, 4 mM Mg-PEP (5 mM free PEP, 4.4 mM free Mg^{2+})	1.48 ± 0.18	1[c]	97
Glc6P, 3 mM PEP	1.79 ± 0.11	3.91 ± 0.86	60
Glc6P, 10 mM $MgCl_2$	3.64 ± 0.27	2.36 ± 0.32	97
Glc6P, 5 mM malate	3.66 ± 0.17	4.03 ± 0.69	29
Glc6P, 10 mM glycine	5.87 ± 0.19	4.00 ± 0.37	100
Glc6P (20% glycerol)[d]	4.64 ± 0.21	3.86 ± 0.36	65
Glc6P (without cosolute)[d]	N.D.[e]	N.D.	N.D.

a. Incubations were carried out as described under "Materials and Methods". 0.8 mM and 0.6 mM PLP were used in the active and allosteric site experiments, respectively.
b. The concentration of free PEP was hold constant at 5 mM.
c. Data were fitted to the equation of a rectangular hyperbola.
d. In these experiments polyethylene glycol was not included in the incubation medium.
e. N.D. : binding could not be detected.

In the absence of polyethylene glycol, the binding parameters for Mg-PEP are similar to those in the presence of cosolute, but Glc6P was unuable to protect against desensitization, what may be due to either lack of binding of Glc6P to the allosteric site or binding of Glc6P to a region far from the PLP-modified lysyl residue. Contrary to polyethylene glycol, glycerol has a dramatic effec on the affinity of the active site for Mg-PEP and abolishes the cooperativity observed in its absence, while has no effect on the binding of Glc6P to the allosteric site. Therefore, the effect of glycerol abolishing the cooperativity and greatly increasing the affinity of ligands of the Glc6P site observed in initial velocity studies (C. Mújica-Jiménez and R.A. Muñoz-Clares, manuscript in preparation) seems to be indirectly achieved through the effect that saturation of the active site exerts on the affinity of the allosteric site for its ligands.

Under our experimental conditions the ligands of the active site always afforded total protection against inactivation, but Glc6P only provided total protection when the enzyme is saturated by Mg-PEP, free Mg^{2+} or by glycine. The different degree of protection achieved by Glc6P in the presence of other ligands of the enzyme is a clear indication of the different conformational states elicited by the binding of these ligands. Thus, it is interesting to note that the lowest and the highest maximal protection were found when malate or Mg-PEP, respectively, were included together with Glc6P in the incubation medium. Therefore, it is important to consider not only the affinity of the allosteric site for Glc6P, but also the way in which this activator binds, which could lead to a productive or nonproductive binding. Accordingly with these results, previous reports show the failure of Glc6P to provide total or even partial protection against modification of residues involved in the response of PEPC to this activator (6,12,13).

3.4. *Mechanism of Allosteric Regulation of Maize-Leaf PEPC*- It has been proposed that the mechanism underlying the homotropic and heterotropic effects observed in initial velocity studies is the association-dissociation process that may take place at the low enzyme concentrations used in the kinetics studies (11). At this respect, the ability of the substrate PEP (12), of the allosteric activator Glc6P (12) and of high concentrations of glycerol (11) to shift the dimer-tetramer equilibrium toward the tetrameric active form is well established. However, the results of the binding studies reported here show that oligomerization alone is not sufficient to cause the substrate and Glc6P-induced allosteric activation. To account for our results, a model involving several ligand-induced conformational states of the tetrameric maize-leaf PEPC need to be postulated.

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6.1.2.2. Resultados complementarios no incluídos en la sección 6.1.2.1.

Como se explica en el apartado anterior, los experimentos de unión fueron hechos con el fin de medir los efectos de unión homotrópicos y heterotrópicos del sitio activo y del sitio alostérico para Glc6P. Sin embargo, faltó evaluar el efecto heterotrópico de Gly sobre el sitio activo, que es mostrado en este inciso.

Además, una importante ventaja del método utilizado para medir la unión tanto al sitio activo como al sitio alostérico para Glc6P es que nos permitió medir la unión de PEP al sitio activo (sección 6.1.2.1.) y al sitio alostérico activador para Glc6P (esta sección). Lo cual era importante considerando la sugerencia del posible papel de fPEP como activador alostérico de la PEPC [Jenkins *et al.*, 1986; Rustin *et al.*, 1988; Rodríguez-Sotres & Muñoz-Clares, 1990; Rustin *et al.*, 1991].

Los resultados se muestran en la Tabla 6.1.

Tabla 6.1. *Parámetros de la unión de ligandos de la PEPC, medidos por protección contra inactivación ó insensibilización a Glc6P por modificación química con PLP*[a].

Ligando	$L_{0.5}$ (mM)	n	Protección máx. (%)
Sitio Activo			
MgPEP (a fPEP 5 mM			
y Gly 10 mM, contantes)	0.20 ± 0.04	1[b]	100
Sitio Alostérico			
PEP	21.6 ± 1.7	2.40 ± 0.34	100

[a] La incubación de la enzima fue realizada como se indica en Material y Métodos (sección 5.6.), con PLP 0.8 mM en presencia de PEG al 6 % (p/v).

[b] Datos ajustados a la ecuación de una hipérbola rectangular.

6.1.2.3. Discusión de resultados de los estudios de unión

La regulación de la PEPC por un proceso de asociación-disociación inducido por los ligandos de la enzima es poco probable dada la elevada concentración del tetrámero *in vivo* (37.5 μM) [Jiao & Chollet, 1991]. Por lo cual pensamos que la forma tetramérica de la enzima posee propiedades alostéricas, tanto homotrópicas como heterotrópicas. Para demostrar esta hipótesis, nos propusimos evaluar las propiedades alostéricas de la PEPC no fosforilada en una preparación que fuese 100% tetramérica. Estas propiedades fueron evaluadas midiendo la unión de ligandos del sitio activo y del sitio alostérico para Glc6P usando la protección que ofrecen los ligandos frente a modificación química, siguiendo el método que reportamos recientemente [Tovar-Méndez *et al.* 1997].

Efecto de PEG y de glicerol sobre la unión de los ligandos del sitio activo y del sitio alostérico activador para Glc6P

La cooperatividad observada en la saturación del sitio activo por PEP (en presencia de magnesio total constante) o por MgPEP en estudios cinéticos de estado estacionario [referencias en antecedentes y datos en Artículo 1 y 2] también fue observada en la cinética de unión de MgPEP al sitio activo en el tetrámero en ausencia de cosolutos, indicando que la cooperatividad presente en la saturación de la PEPC por MgPEP son reflejo de efectos de unión homotrópicos del sitio activo.

La presencia de PEG al 6% (p/v) aumentó ligeramente la afinidad del sitio activo por MgPEP, sin eliminar la cooperatividad de la unión de MgPEP. Un efecto similar se observó en estudios cinéticos de estado estacionario [resultados no mostrados]. En cuanto a glicerol, éste cosoluto aumentó considerablemente la afinidad del sitio activo por el substrato MgPEP y eliminó la cooperatividad de la unión de este substrato, efecto bien establecido en estudios cinéticos de estado estacionario [Uedan & Sugiyama, 1976; Mújica-Jiménez *et al.*, 1998].

El efecto activador de glicerol observado en estudios cinéticos de la PEPC ha sido interpretado por ciertos investigadores [Selenioti *et al.*, 1987; Podestá & Andreo, 1989] como el resultado de que este compuesto favorece la formación del tetrámero a

partir de los dímeros que suponen existirían a la alta dilución de la enzima a la que se realizan los estudios cinéticos [Podestá & Andreo, 1989], en acuerdo con la teoría del "volumen de exclusión" descrita por Gekko & Timasheff (1981). Sin embargo, nuestros resultados indican que el efecto de glicerol se observa también en la PEPC tetramérica. A este respecto se sabe que, adicionalmente, cosolutos inertes a las proteínas [Colombo *et al.*, 1992; Rand, *et al.*, 1993] afectan la afinidad de ligandos que producen cambios en el estado de solvatación de las proteínas a las que se unen. Esto se ha explicado como el resultado de que la exclusión del agua de la superficie de la proteína causada por la presencia del cosoluto produce una conformación deshidratada de la proteína en la que se minimiza el área de superficie expuesta al agua, de manera que la afinidad de la proteína por el ligando en presencia del cosoluto aumenta si la conformación de la proteína inducida por el ligando tiene expuesta al agua una menor superficie [Rand, *et al.*, 1993] y viceversa [Colombo *et al.*, 1992]. Es posible que éste sea el mecanismo mediante el que glicerol y PEG afectan la unión de los ligandos del sitio activo y la unión de Glc6P al tetrámero de la PEPC.

En ausencia de cosoluto, la unión de Glc6P a la PEPC tetramérica no se observó en un intervalo de concentración de 0 a 10 mM, sugiriendo que la forma libre de la PEPC no une o tiene muy baja afinidad por Glc6P. Sin embargo, la unión de Glc6P a la PEPC sí se observó en presencia de PEG ó de glicerol, en el mismo intervalo de concentración; aunque la protección por Glc6P frente a insensibilización por modificación con PLP no fue total. La unión de Glc6P en presencia de cualquiera de los dos cosolutos es muy similar, con un alto grado de cooperatividad como lo indica un número de Hill de casi cuatro.

Dado que en la presencia de PEG la unión de MgPEP a la enzima fue cualitativamente idéntica a la unión en su ausencia y puesto que, sólo en presencia de cosoluto, pudimos medir la unión de Glc6P a la enzima libre, los experimentos realizados para evaluar el efecto de otros ligandos sobre la unión de Glc6P fueron realizados en presencia de PEG.

Efectos heterotrópicos de unión determinados en presencia de PEG.

La afinidad del sitio activo (medida como $L_{0.5}$) por MgPEP se incrementó fuertemente por la presencia de Glc6P 10 mM, como lo indica el cambio de $L_{0.5}$ de 0.45 a 0.007 mM inducido por Glc6P. Además, Glc6P eliminó la cooperatividad en la unión de MgPEP. Estos resultados concuerdan con el efecto de Glc6P sobre la cinética de saturación por MgPEP de la PEPC [datos en Artículo 1 y 2] o por PEP (en presencia de magnesio total constante) [Coombs *et al.*, 1973; Huber & Edwards, 1975; Uedan & Sugiyama, 1976].

En la presencia de Gly 10 mM el $L_{0.5}$ para MgPEP (0.2 mM) disminuye sólo dos veces, pero la cooperatividad de la unión de MgPEP al sitio activo desaparece. Estos resultados están en acuerdo con el pobre efecto de Gly 5 mM [Uedan & Sugiyama, 1976] ó 10 mM [Gillinta & Grover, 1995] sobre el $S_{0.5\,(PEP)}$ determinado en estudios cinéticos de estado estacionario (cinética de saturación por PEP determinada a 10 mM de magnesio total constante). Sin embargo y en contraste con nuestros resultados, los mismos estudios de estado estacionario indican que Gly (5 y 10 mM) no elimina la cooperatividad de la saturación por PEP [Uedan & Sugiyama, 1976; Gillinta & Grover, 1995]. El efecto diferencial de Gly 10 mM sobre la cooperatividad cinética de unión y de estado estacionario de MgPEP, puede ser explicado por que la saturación de la enzima con Gly no elimina la activación de la enzima por fPEP; por lo que, en presencia de concentraciones saturantes de Gly, la saturación de la PEPC con PEP (a magnesio constante) tiene cooperatividad positiva aparente, mientras que la saturación de la enzima con magnesio (a PEP constante) es hiperbólica [resultados en Artículo 1].

Nuestros resultados demuestran que los efectos heterotrópicos de MgPEP y de Gly sobre la unión de Glc6P a su sitio alostérico, observados en los estudios cinéticos de estado estacionario [Gillinta & Grover, 1995; Duff *et al.*, 1995], también se presentan en la PEPC tetramérica, por lo que no pueden ser interpretados en términos de cambios en el equilibrio dímero-tetrámero. Sin embargo, existen algunas particularidades interesantes.

La saturación del sitio activo con MgPEP sólo disminuye el $L_{0.5}$ para Glc6P en aproximadamente tres veces, que es mucho menor que el efecto que tiene la saturación del sitio alostérico para Glc6P sobre la afinidad del sitio activo por MgPEP (60 veces); además, la enzima con el sitio activo saturado con MgPEP, fue protegida totalmente por Glc6P frente a insensibilización por modificación con PLP. Estos resultados sugieren que (al menos en presencia de PEG al 6% p/v) la conformación de la PEPC inducida por MgPEP es diferente de la inducida por Glc6P. Aunque en ambos casos la saturación de un sitio elimina la cooperatividad de unión al otro sitio.

De igual manera, mientras Gly 10 mM aumenta la afinidad del sitio activo por MgPEP y elimina la cooperatividad de la unión de MgPEP (como se indicó anteriormente), Gly 10 mM no afecta significativamente la cinética de unión de Glc6P, aunque la presencia de Gly permite que Glc6P proteja totalmente a la enzima frente a insensibilización por modificación con PLP. Estos resultados sugieren que (al menos en presencia de PEG al 6% p/v) la conformación de la PEPC inducida por MgPEP es diferente de la inducida por Gly.

Unión de PEP libre a la PEPC en presencia de PEG

PEP es capaz de unirse al sitio activo de la enzima en ausencia de iones magnesio como lo demuestra la protección ejercida por PEP frente a inactivación de la enzima por PLP [Tabla I en las memorias en extenso], aunque la afinidad del sitio activo por PEP es 10 veces menor que su afinidad por MgPEP. En estas condiciones, PEP también se une al sitio alostérico para Glc6P, aunque con una afinidad 4 veces menor a la de Glc6P [Tabla I en memorias en extenso y Tabla 6.1. en página 26]. La protección frente a la insensibilización a Glc6P por modificación de la PEPC con PLP, ejercida por PEP en ausencia de iones magnesio [Tabla 6.1. en página 26], no la ofrece el complejo MgPEP. Por el contrario MgPEP desprotege el sitio alostérico para Glc6P, además, Glc6P desprotege el sitio activo [Tovar-Méndez *et al.*, 1997], lo cual es consistente con que Glc6P aumenta la afinidad por el substrato y viceversa [Coombs *et al.*, 1973; Huber & Edwards, 1975; Uedan & Sugiyama, 1976; Duff *et al.*, 1995]. Estos resultados en conjunto evidenciaron que el sitio activo une mejor al

complejo MgPEP que a fPEP, y que el sitio alostérico activador para Glc6P sólo une a fPEP, como posteriormente fue confirmado en estudios cinéticos llevados a cabo en nuestro laboratorio [datos en Artículo 1; Mújica-Jiménez *et al.*, 1998].

La unión de fPEP al sitio alostérico para Glc6P de la PEPC reforzó la idea del posible papel de fPEP como activador alostérico de esta enzima [Jenkins *et al.*, 1986; Rustin *et al.*, 1988; Rodríguez-Sotres & Muñoz-Clares, 1990; Rustin *et al.*, 1991; y resultados en el Artículo 1], lo que comprobamos posteriormente en este trabajo de tesis [datos en Artículo 1].

Finalmente, un resultado destacable de estos estudios es que Glc6P se une a la enzima en presencia de malato, como lo indica el hallazgo de que la cinética de unión de Glc6P en presencia de PEG no se afecta por la presencia de malato 5 mM, aunque la protección máxima ejercida por Glc6P es mucho menor que la observada en ausencia de malato [Tabla I de memorias en extenso]. Lo anterior sugiere que Glc6P puede unirse al complejo enzima-malato y viceversa (malato puede unirse al complejo enzima-Glc6P), pero la unión de Glc6P produce un cambio conformacional en la enzima diferente al que produce en ausencia de malato. Esta hipótesis es apoyada por el hecho de que la inhibición de la enzima impuesta por malato no es contrarrestada por la saturación de la enzima con Glc6P [Figura 5 en Artículo 2].

Re-examination of the roles of PEP and Mg^{2+} in the reaction catalysed by the phosphorylated and non-phosphorylated forms of phosphoenolpyruvate carboxylase from leaves of *Zea mays*

Effects of the activators glucose 6-phosphate and glycine

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To study the effects of phosphoenolpyruvate (PEP) and Mg^{2+} on the activity of the non-phosphorylated and phosphorylated forms of phosphoenolpyruvate carboxylase (PEPC) from *Zea mays* leaves, steady-state measurements have been carried out with the free forms of PEP (*f*PEP) and Mg^{2+} (*f*Mg^{2+}), both in a near-physiological concentration range. At pH 7.3, in the absence of activators, the initial velocity data obtained with both forms of the enzyme are consistent with the exclusive binding of MgPEP to the active site and of *f*PEP to an activating allosteric site. At pH 8.3, and in the presence of saturating concentrations of glucose 6-phosphate (Glc6P) or Gly, the free species also combined with the active site in the free enzyme, but with dissociation constants at least 35-fold that estimated for MgPEP. The latter dissociation constant was lowered to the same extent by saturating Glc6P and Gly, to approx. one-tenth and one-sixteenth in the non-phosphorylated and phosphorylated enzymes respectively. When Glc6P is present, *f*PEP binds to the active site in the free enzyme better than *f*Mg^{2+}, whereas the metal ion binds better in the presence of Gly. Saturation of the enzyme with Glc6P abolished the activation by *f*PEP, consistent with a common binding site, whereas saturation with Gly increased the affinity of the allosteric site for *f*PEP. Under all the conditions tested, our results suggest that *f*PEP is not able to combine with the allosteric site in the free enzyme, i.e. it cannot combine until after MgPEP, *f*PEP or *f*Mg^{2+} are bound at the active site. The physiological role of Mg^{2+} in the regulation of the enzyme is only that of a substrate, mainly as part of the MgPEP complex. The kinetic properties of maize leaf PEPC reported here are consistent with the enzyme being well below saturation under the physiological concentrations of *f*Mg^{2+} and PEP, particularly during the dark period; it is therefore suggested that the basal PEPC activity *in vivo* is very low, but highly responsive to even small changes in the intracellular concentration of its substrate and effectors.

INTRODUCTION

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) from maize leaves catalyses the essentially irreversible formation of oxaloacetate and P_i from phosphoenolpyruvate (PEP) and bicarbonate. This reaction is the first step in the assimilation pathway of atmospheric CO_2 in C_4 plants such as maize. The importance of this step in the photosynthetic metabolism of C_4 plants is underscored by the abundance of the PEPC protein in mesophyll cells of leaves of these plants, accounting for approx. 10–15% of the total soluble protein [1,2].

At physiological pH, PEPC from leaves of C_4 plants is subjected to complex allosteric regulation involving homotropic co-operativity and heterotrophic effects by a number of physiological effectors. The enzyme is also subject to covalent modification consisting of the phosphorylation–dephosphorylation of an N-terminal Ser residue [3]. Regarding the positive effectors, it is considered that the enzyme is activated by two kinds of metabolite: (1) hexose and triose phosphates [4–9], which bind to the so-called glucose 6-phosphate (Glc6P) allosteric site [10], and 2) neutral amino acids, mainly Gly, Ala and Ser [7,9,11–14], which bind to the Gly allosteric site [13]. In addition, several authors have suggested that PEP itself might behave as an allosteric activator of the non-phosphorylated form of the enzyme, on the basis of the observed changes in fluorescence on binding of ligands to PEPC [15] and of steady-state kinetic studies ([16]; C. Mújica-Jiménez, A. Castellanos-Martínez and R. A. Muñoz-Clares, unpublished work). The fact that some PEP analogues are good activators of the non-phosphorylated form of the enzyme [16–18] gives additional support to that proposal.

Mg^{2+} ions are essential for the activity of PEPC [19]. The chemical mechanism of the PEPC-catalysed reaction involves the formation of the enolate form of pyruvate, which is stabilized by Mg^{2+} [20]. In addition, Mg^{2+} can form a binary complex with PEP with a moderate stability constant [21–23], raising the question of whether the complex or the free species combines with the catalytic site of PEPC. The answer to this question, although of great importance to the understanding of the *in vivo* regulation of the enzyme, is still a matter of debate. From kinetic studies of the non-phosphorylated enzyme performed with total PEP and Mg^{2+} concentrations, it has been concluded that the trianionic form of PEP binds to the active site of maize leaf PEPC [24] and that Mg^{2+} binds before PEP [25]. No activation by free PEP (*f*PEP) was detected in these studies. In contrast, from steady-state studies, also of the non-phosphorylated enzyme, in which the MgPEP complex was considered the variable substrate [16,26–28], it was concluded that the binary MgPEP complex is the substrate of the reaction and that either free Mg^{2+} (*f*Mg^{2+}) [26] or *f*PEP [16] behaves as an activator. Thus the role that Mg^{2+}, PEP or MgPEP has in the kinetics of the maize leaf PEPC-catalysed reaction is not clear at present. In addition,

Abbreviations used: *f*Mg^{2+}, free Mg^{2+}; *f*PEP, free PEP; Glc6P, glucose-6-phosphate; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase.

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either of these studies could be considered conclusive, given that the enzyme used in them was prepared in the absence of protease inhibitors, and it is known that the removal of an N-terminal peptide [20] in the first minutes after extraction alters the kinetic properties of the enzyme [29,30].

Because in order to achieve a complete understanding of the operation of PEPC *in vivo* is of pivotal importance to establish its kinetic mechanism, we studied the non-truncated enzyme by performing steady-state measurements with controlled concentrations of *f*PEP and *f*Mg^{2+} to determine: (1) the relative affinities of the active site for MgPEP and the free species, (2) if appropriate, the order of binding of the free species, and (3) activation by *f*PEP. In the present study we also examined the effects of saturating concentrations of Glc6P and Gly on the above-mentioned kinetic properties of the enzyme, to gain further insight into the mechanism of the allosteric regulation and to determine the allosteric site involved in the *f*PEP activation. In addition we considered it of interest to investigate not only the non-phosphorylated form of the enzyme, which is present in leaves during the dark period of the diurnal cycle [31,32], but also the phosphorylated, day-time form of the enzyme [31,32]. Our results provide experimental evidence indicating that the Mg^{2+} complex of PEP is the true substrate of the reaction catalysed by PEPC from maize leaves in the absence of activators and is the preferred substrate in their presence, and that *f*PEP is an activator that binds to the Glc6P allosteric site. The possible physiological implications of these findings are discussed. A preliminary account of part of this work has been published [33].

MATERIALS AND METHODS

Chemicals and biochemicals

PEP (monocyclohexylammonium salt), NADH (disodium salt), Glc6P, Gly, pig heart malic dehydrogenase and Hepes were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). EDTA (disodium salt) were from Merck KGaA (Darmstadt, Germany). All other chemicals of analytical grade were from standard suppliers.

Enzyme purification and assay

The non-phosphorylated night form of PEPC was purified from *Zea mays* L. leaves kept in darkness for 10 h before extraction as described elsewhere [10]. The sensitivity of the enzyme to malate, measured as IC_{50}, under the conditions described in [30], was typical of the non-truncated, non-phosphorylated form [30], and did not change on incubation with alkaline phosphatase under the conditions described in [31]. The specific activity of the enzyme preparation used, determined in a standard assay in the presence of 5 mM total PEP and 10 mM total Mg^{2+} at pH 7.3 and 30 °C, was 33 units/mg of protein. This enzyme preparation was phosphorylated *in vitro* by the method described in [34]. The degree of phosphorylation was assessed by (1) the change in IC_{50} for malate, which increased from 0.18 mM for the non-phosphorylated form to a maximum of 1.54 mM after prolonged incubation with the kinase, and (2) the activity at the subsaturating concentrations at which the IC_{50} was determined, which approximately doubled in the phosphorylated form. The addition of fresh ATP and kinase did not cause further increases in the maximum IC_{50} value reached. After exhaustive incubation with alkaline phosphatase, the IC_{50} for malate and the activity at the subsaturating substrate concentrations reverted to those of the non-phosphorylated enzyme. The specific activity of the enzyme determined at saturating substrate did not change upon phosphorylation.

Kinetic studies

Steady-state initial velocity studies were performed at 30 °C in a final volume of 0.5 ml of 100 mM Hepes/KOH buffer, pH 7.3 or 8.3, containing 1 mM EDTA, 10 mM $NaHCO_3$, 0.2 mM NADH, 4 units of malate dehydrogenase, varied concentrations of *f*PEP and *f*Mg^{2+}, both in the range from 0.25–4 mM, and the concentrations of Glc6P or Gly stated for each experiment. The amounts of total magnesium (as $MgCl_2$) and total PEP used to give the desired concentrations of the free species were calculated as described in [16]. The ligand–Mg dissociation constants used were 5.55 mM for PEP [21], 15.4 mM for Glc6P [23] and 50.12 mM for Gly [35]. The other dissociation constants used were as in [16]. Typically, assays were initiated by the addition of 15 μg of PEPC. A thermostatically controlled Beckman DU-7500 spectrophotometer, equipped with a kinetics software package, was used for these measurements. Initial velocities are expressed in units (μmol of product formed per minute). Each point shown in the figures is the average of duplicate or triplicate determinations.

Data analysis

PEPC kinetic data were analysed by non-linear regression calculations with a commercial computing program formulated with the algorithm of Marquardt [36]. Initial velocity data at several concentrations of the fixed substrate were first individually fitted to either the Michaelis–Menten equation [eqn. (1)] for hyperbolic kinetics, or to the Hill equation [eqn. (2)] for sigmoidal kinetics:

$$v = V[S]/(K_s + [S]) \quad (1)$$

$$v = V[S]^h/(S_{0.5}{}^h + [S]^h) \quad (2)$$

where v is the experimentally determined initial velocity; V is the maximal velocity; [S] is the concentration of the variable substrate, K_s and $S_{0.5}$ are the concentration of substrate at half-maximal velocity, and h is the Hill coefficient.

On the basis of the corresponding double-reciprocal plots, the mechanism was identified, and each data set was globally fitted to the corresponding initial velocity equation derived from that particular mechanism, always assuming rapid equilibrium conditions. The rapid equilibrium assumption needs to be validated by additional experiments, although, as shown below, the kinetic data in this paper are consistent with rate equations derived for rapid equilibrium models. Eqns. (3) and (4) correspond to mechanisms where there is exclusive binding of the MgPEP complex to the active site and activation by *f*PEP or competitive inhibition by *f*Mg^{2+} respectively:

$$v = V([S][M]/K_d)^n/[K_{sm}{}^n/(1 + [S]/K_a) + ([S][M]/K_d)^n] \quad (3)$$

$$v = V([S][M]/K_d)^n/(K_{sm}{}^n(1 + [M]/K_i) + ([S][M]/K_d)^n) \quad (4)$$

where [S] is the concentration of *f*PEP, [M] the concentration of *f*Mg^{2+}, K_d the dissociation constant of the substrate–metal complex, K_{sm} the concentration of the substrate–metal complex at half-maximal velocity, K_a the activation constant for *f*PEP, i.e. the dissociation constant of *f*PEP from the allosteric site, and K_i the inhibition constant for *f*Mg^{2+}.

Eqns. (5) to (8) were used when the substrate–metal complex and the free species bound to the active site, either in an ordered fashion with the substrate combining first and with [eqn. (5)] or without [eqn. (6)] activation by *f*PEP, or in a random fashion and with [eqn. (7)] or without [eqn. (8)] activation by *f*PEP:

$$= V[S][M]/(\alpha K_s K_m(1+[S]/K_s)/(1+[S]/K_a)+[S][M]) \quad (5)$$

$$= V[S][M]/(\alpha K_s K_m(1+[S]/K_s)+[S][M]) \quad (6)$$

$$= V[S][M]/(\alpha K_s K_m(1+[S]/K_s+[M]/K_m+[S][M]/K_s \times K_m+[S]^2/K_s K_a)/(1+[S]/K_a)+[S][M]) \quad (7)$$

$$= V[S][M]/(\alpha K_s K_m(1+[S]/K_s+[M]/K_m)+[S][M]) \quad (8)$$

here K_s and K_m are the concentrations of /PEP and /Mg^{2+} at lf-maximal velocity respectively and α is the interaction factor at describes the influence that the binding of one of the ligands, ee substrate or free metal ion, has on the binding of the other gand.

Eqn. (9) is a modification of eqn. (6) containing additional rms to account for inhibition by Mg^{2+} and was used to analyse e initial velocity patterns of the non-phosphorylated enzyme in e presence of Glc6P at pH 8.3:

$$= V[S][M]/(\alpha K_s K_m(1+[S]/K_s+[M]/K_i+[S][M]/K_s \times K_i)/(1+[M]/\delta K_i)+[S][M]) \quad (9)$$

here K_i is the inhibition constant for the metal, i.e. the ssociation constant from the allosteric site, and δ the interaction ctor describing the influence that the binding of the non-roductive metal ion has on the binding of the productive metal n or on the binding of the substrate–metal complex, and vice ersa.

In the experiments in which the concentration of the activator as varied at constant concentration of substrates, eqn. (10) was sed:

$$v_a - v_0)/v_0 = \mathrm{Act}_{max}[A]^h/(A_{0.5}{}^h + [A]^h), \quad (10)$$

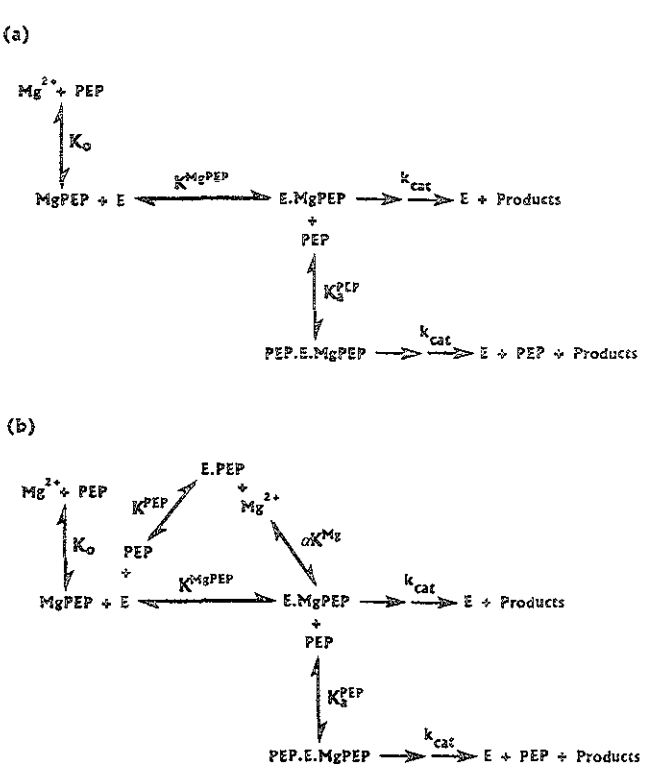
here v_0 and v_a are the initial velocities in the absence and resence of activator respectively, Act_{max} is the maximum ac-vation, [A] is the activator concentration, and $A_{0.5}$ is an apparent ctivation constant that equals the concentration of activator iving half-maximal activation at a given substrate concentration.

The points in the figures are the experimentally determined alues; the curves are calculated from the global fit of these data o the appropriate equation. The best fits were determined by the elative fit error, error of the constants and absence of significant orrelation between the residuals and other relevant variables uch as observed velocities, substrate concentration and data umber

ESULTS

inetics in the absence of activators

nitial velocity studies were performed by varying /PEP con-entration while keeping /Mg^{2+} concentration at different fixed evels, and vice versa. The concentration range used, from 0.25 to mM in both cases, was chosen to include and not greatly exceed ne physiological concentrations of PEP [9] and /Mg^{2+} [37]. At H 7.3, the saturation curves of both forms of PEPC for either PEP or /Mg^{2+} were sigmoidal and yielded maximal velocity alues that were independent of the concentration of the fixed ubstrate when individually fitted to the Hill equation [eqn. (2)] esults not shown), suggesting that the MgPEP complex is the ue substrate of the enzyme. However, if MgPEP were the only inetically significant species, we should obtain similar apparent Hill coefficients (h) when the concentrations of the fixed species, ither /PEP or /Mg^{2+}, were the same, but we consistently bserved higher h values when the variable substrate was /PEP han when it was /Mg^{2+} (results not shown). Lower Hill oefficients in the metal saturation curves than in the PEP aturation curves were also observed previously by others [38].



Scheme 1 PEP and Mg^{2+} binding to maize leaf PEPC in the absence of activators

Proposed mechanism for the non-phosphorylated and phosphorylated forms of the enzyme at pH 7.3 (**a**), and for the non-phosphorylated form of the enzyme at pH 8.3 (**b**)

These differences in the behaviour of the free species could be accounted for by the mechanism shown in Scheme 1(a), in which there is exclusive binding of the complex MgPEP to the catalytic site and of /PEP to an allosteric site in an obligate order, i.e. /PEP cannot add to the allosteric site until after the substrate MgPEP has added to the active site. Binding of /PEP to the allosteric site results in activation, because the apparent K^{MgPEP} is decreased by the factor $(1+[/PEP]/K_a^{PEP})$ but has no effect on the catalytic constant. As an approximation to allow mathematical treatment of the experimental data, the initial velocity data were analysed by using the velocity equation that accounts for the main features of this model, modified to allow for the co-operativity of substrate binding [eqn. (3)]. A good fit was obtained for both enzyme forms, non-phosphorylated and phosphorylated, which shows that the simplified analysis is valid at least for determining the maximal velocity and half-saturating concentrations of MgPEP and /PEP. Figures 1(a) and 1(b) show the data and the fits of the non-phosphorylated enzyme, and the estimated kinetic constants are given in Table 1.

At pH 8.3, the saturation curves for /Mg^{2+} of the non-phosphorylated form of PEPC were hyperbolic and the individual fit of each line to the Michaelis–Menten equation gave apparent V values independent of the concentration of /PEP (results not shown). Interestingly, saturation curves for /PEP were sigmoidal. This non-symmetrical sigmoidicity is suggestive of the same kinetic mechanism as that found at pH 7.3 (Scheme 1a), with the only difference that the binding of MgPEP to the active site is now non-co-operative. Binding of /PEP to an allosteric site will introduce $[/PEP]^2$ terms in the numerator and denominator of the velocity equation, accounting for the sigmoidicity of the

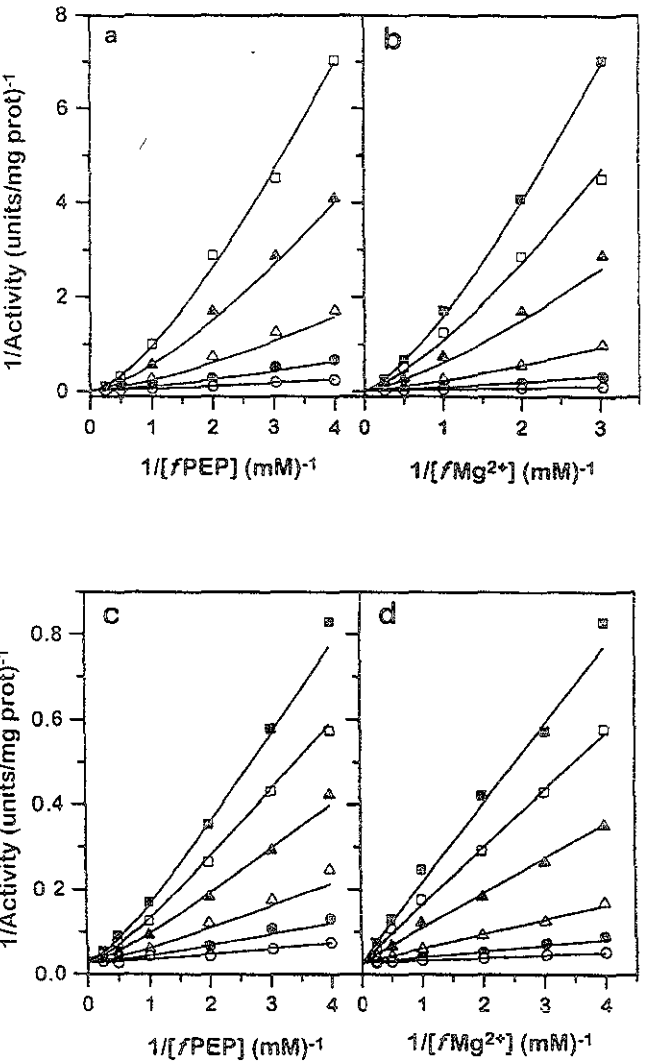


Figure 1 Double-reciprocal plots of initial velocity of the non-phosphorylated form of maize leaf PEPC at saturating bicarbonate concentration

Initial velocities were measured at pH 7.3 (**a**, **b**) or pH 8.3 (**c**, **d**) with *f*PEP (**a**, **c**) or *f*Mg^{2+} (**b**, **d**) as the variable substrate at the following fixed concentrations of the other: 0.25 (■), 0.33 (□), 0.5 (▲), 1.0 (△), 2.0 (●) and 4.0 (○) mM. Assays were performed under the standard conditions described in the Materials and methods section. The points are the experimental values and the curves are the best fit of the experimental data to eqn. (3) (**a**, **b**) or eqn. (5) (**c**, **b**).

*f*PEP saturation curves. In fact, the data obtained at pH 8.3 fit very well to eqn. (3), giving $V = 36.1 \pm 0.9$ units/mg of protein, $K^{MgPEP} = 0.30 \pm 0.02$ mM, $K_a^{PEP} = 3.7 \pm 0.9$ mM and $h = 1.06 \pm 0.07$. It can be seen that the h was close to 1 at this pH, and that the affinities of the active and allosteric sites for their respective ligands were increased by the same factor (approx. 3.5-fold) compared with those obtained at pH 7.3.

Attempts were made to fit the initial velocity data obtained at pH 7.3 and 8.3 to velocity equations derived for alternative mechanisms, such as those in which there is binding of the free species to the active site in a random or ordered fashion. All of these equations gave poor fits of the initial velocity data obtained at pH 7.3, with undefined kinetic constants. However, at pH 8.3 we obtained a good fit of the data to eqn. (5), which described an ordered mechanism in which PEP adds to the active site in rapid equilibrium before Mg^{2+} and there is activation by *f*PEP (Scheme 1b). In the latter fit, the error affecting the dissociation constant of PEP from the active-site–PEP complex, K^{PEP}, or of Mg^{2+} from the active-site–PEP–Mg complex, αK^{Mg}, were high (Table 1), which is understandable if we consider that the highest *f*PEP concentration used in this study is one-fifth of the estimated dissociation constant of the active-site–PEP complex. The absence of a term for the dissociation constant of Mg^{2+} from the active-site–Mg binary complex does not necessarily indicate that this complex does not form, but rather than the Mg^{2+} concentrations used in our study might not have been high enough to observe it. Similarly, at pH 7.3 the dissociation constant of the possible complexes of the free species from the active site of the enzyme might have been so high that the levels of these complexes were not kinetically significant in the concentration range used by us.

Kinetics in the presence of saturating concentrations of Glc6P

The results shown above support that *f*PEP binds to an activating allosteric site as a non-essential activator. Binding of *f*PEP to the Glc6P-allosteric site was proposed previously as a result of studies on changes in the fluorescence on binding of ligands to PEPC [15], or of kinetic studies with PEP analogues as PEPC activators [16–18]. To test whether Glc6P competes for the *f*PEP-binding site, we performed steady-state measurements of PEPC in the presence of a saturating concentration of Glc6P. Because the concentration at which Glc6P saturates the allosteric site depends on the degree of saturation of the active site [34], we first investigated the saturation kinetics of both forms of PEPC by Glc6P at the lowest and highest substrate concentrations used in our experiments. On the basis of the results obtained, given in

Table 1 Kinetic constants of maize leaf PEPC at fixed, saturating bicarbonate concentration

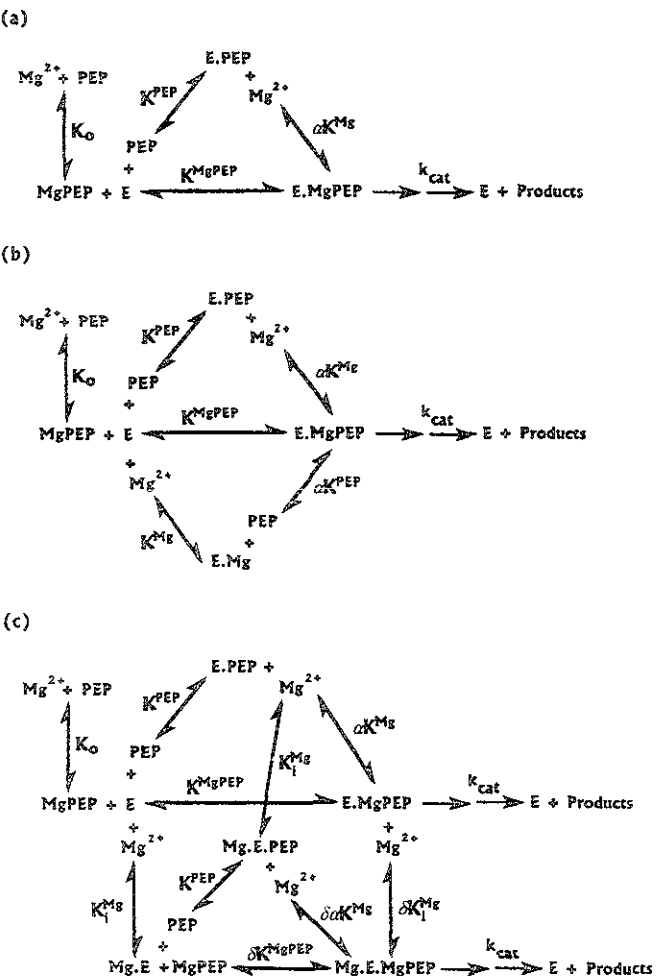
Means ± S.E.M. for the kinetic constants were calculated from a fit of the initial velocity data obtained at pH 7.3 to eqn. (3), or of those obtained at pH 8.3 to eqn. (5) ($K^{MgPEP} = K^{PEP}\alpha K^{Mg}/K_a = \alpha K^{PEP}K^{Mg}/K_a$, K_a (dissociation constant of the MgPEP complex) = 5.55 mM [21]).

Constant	Description	pH	Non-phospho-PEPC 7.3	Non-phospho-PEPC 8.3	Phospho-PEPC 7.3
V (units/mg of protein)			32.0 ± 0.5	36.2 ± 0.9	31.8 ± 0.4
K^{MgPEP} (mM)	Dissociation constant of MgPEP from E.MgPEP		0.83 ± 0.03	0.36 ± 0.05	0.55 ± 0.02
K^{PEP} (mM)	Dissociation constant of PEP from E.PEP		–	19.7 ± 5.8	–
αK^{Mg} (mM)	Dissociation constant of Mg^{2+} from E.MgPEP		–	0.10 ± 0.03	–
K_a^{PEP} (mM)	Dissociation constant of PEP from the allosteric site		4.78 ± 0.45	1.32 ± 0.51	7.00 ± 0.82
h	Hill coefficient		1.35 ± 0.03	1.03 ± 0.05	1.26 ± 0.02

Table 2 Kinetic constants for maize leaf PEPC activator Glc6P

Means ± S.E.M. for the kinetic constants were calculated from a fit of the initial velocity data to eqn. (10). *f*PEP and *f*Mg^{2+} were used at equal concentrations. Bicarbonate concentration was fixed at 10 mM.

Enzyme form	Assay conditions			Kinetic constants		
	pH	[Free species] (mM)	[MgPEP] (mM)	$A_{0.5}$ (mM)	Act_{max}	h
Non-phospho-PEPC	7.3	0.25	0.011	3.7 ± 0.2	39.0 ± 1.2	1.81 ± 0.13
		4	2.88	0.77 ± 0.09	0.37 ± 0.01	1.24 ± 0.29
	8.3	0.25	0.011	1.5 ± 0.1	11.3 ± 0.2	1.70 ± 0.11
		4	2.88	0.57 ± 0.05	0.39 ± 0.01	1.05 ± 0.10
Phospho-PEPC	7.3	0.25	0.011	4.1 ± 0.3	28.9 ± 0.9	1.97 ± 0.20
		4	2.88	1.9 ± 0.1	0.46 ± 0.02	1.08 ± 0.17



Scheme 2 PEP and Mg^{2+} binding to maize leaf PEPC in the presence of Glc6P

Proposed mechanism for the non-phosphorylated (**a**) and phosphorylated (**b**) forms of the enzyme at pH 7.3, and for the non-phosphorylated form at pH 8.3 (**c**).

Table 2, we decided to use 20 mM Glc6P at pH 7.3 and 10 mM Glc6P at pH 8.3. Concentrations of Glc6P above 20 mM could not be used at pH 7.3 because they produced significant inhibition at the lowest substrate concentration. The same Glc6P con-

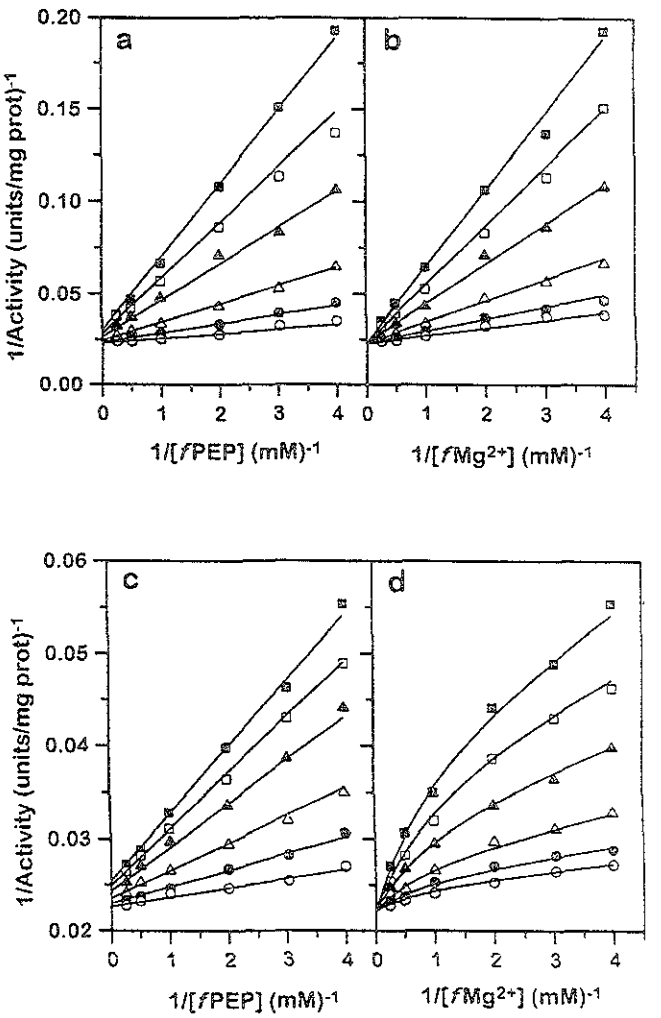


Figure 2 Double-reciprocal plots of initial velocity of the non-phosphorylated form of maize leaf PEPC at saturating bicarbonate concentration in the presence of Glc6P

Initial velocities were measured at pH 7.3 (**a**, **b**) or pH 8.3 (**c**, **d**) with *f*PEP (**a**, **c**) or *f*Mg^{2+} (**b**, **d**) as the variable substrate at the following fixed concentrations of the other: 0.25 (▩), 0.33 (□), 0.5 (▲), 1.0 (△), 2.0 (●) and 4.0 (○) mM. Assays were performed under the standard conditions described in the Materials and methods section in the presence of 20 (**a**, **b**) or 10 (**c**, **d**) mM Glc6P. The points are the experimental values and the curves are the result of the overall fit of the experimental data to eqn. (6) (**a**, **b**) or eqn. (9) (**c**, **d**).

Table 3 Kinetic constants for maize leaf PEPC at fixed, saturating concentrations of bicarbonate and Glc6P

The concentration of Glc6P in the assay medium was 20 mM at pH 7.3 and 10 mM at pH 8.3. Means ± S.E.M. for the kinetic constants were calculated from a fit of the initial velocity data obtained at pH 7.3 to eqn. (6) or eqn. (7) for the non-phosphorylated or phosphorylated enzymes respectively, and of those obtained at pH 8.3 to eqn. (9). See Table 1 for a description of K^{MgPEP}.

Constant	Description	pH	Non-phospho-PEPC 7.3	Non-phospho-PEPC 8.3	Phospho-PEPC 7.3
V (units/mg of protein)			44.0 ± 0.4	45.1 ± 0.3	44.9 ± 0.4
K^{MgPEP} (μM)	Dissociation constant of MgPEP from E.MgPEP		79 ± 2	7 ± 1	33 ± 1
K^{PEP} (mM)	Dissociation constant of PEP from E.PEP		5.7 ± 0.7	2.2 ± 0.2	1.7 ± 0.1
K^{Mg} (mM)	Dissociation constant of Mg^{2+} from E.Mg		–	–	4.9 ± 0.8
K^{Mg} (μM)	Dissociation constant of Mg^{2+} from E.MgPEP		76 ± 9	18 ± 4	107 ± 2
K^{PEP} (μM)	Dissociation constant of PEP from E.MgPEP		–	–	37 ± 3
K^{Mg}_{iv} (mM)	Dissociation constant of Mg^{2+} from the non-catalytic site of the free enzyme (Mg.E)		–	0.15 ± 0.05	–
K^{MgPEP} (μM)	Dissociation constant of MgPEP from the complex Mg.E.MgPEP		–	38 ± 7	–
αK^{Mg} (μM)	Dissociation constant of Mg^{2+} from the catalytic site of the complex Mg.E.MgPEP		–	96 ± 20	–
K^{MI} (mM)	Dissociation constant of Mg^{2+} from the non-catalytic site of the complex Mg.E.Mg or Mg.E.MgPEP		–	0.80 ± 0.29	–

Table 4 Kinetic constants for maize leaf PEPC activator Gly

Means ± S.E.M. for the kinetic constants were calculated from a fit of the initial velocity data to eqn. (10). fPEP and $f Mg^{2+}$ were used at equal concentrations. Bicarbonate concentration was fixed at 10 mM. Abbreviation: n.d., activation not detected.

Enzyme form	Assay conditions: pH	[Free species] (mM)	[MgPEP] (mM)	Kinetic constants: $A_{0.5}$ (mM)	Act_{max}	h
Non-phospho-PEPC	7.3	0.25	0.011	22.9 ± 1.4	40.8 ± 0.9	1.25 ± 0.07
		4	2.88	0.41 ± 0.04	0.28 ± 0.01	1.02 ± 0.12
	8.3	0.25	0.011	5 ± 1.3	5.5 ± 0.7	0.93 ± 0.37
		4	2.88	n.d.	n.d.	n.d.
Phospho-PEPC	7.3	0.25	0.011	19.3 ± 2.2	28.8 ± 1.1	1.17 ± 0.14
		4	2.88	0.61 ± 0.05	0.28 ± 0.01	1.05 ± 0.08

centration was used for the non-phosphorylated as for the phosphorylated forms of the enzyme, because we found only a small increase in $A_{0.5}$ after phosphorylation. The latter finding contradicts the previous report of decreases in the apparent activation constant of Glc6P brought about by phosphorylation [34].

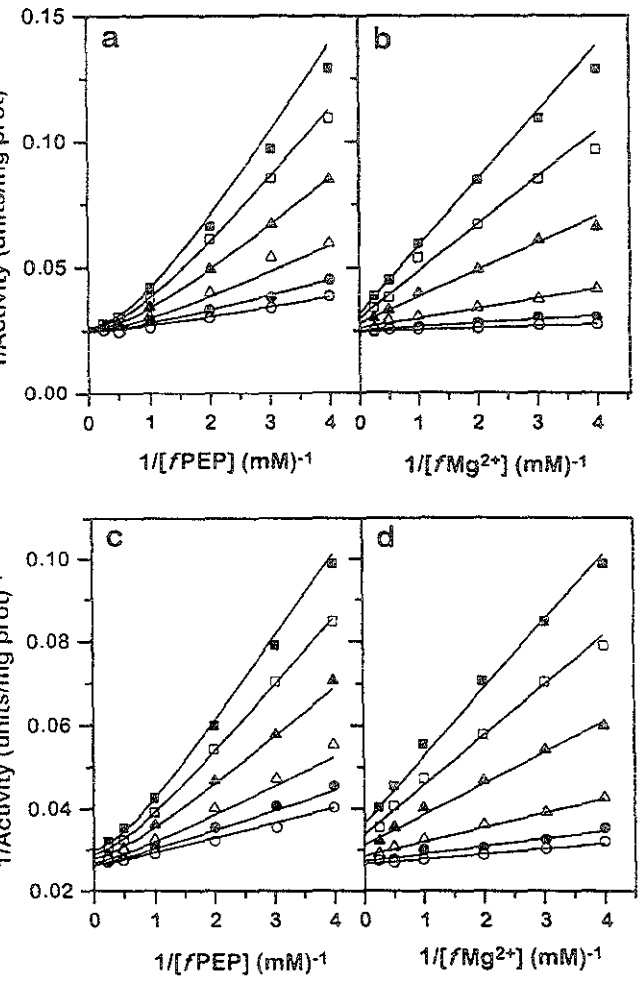
The saturation kinetics of both fPEP and $f Mg^{2+}$ at pH 7.3 in the presence of 20 mM Glc6P were hyperbolic, in line with previous findings of a decrease in the co-operative homotropic effects by this activator [1,14,39]. These observations rule out activation by fPEP, otherwise sigmoidal kinetics would have been obtained when fPEP was the varied substrate. Double-reciprocal plots were constructed for both sets of data. For the non-phosphorylated enzyme, the family of lines obtained when Mg^{2+} was varied and fPEP was held constant intersected on the $1/v$-axis, whereas the family of lines obtained when fPEP was varied and $f Mg^{2+}$ was held constant intersected to the left of the $1/v$-axis. These results are consistent with a rapid-equilibrium ordered mechanism in which fPEP binds before $f Mg^{2+}$ (Scheme 1a). Accordingly we obtained a good global fit of the data to eqn. (6), as shown in Figures 2(a) and 2(b). With the phosphorylated enzyme, both families of lines intersected at the left of the $1/v$-axis (results not shown), suggesting a rapid-equilibrium random binding of the free species (Scheme 2b); the best fit was obtained by using eqn. (7). The estimated kinetic parameters are given in Table 3.

At pH 8.3, double-reciprocal plots of $1/v$ against $1/[f$PEP$]$ at fixed levels of $f Mg^{2+}$ were linear (Figure 2c) but plots of $1/v$ against $1/[f Mg^{2+}]$ were non-linear at all fPEP concentrations (Figure 2d). The best fit of these data, shown in Figures 2(c) and 2(d), was obtained with eqn. (9), which was derived for the mechanism shown in Scheme 2(c). The estimated kinetic parameters are included in Table 3. This mechanism is similar to that described for pH 7.3, but here two Mg^{2+} ions bind to the enzyme, one as a substrate and the other as an inhibitor interfering with the binding of the catalytic Mg^{2+} and of MgPEP, but not with the binding of fPEP. A modified equation, including terms accounting for an effect of the inhibitor Mg^{2+} in the binding of fPEP, gave poorer fits to the experimental data. Therefore it seems that the sites at which the two metal ions bind partly overlap. In this respect it is interesting that two binding sites for metal ions [38] or concave-downward double-reciprocal plots of $1/v$ against $1/[f Mg^{2+}]$ [26] have been reported before for maize leaf PEPC, although the latter report concluded that the non-linearity of the double-reciprocal plots was due to activation by high concentrations of the metal ion.

Interestingly, no activation by fPEP was observed in the presence of saturating Glc6P, regardless of the form of the enzyme or of the pH of the assay, indicating either that binding of fPEP and Glc6P is mutually exclusive or that both activators share the same mechanism of activation.

Kinetics in the presence of saturating concentrations of Gly

The effects of Gly and Glc6P on the kinetics of maize leaf PEPC are additive [13,14], even at saturating concentrations of both



igure 3 Double-reciprocal plots of initial velocity of the non-phosphorylated form of maize leaf PEPC at saturating bicarbonate concentration in the resence of Gly

itial velocities were measured at pH 7 3 (**a**, **b**) or pH 8.3 (**c**, **d**) with /PEP (**a**, **c**) or /Mg^{2+} **b**, **d**) as the variable substrate at the following fixed concentration of the other. 0 25 (■), 0.33 □), 0 5 (▲), 1 0 (△), 2.0 (●) and 4 0 (○) mM. Assays were performed under the andard conditions described in the Materials and methods section in the presence of 100 **a**, **b**) or 50 (**c**, **d**) mM Gly The points are the experimental values and the lines are the best of the experimental data to eqn. (8)

ctivators (A. Tovar-Méndez and R A. Muñoz-Clares, unublished work). Therefore we expected to observe activation by PEP at saturating concentrations of Gly if /PEP bound to the Glc6P allosteric site. In contrast, if /PEP bound to the same site s Gly, or to a third allosteric site eliciting the same allosteric ansition as Gly, Gly should abolish the activation by /PEP.

To test whether the activation by /PEP could be abolished by aturation of the allosteric Gly site, the response of PEPC to arying the concentration of /PEP and /Mg was studied in the resence of saturating concentrations of the activator As with Glc6P, the affinity of the enzyme for Gly greatly increased as the ubstrate concentration did (Table 4). Therefore, to ensure aturation by the activator in the whole range of substrate oncentrations used in our experiments, the concentrations of ly used in this study were chosen on the basis of the apparent ctivation constants determined at the lowest substrate concenations. Because Gly did not produce enzyme inhibition even at ery high concentrations, we used Gly concentrations that were at least 3-fold the apparent activation constants estimated at the lowest substrate concentration.

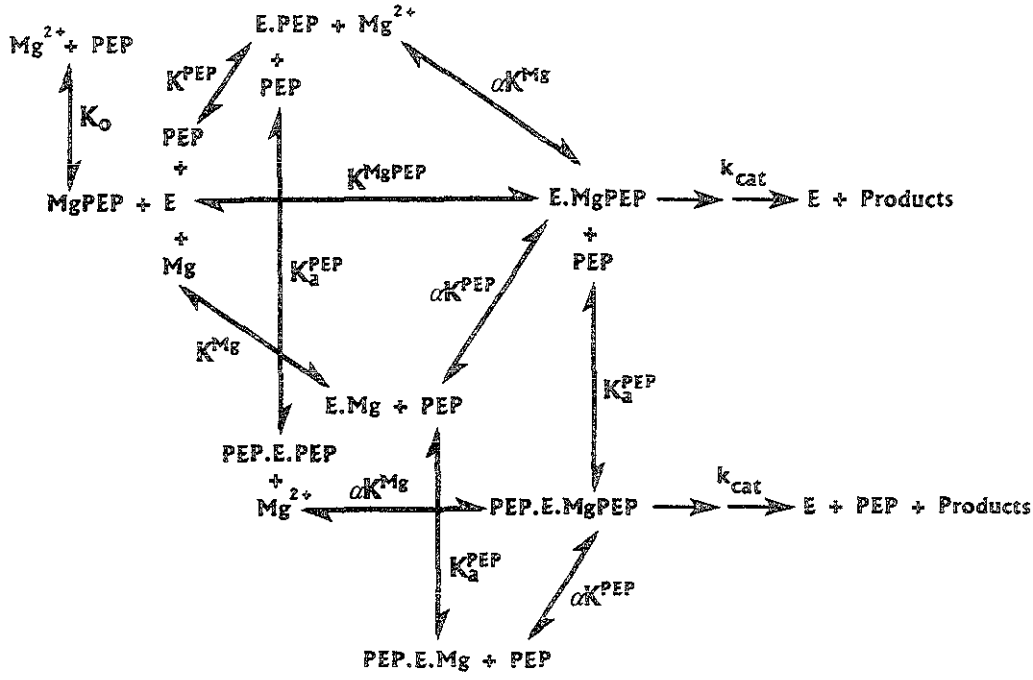
The initial velocity patterns of Figure 3, obtained with the non-phosphorylated enzyme at pH 7 3 in the presence of 100 mM Gly (Figures 3a and 3b) or at pH 8.3 in the presence of 50 mM Gly (Figures 3c and 3d), are consistent with random binding of the MgPEP complex and the free species and with activation by /PEP (Scheme 3). The experimental data were best fitted by eqn (8), derived from the mechanism outlined in Scheme 3, assuming that all ligands interacted with the enzyme in a rapid-equilibrium fashion. Saturation with Gly eliminated the positive co-operativity in the binding of the substrate MgPEP or of the free species to the catalytic site. The sigmoidal nature of the saturation kinetics observed only when /PEP was the variable substrate [Figures 3(a) and 3(c) show the corresponding double-reciprocal plots] arises from the heterotropic effects produced by the binding of /PEP to the allosteric site, as discussed above. Similar results were obtained with the phosphorylated form of the enzyme (results not shown). The kinetic parameters in Table 5 show that the effect of Gly on the affinity of the active site for MgPEP is similar to that of Glc6P, lowering the K^{MgPEP} to approx. one-tenth and one-sixteenth in the non-phosphorylated and phosphorylated forms respectively. Glycine also increased the affinity of the active site for the free species, mostly for /Mg^{2+}, which resulted in a preferred pathway of binding of /Mg^{2+} before /PEP. In contrast, binding of Glc6P favoured the binding of /PEP to the free enzyme, as shown above. It is then very clear that occupation of the Glc6P allosteric site has different consequences on the kinetics of the enzyme from occupation of the Gly-allosteric site, although both are activating sites This finding indicates that the allosteric properties of maize leaf PEPC cannot be explained by a two-state model, as suggested previously by binding studies [10,40].

Interestingly, Gly notably increases the affinity of the allosteric site for /PEP Thus, at pH 7.3, the estimated activation constants for /PEP in the non-phosphorylated and phosphorylated forms were approx. one-sixth and one-quarter in the presence of Gly of those in its absence, and approx. one-quarter at pH 8.3 in the non-phosphorylated enzyme. This result supports that Gly increases the binding of ligands to both the active and the Glc6P-allosteric sites, as suggested before [14]. The finding that /PEP behaves as an activator even in the presence of saturating Gly concentrations shows clearly that Gly and /PEP do not share either the same binding site or the same mechanism of activation

DISCUSSION

MgPEP is the true substrate of maize leaf PEPC at the physiological concentrations of PEP, Mg^{2+} and H^+ in the absence of activators, and the preferred substrate in their presence

Divalent metal ions, mainly Mg^{2+}, are essential activators of all known PEPCs. But whether the metal activates them by complexing with the substrate PEP or by complexing with the enzyme is still a matter of debate. Several studies on PEPC from maize leaves concluded that the MgPEP complex is the true substrate of the enzyme [16,26–28] However, a more recent study claimed that PEPC binds the free species in an ordered fashion, with Mg^{2+} binding before PEP [25]. This conclusion was drawn from results obtained in initial velocity studies performed on the non-phosphorylated, truncated enzyme at non-physiological pH 7.8, with varied total PEP concentrations at several fixed total Mg^{2+} concentrations. They argued that the previous claims of preferential binding of the MgPEP complex to the enzyme were based on a misinterpretation of the experimental



Scheme 3 PEP and Mg^{2+} binding to the non-phosphorylated and phosphorylated forms of maize leaf PEPC in the presence of Gly at pH 7.3 and 8.3

Table 5 Kinetic constants of maize leaf PEPC at fixed, saturating concentrations of bicarbonate and Gly

The concentration of Gly in the assay medium was 100 mM at pH 7.3 and 50 mM at pH 8.3. Means ± S.E.M. for the kinetic constants were calculated from a fit of the initial velocity data to Eqn (8). See Table 1 for a description of K^{MgPEP}.

Constant	Description	pH	Non-phospho-PEPC		Phospho-PEPC
			7.3	8.3	7.3
V (units/mg of protein)			41.3 ± 0.5	38.7 ± 0.3	40.2 ± 0.4
K^{MgPEP} (μM)	Dissociation constant of MgPEP from E.MgPEP		68 ± 7	33 ± 2	34 ± 2
K^{PEP} (mM)	Dissociation constant of PEP from E.PEP		19.6 ± 5.4	4.7 ± 0.6	9.8 ± 2.9
K^{Mg} (mM)	Dissociation constant of Mg^{2+} from E.Mg		4.9 ± 0.9	1.8 ± 0.2	1.2 ± 0.3
αK^{PEP} (μM)	Dissociation constant of PEP from E.MgPEP		78 ± 21	99 ± 13	160 ± 47
αK^{Mg} (μM)	Dissociation constant of Mg^{2+} from E.MgPEP		19 ± 4	37 ± 4	19 ± 2
K_a^{PEP} (mM)	Dissociation constant of PEP from the allosteric site		0.6 ± 0.1	1.2 ± 0.4	1.7 ± 0.6

ata that arose when the high degree of synergism in the binding f PEP to the enzyme–metal complex was not taken into account. Ve did find such a synergism, which could be assessed by the stimated interaction factors in the cases in which there is andom addition of the free species. These interaction factors ere 0.02 and 0.016 for the phosphorylated enzyme at pH 7.3 in ie presence of Glc6P or Gly respectively, and 0.004 and 0.020 or the non-phosphorylated enzyme in the presence of Gly at H 7.3 and 8.3 respectively. Therefore the binding of one of the ee species to the active site enhances the binding of the second t least 50-fold. However, it is clear from the results of steady-tate studies reported here that in the absence of effectors and at hysiological pH values and concentrations of free species, either of the free species, PEP or Mg^{2+}, binds to the active site f maize leaf PEPC; only the complex MgPEP does, regardless of ie state of phosphorylation of the enzyme. When the affinity f the active site for its ligands was increased by raising the H of the assay medium to 8.3, or by including Glc6P or Gly, we observed the binding of the free species to the active site. However, even under these conditions the MgPEP complex was still the substrate preferentially bound for the free enzyme. Thus the dissociation constants of the MgPEP complex from the free non-phosphorylated and phosphorylated forms of the enzyme are around 1/35 to 1/70 those of fPEP or $f Mg^{2+}$ (Tables 1, 3 and 5). Given the stability constant value of the MgPEP complex [21–23] and the concentration of $f Mg^{2+}$ present in the cytoplasm of vegetal cells (0.4 mM) [37], the cytosolic concentration of fPEP is only around 10-fold that of MgPEP, so formation of the active-site–MgPEP complex would be favoured over formation of the active-site–fPEP complex. However, it is clear that if the concentration of both free species and their respective dissociation constants from the active site are considered, equal amounts of the enzyme–MgPEP complex are formed through the pathway involving MgPEP and through the pathway involving the free species, because there is a high synergism in the binding of the second species once the other has been bound.

At pH 8.3 in the absence of Glc6P and Gly, the results were nsistent with a rapid-equilibrium ordered addition of /PEP fore Mg^{2+}, which is the same mechanism as that found in the esence of Glc6P, as would be expected if /PEP and Glc6P und to the same allosteric site. This mechanism is opposite to e ordered binding of /Mg^{2+} before /PEP found by Janc et al 5] The reason for the discrepancies between our results and ose of these authors are not clear at present, although they ght be related to their use of total concentrations of PEP and g^{2+} in their kinetic studies or, most probably, to differences in is respect between the non-truncated and truncated enzymes. ie N-terminal region of maize leaf PEPC might therefore fluence not only the phosphorylation status of the enzyme ,42] and therefore its sensitivity to malate [29] but also the lative affinities of the active site for MgPEP, PEP and Mg^{2+}.

EP activates maize leaf PEPC by binding to the Glc6P osteric site

ur initial-velocity results are consistent with the activation of EP of maize leaf PEPC regardless of the phosphorylation status the enzyme, the pH of the assay medium and the presence of tivators other than those that bind to the Glc6P site. An ternative mechanism, in which MgPEP is the only substrate d /Mg^{2+} inhibits the reaction by competition with MgPEP, uld also account for our results in the absence of activators. In is mechanism, the metal ion might bind to an allosteric site or the active site, but in the latter case binding of /Mg^{2+} would ot lead to a productive enzyme–Mg complex, i.e. /Mg^{2+} ould not be the first substrate to add, but an inhibitor that ould form a true dead-end enzyme–Mg complex unable to bind EP. Because at fixed MgPEP the concentration of /Mg^{2+} ecreases by a fixed factor when the concentration of /PEP creases, and vice versa, it is not experimentally feasible to parate the effects of /Mg^{2+} from those of /PEP. On the basis χ^2 values and error of the constants, poorer fits of the data ere obtained with eqn. (4), derived from the mechanism in hich there is inhibition by /Mg^{2+}, than with eqn. (3), which scribes activation by /PEP. Moreover, the results obtained in e presence of saturating Gly could not be fitted by equations in hich the terms corresponding to activation by /PEP were iminated and terms corresponding to inhibition by /Mg^{2+} were cluded Additional experimental evidence supporting activation / /PEP was obtained in independent experiments in which sensitization of the enzyme to activation by Glc6P by means of emical modification with PLP [10] also abolished the activation / /PEP (results not shown).

In none of the conditions in which activation by /PEP is detected, the data could be fitted to rate equations corsponding to mechanisms in which /PEP adds to the allosteric e in the free enzyme, suggesting that this complex does not rm at the concentrations of /PEP used in our study. Our sults do not allow us to conclude whether /PEP is able or not add to the allosteric site in the enzyme–Mg complex, which rms in the presence of saturating Gly, because similar fits were tained in both cases, although it was clear that /PEP binds to e enzyme–PEP and enzyme–PEPMg complexes. We therefore ncluded that under physiological conditions /PEP cannot bind the allosteric site until a ligand of the active site, whether gPEP, PEP or Mg^{2+}, is bound In such a mechanism, binding /PEP to the allosteric site results in activation because the parent dissociation constant of the complex MgPEP is lowered the factor $(1 + [/PEP]/K_i^{/PEP})$ resulting from the formation of e complex PEP–enzyme–MgPEP. In addition to this activating ect, due exclusively to the kinetic mechanism, it might be expected that /PEP could induce a conformational change in subunit to which it is bound, as most allosteric activators do, leading to (1) an increase in the affinity of the enzyme for the second substrate bicarbonate, which has been shown to bind after MgPEP [25], (2) an increase in the k_{cat}, i.e. an increase in the rate-limiting step of the reaction, or (3) increased affinities of the other subunits for MgPEP or allosteric activators. Although, before ruling out any effect of the binding of /PEP to the allosteric site on the binding of bicarbonate, experiments at subsaturating concentrations of this substrate must be performed, the good fit of our data to the equation derived from the mechanisms outlined in Schemes 1 and 3 do not support the suggestion that /PEP either induces an allosteric transition or affects the allosteric transition triggered by the substrate MgPEP Therefore the activating effects of /PEP seem to arise exclusively from the kinetics of the reaction.

Physiological implications of the kinetic mechanism of maize leaf PEPC

The /Mg^{2+} concentration in the cytoplasm of leaf cells has been reported to be approx. 0.4 mM [37] and that of total PEP approx. 0.1 and 4 mM during the dark and light periods respectively [9]. As discussed above, these concentrations are certainly consistent with exclusive binding of MgPEP to the active site of the non-phosphorylated and phosphorylated forms of maize leaf PEPC in the absence of effectors. The physiological role of Mg^{2+} in regulating of the enzyme activity is only that of a substrate, mainly as part of the MgPEP complex, whereas that of PEP is as a substrate and activator of the enzyme

Given the low concentrations of MgPEP *in vivo*, and the kinetic properties of the enzyme described here, the degree of saturation of the enzyme will be very low, particularly during the dark period. Therefore the enzyme will be almost inactive when the C_4 cycle is not in operation, avoiding an unnecessary use of PEP. In addition the enzyme would be highly responsive to the increases in the levels of substrate and allosteric activators, which affect mainly the affinity for the substrate, brought about by illumination.

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It has been a common practice to assay phospho*enol*pyruvate carboxylase at magnesium and bicarbonate concentrations much higher than the physiological ones. We have now performed kinetic studies on the enzyme from *Zea mays* leaves at 0.4 mM free Mg^{2+} and 0.1 mM bicarbonate, and found that both the nonphosphorylated and phosphorylated enzymes exhibited a high degree of cooperativity in the binding of phospho*enol*pyruvate, a much lower affinity for this substrate and for activators, and a greater affinity for malate than at high concentrations of these ions. Inhibition by malate was overcome by glycine or alanine but not by glucose-6-phosphate, either in the absence or presence of high concentrations of glycerol. Alanine caused significant activation at physiological concentrations, suggesting a pivotal role for this amino acid in regulating maize-leaf phospho*enol*pyruvate carboxylase activity. Our results showed that the maximum enzyme activity attainable in vivo would be less than 50% of that attainable in vitro under optimum conditions. Therefore, the high levels of phospho*enol*pyruvate carboxylase protein in the cytosol of C_4-mesophyll cells might be an adaptation for sustaining the steady-state rate of flux through the photosynthetic CO_2 assimilation pathway despite the limitations imposed by the phospho*enol*pyruvate carboxylase kinetic properties and the conditions of its environment.

In leaves of C_4 plants, the initial reaction in the assimilation pathway of atmospheric CO_2 is the essentially irreversible carboxylation of PEP by PEPC (EC 4.1.1.31), which requires Mg^{2+} for its activity (Bandurski, 1955). As in the case of enzymes catalyzing reactions involving ATP and ADP, the substrate of the PEPC catalyzed reaction is a complex between Mg and substrate (Wedding et al., 1988; Rodríguez-Sotres and Muñoz-Clares, 1990; Tovar-Méndez et al., 1998). This is surprising considering that the stability constant of MgPEP (0.18 mM) (Wold and Ballou, 1957) is very low compared to the stability constants of MgATP or MgADP (63 and 4 mM, respectively) (Dawson et al., 1986). Because the amount of fMg^{2+} estimated to be in the plant cytosol is only 0.4 mM, which it is not believed to drastically change under any plausible physiological condition (Yazaki et al., 1988), the cytosolic MgPEP concentration will be only about one tenth of the PEP concentration. We have recently proposed that the main features of the kinetics of maize leaf PEPC would lead to an enzyme mostly inactive under the physiological concentrations of the substrate if the concentrations of allosteric activators are low (Tovar-Méndez et al., 1998).

The PEPC catalyzed reaction had been regarded as a non rate-controlling step of the CO_2 assimilation pathway in leaves of maize plants, because the extractable PEPC activity was in great excess of that needed for the observed flux of this photosynthetic process (Avdeva and Andreeva, 1973; Usuda, 1984). However, the importance of this step in the photosynthetic metabolism of C_4 plants is underscored by the complex regulation of the activity of the C_4 PEPC isoenzyme, which indicates an important role in the control of the rate of CO_2 assimilation. At physiological pH, C_4 PEPC is activated homotropically by its substrate MgPEP (Tovar-Méndez et al., 1998) and heterotropically by phosphorylated sugars (Coombs et al., 1973; Wong and Davies, 1973) and neutral amino acids (Nishikido and Takanashi, 1973; Bandarian et al., 1992), and inhibited by dicarboxylic acids (Huber and Edwards, 1975). The enzyme is additionally controlled by phosphorylation on an N-terminal Ser residue (Jiao and Chollet, 1988) which causes a decrease in

affinity for the dicarboxylic acids (Jiao and Chollet, 1988; Echevarría et al., 1994) and an increase in affinity for PEP (Duff et al., 1995) or MgPEP (Tovar-Méndez et al., 1998). Recently, in a study of *Amaranthus edulis* mutants that have reduced amounts of PEPC, control coefficients of 0.26 and 0.39 were determined for the wild-type and mutant enzymes, respectively, at high light and ambient CO_2 concentration (Dever et al., 1997), implying that PEPC protein is not in excess.

To understand the role of PEPC in varying the flux through the CO_2 assimilation pathway of C_4 plants, under a wide range of conditions, one needs to understand the degree to which the enzyme is sensitive to changes in concentration of substrates and putative regulators in vivo. In some studies, care has been taken to simulate in vivo conditions by assaying the enzyme in the presence of the estimated cytosolic concentrations of PEP and allosteric effectors (Doncaster and Leegood, 1987; Echevarría et al., 1994; Gao and Woo, 1996) or in the presence of high concentrations of glycerol, to simulate conditions of high protein concentration (Stamatakis et al., 1990). However, these studies were carried out at high, nonphysiological concentrations of Mg^{2+} and, consequently, substrate MgPEP. Moreover, the other substrate of the PEPC reaction, bicarbonate ion, has also been used at much higher concentrations than its estimated concentration (77 μM) in mesophyll cell cytosol under air and normal illumination conditions (Jenkins et al., 1989). Because regulation of PEPC activity by metabolite effectors (Doncaster and Leegood, 1987) or by post-translational modificatic · is mostly exerted at subsaturating concentrations of substrate (Huber and Sugiyama, 1986; Echevarría et al., 1994), the use of high substrate concentrations would lead to erroneous estimates of in vivo PEPC activity and confusion about the role and relevance of its mechanisms of regulation.

The study presented here investigated the kinetics features of maize-leaf PEPC at concentrations of fMg^{2+} and bicarbonate close to those existing in vivo, in an attempt to understand how the kinetic features determine the responses of the enzyme to changes in the environment.

Our results showed that (i) inhibition by malate could be overcome only by Gly or Ala but not by Glc6P, even when the enzyme is fully phosphorylated and in the presence of high concentrations of glycerol, (ii) the nonphosphorylated enzyme may exhibit appreciable activity even at low concentration of tPEP if the activators are present, and (iii) the maximal enzyme activity attainable in vivo if PEP and activators are saturating would be less than 50% of the maximum activity attainable in vitro under the same conditions with saturating bicarbonate.

MATERIALS AND METHODS

Chemicals and Biochemicals

PEP (monocyclohexylammonium salt), NADH (disodium salt), Glc6P, malate, Gly, Ala, porcine heart malic dehydrogenase, and Hepes were purchased from Sigma Chemical Co (St. Louis, MO). EDTA (disodium salt) was from Merck KGaA (Darmstadt, Germany). All other chemicals of analytical grade were from standard suppliers.

Enzyme Purification and Assay

The purification procedure of the nontruncated and nonphosphorylated, night-form, PEPC from *Zea mays* L. cv Chalqueño leaves and the storage conditions of the pure enzyme were as described elsewhere (Tovar-Méndez et al., 1997). PEPC activity was measured spectrophotometrically with a coupled enzyme assay using malate dehydrogenase, and following the oxidation of NADH at 340 nm with a Beckman DU-7500 spectrophotometer equipped with a kinetics software package as described (Tovar-Méndez et al., 1998). The specific activity of the enzyme preparation used, determined in a standard assay in the presence of 5 mM tPEP and 10 mM tMg^{2+} at pH 7.3 and 30°C, was 33 μmol min^{-1} mg $protein^{-1}$. The enzyme preparation was fully phosphorylated in vitro by the method described by Duff et al. (1995). The phosphorylation status of PEPC was assessed as described in Tovar-Méndez et al. (1998).

Kinetic Studies

Steady state initial velocity studies were performed at 30°C in a final volume of 0.5 mL of 100 mM Hepes-KOH buffer (pH 7.3), 1 mM EDTA, 0.2 mM NADH, and 8 units $mL^{-1}$of malate dehydrogenase, with the concentrations of $NaHCO_3$, tPEP, fMg^{2+}, malate, Glc6P, Gly, Ala or glycerol stated in each experiment. The amounts of total magnesium (as $MgCl_2$) and tPEP used to give the desired concentrations of the free species were calculated using the procedure and dissociation constants of MgPEP, MgGlc6P, and MgGly complexes described by Tovar-Méndez et al. (1998). The dissociation constants used for Mgmalate and MgAla were 28.2 mM and 10 mM respectively (Dawson et al., 1986). No exogenous bicarbonate was added to the assays in which the concentration of bicarbonate was 0.1 mM. Theoretical calculations (Segel, 1976) assuming a partial pressure of CO_2 of 300 μbar give a concentration of bicarbonate of 107 μM in aqueous solutions at pH 7.3 in equilibrium with air. We confirmed this theoretical value by end-point determinations following a modification of the method described by Bauwe (1986). Briefly, we determined the amount of NADH produced in an coupled assay at 30°C, using pure maize leaf PEPC and saturating concentrations of PEP and Mg^{2+} without adding exogenous bicarbonate. Assays were initiated by the addition of 10 to 15 μg of PEPC to cuvettes with 10 mM bicarbonate and 50 to 100 μg to cuvettes with 0.1 mM bicarbonate. The progress of the reaction was followed during the first 30 s. To avoid cold inactivation (Kleczkowski and Edwards, 1990), the enzyme was kept at room temperature throughout the experiments, which were started at least 3 h after thawing the frozen enzyme preparation. We used an enzyme preparation in which PEPC is tetrameric, as assesed by exclusion chromatography and indicated by the lack of hysteresis in assays in which the reaction was allowed to proceed for several min. PEPC cannot significantly dissociate during the assay procedure in any of the conditions tested, given that the half-time for dissociation in the

incubation mixture in the absence of substrates is very long (30 min in the presence of 20 mM malate and 190 min in its absence) (Tovar-Méndez and Muñoz-Clares, unpublished results). Each point shown in the figures is the average of duplicate determinations. Initial velocities are expressed in units (μmol of product formed min^{-1}). We display the results of the kinetics of saturation of the enzyme by its substrate PEP by considering tPEP as the variable substrate, instead of fPEP or MgPEP to facilitate the evaluation of the effects of the different conditions tested in the physiological range of concentration of this metabolite.

Data Analysis

PEPC kinetic data were analyzed by nonlinear regression calculations using a commercial computing program formulated with the algorithm of Marquardt (1963). Kinetic data depending upon varied concentration of substrate were fitted to the Michaelis-Menten equation (equation 1) for hyperbolic kinetics, to the Hill equation (equation 2) for sigmoidal kinetics, or to the substrate inhibition equation (equation 3),

$$v = V_{max}.[S]/(K_s + [S]) \quad (1)$$

$$v = V_{max}.[S]^n/(S_{0.5}{}^n + [S]^n) \quad (2)$$

$$v = V_{max}.[S]/\{K_m + [S](1 + [S]/K_{IS})\}, \quad (3)$$

where v is the experimentally determined initial velocity, V_{max} the maximum velocity, [S] the concentration of the variable substrate, K_s and $S_{0.5}$ the concentration of substrate that gives half-maximum velocity, K_{IS} the inhibition constant for the substrate, and n the Hill number.

In the experiments in which the concentration of the activator was varied at constant concentration of substrates, equations 4 or 5 were used to fit the data to hyperbolic or sigmoidal saturation curves.

$$v_a = \{(v_{amax} - v_0)[A]/(A_{0.5} + [A])\} + v_0, \quad (4)$$

$$v_a = \{(v_{amax} - v_o)[A]^n/(A_{0.5}{}^n + [A]^n)\} + v_o, \quad (5)$$

where v_a and v_o are the initial velocities in the presence and absence of activator, respectively, v_{amax} is the highest velocity obtained at saturating activator concentrations, [A] is the activator concentration, and $A_{0.5}$ is the concentration of activator that gives half-maximum activation at fixed concentrations of substrates.

When the concentration of inhibitor was varied at constant concentration of substrates, the experimental data were fitted to equation 6 or 7 for data conforming to hyperbolic or to sigmoidal binding of the inhibitor, respectively:

$$v_i = v_o I_{50}/(I_{50} + [I]) \quad (6)$$

$$v_i = v_o I_{50}{}^n/(I_{50}{}^n + [I]^n) \quad (7),$$

where v_i and v_o are the initial velocities in the presence and absence of inhibitor, respectively, I_{50} is the concentration of inhibitor that gives half-maximum inhibition, and [I] is the inhibitor concentration.

The points in the figures are the experimentally determined values, whereas the curves are calculated from fits of these data by the appropriate equation. The best fits were determined by the relative fit error, error of the constants and absence of significant correlation between the residuals, and other relevant variables like observed velocities, substrate concentration, and data number.

RESULTS

Effects of Mg^{2+} on the Kinetics of Saturation of PEPC by PEP

Figure 1 shows the saturation kinetics of nonphosphorylated PEPC by tPEP at 0.4 and 10 mM fMg^{2-} and at saturating bicarbonate (10 mM). The assays were carried out at pH 7.3, which is the reported pH of the cytosol of C_4-mesophyll cells (Rajagopalan et al., 1993). As expected from

the fact that MgPEP is the reaction substrate, the kinetics of saturation by tPEP at 0.4 mM fMg^{2+} were clearly different from those at 10 mM, both in the absence and in the presence of effectors. V_{max} values at 0.4 mM fMg^{2+} were very similar to those at 10 mM under all conditions tested, but the apparent $S_{0.5}$ for tPEP values were around 4 fold higher at 0.4 than at 10 mM fMg^{2+} (results not shown). There were important differences in the degree of cooperative binding of PEP by the enzyme, as indicated by the Hill number, particularly in the presence of 5 mM malate where we estimated a $S_{0.5}$(tPEP) value of 13.4 mM and a n value of 3.4 ± 0.2 at the low Mg^{2+} concentration. Even in the absence of malate, PEPC, which exhibited a poor cooperativity at 10 mM fMg^{2+} (n = 1.4 ± 0.1), becomes an enzyme quite responsive to changes in tPEP concentrations when assayed at 0.4 mM fMg^{2+} (n = 2.1 ± 0.2). Qualitatively similar results were obtained with the phosphorylated form (results not shown).

Given the kinetic properties of PEPC, the differences in initial velocity between the two magnesium concentrations were important in the tPEP concentration range of 0.1 to 3 mM (Fig. 1, shaded area), especially in the presence of the inhibitor malate. These two concentrations of PEP are believed to be close to those existing in the cytosol of the mesophyll cells during the dark and light periods, respectively (Leegood, 1985; Stitt and Heldt, 1985; Doncaster and Leegood, 1987). It is clear that the potential in vivo PEPC activity would be greatly overestimated if assays were carried out at Mg^{2+} concentrations higher than the physiological ones.

Effects of fMg2+ on the Kinetics of Saturation of PEPC by Effectors

The known effectors of PEPC, activators such as Glc6P and Gly, and inhibitors such as malate, exert their action mainly at low concentrations of substrate (Doncaster and Leegood, 1987). Because of that, and since the true substrate of maize leaf PEPC is the MgPEP complex, the evaluation of their effects should be greatly dependent on the concentration of the metal ion

used in the enzyme assays. This was indeed found to be the case. As can be seen in Figure 2, the enzyme activities measured in the presence of Glc6P (Fig. 2, A and C) or Gly (Fig. 2, B and D) were much lower at physiological than at high fMg^{2+} concentration, especially at 0.1 mM tPEP (Fig. 2, A and B) where they were very low even at saturating concentrations of any of the activators. However, when both activators were present, the activities of the enzyme measured at 0.4 mM fMg^{2+} were slightly higher than those at 10 mM fMg^{2+} in the absence of activators. Concentrations of Glc6P higher than 15 to 20 mM resulted in inhibition of the enzyme (results not shown) as previously reported (Mújica-Jiménez et al., 1998). No inhibition by Gly was observed even at very high concentrations.

The concentration of the metal ion in the assay medium also affected the $A_{0.5}$ values, which measure the apparent affinity of the activators for the enzyme, and the degree of cooperativity in their binding, as assessed by the Hill number. The affinity of the enzyme for Gly was much lower than for Glc6P at 0.1 mM tPEP but it was very similar for both activators at 3 mM PEP. Thus, the ratio between the $A_{0.5}$ for Glc6P values determined at 0.1 and 3 mM tPEP was 2.5 whereas the same ratio in the case of Gly was almost 13. Given the reciprocity in the heterotropic effects, it is expected that, in the range of concentration of tPEP from 0.1 to 3 mM, Gly causes greater increases in the binding of this substrate to the enzyme than Glc6P when both activators are at equimolar concentrations. This was in fact observed in the experiments shown in Figure 1 (B and C). At 3 mM PEP, saturation of the enzyme with Gly was noncooperative whereas that of Glc6P was still cooperative. In addition, the activity measured at saturating Glc6P was much lower than that at saturating Gly. Again, similar results were found with the phosphorylated form of the enzyme (results not shown).

The kinetics of saturation by malate were also greatly affected by the concentration of fMg^{2+} (Fig. 3). The inhibitor concentration required to inhibit 50% was about 3 to 5 fold lower at low

compared to high fMg^{2+}, depending on the concentration of tPEP. Under our experimental conditions, the binding of malate to the enzyme was noncooperative, except at 3 mM tPEP and 10 mM fMg^{2+} when the best fit of the data was achieved using equation 8 (yielding a Hill number of 1.6 ± 0.1). This is an indication that the enzyme may exist in at least two states in equilibrium, one of which would be stabilized by the substrate and the other by malate. The high concentration of substrate in the latter experiment would displace the equilibrium towards the enzyme form not able to bind malate.

Taken together, these results show that the effects of the activators are overestimated and the effects of the inhibitor underestimated if a high, nonphysiological concentration of the metal ion is used in the assays.

Effects of Activators on the Inhibition by Malate at 0.4 mM fMg^{2+}

It has been reported that Glc6P effectively overcomes the inhibition by malate (Huber and Edwards, 1975; Echevarria et al., 1994). On the other hand, Gly has been found to be more effective than Glc6P in this respect (Gao and Woo, 1996). Because these studies were carried out at high concentrations of Mg^{2+}, we were interested in examining the effects of Glc6P and Gly, alone and in combination, on the I_{50} for malate of the nonphosphorylated and phosphorylated PEPC forms at 0.4 mM fMg^{2+}. In Table I are given the results of these experiments carried out at the same two fixed concentration of tPEP as above: 0.1 mM and 3 mM. Under all the conditions tested, Gly was much more effective in preventing inhibition by malate than was Glc6P, confirming the report of Gao and Woo (1996). The effects of 10 mM Gly were especially significant at 3 mM PEP, where the activator increased the I_{50} for malate more than 10-fold in both enzyme forms. At the same PEP concentration, 10 mM Glc6P caused only a 2 to 3 fold increase. When both activators were present, the I_{50} value was increased with respect to the value in the absence of activators 17 and

23 fold in the nonphosphorylated and phosphorylated forms, respectively. Interestingly, the ratios of the I_{50} values of the phosphorylated form to the I_{50} values of the nonphosphorylated form are 2 to 3 fold lower at low compared to high PEP concentration. Thus, the partial desensitization of the enzyme to the inhibitor malate caused by phosphorylation is increased by high PEP concentrations and by the presence of activators (i.e. under the conditions presumably prevailing during the light period of the day).

Kinetics of Saturation of PEPC by PEP, Glc6P, Gly, or Malate at 0.4 mM fMg^{2+} and 0.1 mM Bicarbonate

The concentration of bicarbonate in the cytosol of maize mesophyll cells has been estimated to be as low as 77 μM (Jenkins et al., 1989). This concentration is much lower than those commonly used in the assays of PEPC, which were 1 (Echevarría et al., 1994; Duff et al., 1995; Gao and Woo, 1996; Ogawa et al., 1997), or 10 mM (Uedan and Sugiyama, 1976; Doncaster and Leegood, 1987; McNaughton et al., 1989; Gillinta and Grover, 1995; Dong et al., 1997; Tovar-Méndez et al., 1998), concentrations that are at least 10 to 100 fold the K_m for bicarbonate of the C_4 PEPC (Uedan and Sugiyama, 1976; Bauwe, 1986; Janc et al., 1992; Dong et al., 1997). To see whether our conclusions might be affected qualitatively and/or quantitatively by low bicarbonate concentration, we studied the kinetics of saturation of the nonphosphorylated and phosphorylated forms of PEPC by tPEP or by Glc6P, Gly, or malate at 0.4 mM fMg^{2+} and 0.1 mM bicarbonate. Although we are aware that this concentration is still higher than the physiological one, we chose it to simplify the experiments. No exogenous bicarbonate had to be added to the cuvette, thus avoiding possible errors in estimating its concentration, and no further precautions, such as extensive degassing and isolation of the samples from air, are required. The results are shown in Figure 4, and the apparent kinetic parameters obtained by the best nonlinear fit of the experimental initial velocity data to the

appropriate equations are given in Table II. Assaying the enzyme at low concentrations of bicarbonate resulted in increases in the estimated values of the $S_{0.5}$ for tPEP and the $A_{0.5}$ for Gly and Glc6P, and decreases in the I_{50} for malate values with respect to those found at a high, saturating bicarbonate concentration. These findings are in agreement with previous reports (Ogawa et al., 1997; Parvathi et al., 1998). Interestingly, the Hill numbers were not affected by lowering the concentration of bicarbonate 100-fold.

As can be seen in Figure 4, the activity of the phosphorylated form was higher than that of the nonphosphorylated form at subsaturating concentrations of tPEP, but slightly lower at saturating and near-saturating concentrations of the substrate. The increase in the affinity of the enzyme for the substrate MgPEP brought about by phosphorylation, and so far observed at high, saturating concentrations of bicarbonate, was therefore also observed at subsaturating concentrations. The different effects of Glc6P and Gly on enzyme activity were also observed at low bicarbonate. Thus, at 3 mM tPEP the binding of Glc6P was still cooperative whereas that of Gly was not. The highest activity measured at saturating concentrations of the activators was indicative of saturation of the enzyme by this substrate concentration in the presence of Gly but not in the presence of Glc6P.

When 20 mM malate was added to the assay medium to simulate near physiological conditions, the kinetic differences between Glc6P and Gly were accentuated. Figure 5A shows the kinetics of saturation of the phosphorylated PEPC by Glc6P or Gly, at 3 mM tPEP, 0.4 mM fMg^{2+} and 0.1 mM bicarbonate in the presence of 20 mM malate. The effects on PEPC activity of Ala, the most abundant neutral amino acid in mesophyll cells (Weiner and Heldt, 1992) was also studied. Saturating the enzyme with Glc6P caused a rise of only about 3-fold in the velocity, which was still well below the one measured at the same substrate concentration but in the absence of malate and Glc6P. Saturating the enzyme with Gly or Ala caused a 150-fold increase, yielding the same enzyme activity observed in the absence of malate and presence of saturating concentrations of

the neutral amino acids. As a consequence, the maximum activity obtained under the conditions of this assay at saturating Glc6P is only about 2% of the maximum activity measured at saturating Gly or Ala. Moreover, the estimated $A_{0.5}$ for Glc6P is only 1.4-fold higher than that determined in the absence of malate and otherwise identical conditions whereas the estimated $A_{0.5}$ for Gly is increased 13-fold. These findings suggest that malate effectively prevents the binding of Gly, and vice versa. Both ligands are mutually exclusive. In contrast, Glc6P and malate appear to bind simultaneously to the enzyme, even though both are mutually competitive to a small degree. The resulting enzyme-malate-Glc6P complex seems not to bind the substrate (i.e., it behaves as an inhibited enzyme form). As it is shown in Figure 5B, qualitatively the same results were obtained when the experiment was performed in the presence of 20% (v:v) glycerol to simulate the low water activity level likely existing in vivo. Saturating concentrations of Gly or Ala completely overcome malate inhibition, whereas saturating Glc6P only caused small increases in the enzyme activity determined in its absence. Moreover, PEPC inhibition occurred when Glc6P was increased above 15 or 20 mM in the absence or presence of glycerol, respectively (not shown). Although the enzyme activity in the absence of activators was notably higher in the presence than in the absence of glycerol, indicating that glycerol opposed to malate inhibition in some degree, it is interesting that the maximum activity reached at saturating concentrations of neutral amino acids in the absence of glycerol was very similar to that in its presence.

Whatever the mechanism of interaction between the two kinds of activators and malate, it is clear from our results that Glc6P by itself is a very inefficient activator of PEPC if malate is present. Thus. the neutral amino acids, particularly Ala, would be much better activators of the enzyme than Glc6P under the conditions prevailing during the day.

As a summary of the results described above, Figure 6 shows the combined effects of phosphorylation, activators, and PEP concentration on the activity of the phosphorylated and

nonphosphorylated forms of the enzyme, at concentrations of fMg^{2+} and bicarbonate close to the physiological ones, in the absence and presence of 20 mM malate. At the lowest PEP concentration, the activities of both enzyme forms were negligible unless high concentrations of both activators were present, particularly in the presence of malate. It can be seen that 10 mM Gly produced higher increases in PEPC activity than 10 mM Glc6P. Also, the activities of the phosphorylated form were always higher than those of the nonphosphorylated form, but the differences between both forms were small in the absence of malate. The advantages of phosphorylation were clearly seen in the presence of the inhibitor. However, phosphorylation by itself, without concomitant increases in tPEP, was not able to cause a significant increase in enzyme activity. Thus, at 0.1 mM tPEP and 20 mM malate, the activity of the phosphorylated enzyme when both Glc6P and Gly (10 mM each) were present was only 2.7% of the maximum activity at saturating MgPEP and bicarbonate. On the other hand, without phosphorylation PEPC activity was equally low at 2.2% of the maximum activity, even at high PEP and activator concentrations (Fig. 6).

DISCUSSION

Effect of Mg^{2+} and PEP in the Response of the Enzyme to its Allosteric Effectors.

The kinetics of PEPC at 0.4 mM fMg^{2+} are quite different from the kinetics at high fMg^{2+} (Figs. 1, 2 and 3). The experimental data shown in Figure 1A are fully consistent with the kinetic model we have recently proposed for maize leaf PEPC (Tovar-Méndez et al., 1998), and they gave a very good fit to this model when fPEP or MgPEP are considered as the variable substrate, yielding identical V_{max} and $S_{0.5}$ for MgPEP values regardless of the metal ion concentration (results not shown). Thus, the results in this paper support the role of magnesium ions in the kinetics of the enzyme as part of the MgPEP complex.

nonphosphorylated forms of the enzyme, at concentrations of fMg^{2+} and bicarbonate close to the physiological ones, in the absence and presence of 20 mM malate. At the lowest PEP concentration, the activities of both enzyme forms were negligible unless high concentrations of both activators were present, particularly in the presence of malate. It can be seen that 10 mM Gly produced higher increases in PEPC activity than 10 mM Glc6P. Also, the activities of the phosphorylated form were always higher than those of the nonphosphorylated form, but the differences between both forms were small in the absence of malate. The advantages of phosphorylation were clearly seen in the presence of the inhibitor. However, phosphorylation by itself, without concomitant increases in tPEP, was not able to cause a significant increase in enzyme activity. Thus, at 0.1 mM tPEP and 20 mM malate, the activity of the phosphorylated enzyme when both Glc6P and Gly (10 mM each) were present was only 2.7% of the maximum activity at saturating MgPEP and bicarbonate. On the other hand, without phosphorylation PEPC activity was equally low at 2.2% of the maximum activity, even at high PEP and activator concentrations (Fig. 6).

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As part of the substrate, Mg^{2+} indirectly affects the binding of the allosteric ligands to the enzyme. The same reasoning applies to PEP. We attempted to determine whether, in addition, fMg^{2+} or fPEP could directly modulate the response of the enzyme to its effectors by comparing the results obtained at 10 mM fMg^{2+} with those obtained at 0.4 mM fMg^{2+}.

For nonessential activators, the relationship between the $A_{0.5}$ value and the normalized substrate concentration ([S]/K_s) is

$$A_{0.5} = \alpha K_a (1 + [S]/K_s)/(\alpha + [S]/K_s) \qquad (8)$$

where α is the interaction factor, which describes the influence that the binding of the substrate has on the binding of the activator and vice versa, and K_a is the activation constant (i.e. the dissociation constant of the activator from the complex enzyme-activator). When there is cooperative binding of the substrate and activator, equation 8 becomes:

$$A_{0.5}{}^{n} = \alpha K_a^{n} \{1 + ([S]/S_{0.5})^{n}\}/\{\alpha + ([S]/S_{0.5})^{n}\} \qquad (9)$$

When bicarbonate is saturating, the reaction catalyzed by PEPC may be considered a single substrate reaction in which the normalized substrate concentration ([S]/$S_{0.5}$), is related to the initial velocity, v_o, by equation 10, which was derived from the Hill equation (equation 2)

$$v_o/(Vmax - v_o) = ([S]/S_{0.5})^{n} \qquad (10)$$

Thus, combining equations 9 and 10, it is possible to relate the $A_{0.5}$ value with the degree of saturation of the enzyme by the substrate, indicated by the ratio of the initial velocity in the absence of inhibitor to the corresponding maximum velocity (v_o/V_{max}):

$$A_{0.5}{}^{n} = K_a^{n}/\{1 + v_o/V_{max}(1/\alpha - 1)\} \qquad (11)$$

Assuming that Glc6P and Gly behave as nonessential activators able to bind to the free enzyme and to the enzyme-substrate complex. and taking into account the degree of saturation of the enzyme by MgPEP (measured as v_o/V_{max} under each condition) and the $A_{0.5}$ obtained at the two

concentration of fMg^{2+}, it is thus possible to estimate theoretical values for K_a for a given value of α using equation 11.

Similarly, the I_{50} value for a competitive inhibitor is an apparent kinetic parameter related to the dissociation constant of the inhibitor-enzyme complex, K_i, and to the normalized concentration of the substrate by the following expression (Segel, 1975):

$$I_{50} = Ki\ (1 + [S]/K_s) \qquad (12)$$

or when there is cooperative binding of the substrate and inhibitor:

$$I_{50}^{\ h} = K_i^{\ h}\{1 + ([S]/K_s)^n\} \qquad (13)$$

Combining equations 10 and 13, we obtain:

$$I_{50}^{\ h} = K_i^{\ h}/(1 - v_o/V_{max}) \qquad (14)$$

Thus, assuming that malate behaves as a competitive inhibitor with respect to MgPEP, we can estimate theoretical K_i values using equation 14 and the experimentally determined I_{50} values.

Under saturating bicarbonate, v_o/V_{max} is a measure of the amount of this complex relative to total enzyme. Therefore, for a given MgPEP concentration, the K_a and K_i values estimated by equations 11 and 14, respectively, should be independent of the concentration of Mg^{2+} and PEP, if these ligands affect the binding of the allosteric effectors only through the formation of the enzyme-MgPEP complex. This was found for malate and Glc6P, within experimental error, but for Gly we found important differences between the theoretical K_a values estimated at low and high fMg^{2+} and also between those estimated at low and high tPEP for any given value of α (not shown). Therefore, the binding of Gly appears not only to be dependent on the steady state level of the enzyme-MgPEP complex but also on the level of the enzyme-Mg or enzyme-PEP complex. This conclusion is consistent with our previous finding that Gly greatly increases the binding of fMg^{2+} and fPEP to the active site of the enzyme (Tovar-Méndez et al., 1998) and might explain why the $A_{0.5}$ for Gly changed to a much greater extent than the $A_{0.5}$ for Glc6P when Mg or PEP were varied

in a given concentration range (Fig. 2). Although Gly promotes the binding of fMg^{2+} to the active site more than that of fPEP, we have also shown that fPEP activates the enzyme by binding to the Glc6P allosteric site (Tovar-Méndez et al., 1998). In fact, Gly increases the affinity of the allosteric site for fPEP. Therefore, it is expected that the fPEP bound to the allosteric site would have a positive effects on the binding of Gly, as has been found for phosphomycin, another ligand of this allosteric site (Mújica-Jiménez et al., 1998).

Effect of Bicarbonate in the Response of the Enzyme to PEP and the Allosteric Effectors.

We observed a decrease in V_{max} and an increase in $S_{0.5}$ for tPEP when the concentration of bicarbonate was reduced from 10 to 0.1 mM at 0.4 mM fMg^{2+}. Kinetic studies of maize leaf PEPC carried out at pH 7.8 indicated that the addition of PEP and bicarbonate to PEPC is random, but the reaction pathway in which bicarbonate adds after PEP is preferred (Janc et al., 1992). In such a mechanism, V_{max} is a function of the concentration of bicarbonate (Segel, 1975):

$$\text{app } V_{max} = V_{max}[\text{bicarbonate}]/\{K_m(\text{bicarbonate}) + [\text{bicarbonate}]\} \qquad (15)$$

Therefore, the 2-fold decrease observed in the V_{max} value when the concentration of bicarbonate was lowered is accounted for by the kinetic mechanism assuming that the K_m(bicarbonate) at pH 7.3 is 0.1 mM (Dong et al., 1997). The decrease in the apparent affinity of the enzyme for tPEP could also be explained by the kinetic mechanism if the value of the dissociation constant of MgPEP from the free enzyme is higher than the value of its dissociation constant from the enzyme-MgPEP-bicarbonate complex in a random mechanism, or if the value of the dissociation constant of MgPEP from the free enzyme (K_{ia}) is higher than K_m(MgPEP) in a steady state ordered mechanism (Segel, 1975). However, in a previous study Janc et al. (1992) found that K_{ia} is considerable lower than K_m(PEP). These discrepancies may arise because these

authors used tPEP and tMg in their experiments and they analyzed their data considering that the PEPC-catalyzed reaction has three substrates, Mg, PEP and bicarbonate.

Assuming that the allosteric transition takes place upon formation of the enzyme-MgPEP complex, the observed effects that decreasing the concentration of bicarbonate had on the affinity of the enzyme for its allosteric regulators could be explained if the steady state levels of the enzyme-MgPEP complex were lower at low compared to high bicarbonate. This would imply some degree of synergism in the binding of both substrates, which is consistent with the observed effects of bicarbonate on the apparent affinity for tPEP at a fixed fMg^{2+} concentration. The finding that changing the concentration of bicarbonate 100-fold did not affect the degree of cooperativity observed in the kinetics of saturation by tPEP rules out a cooperative binding of bicarbonate under our experimental conditions, in agreement with previous results (Uedan and Sugiyama, 1976; Bauwe, 1986; Janc et al., 1992; Dong et al., 1997). However, Parvathi et al. (1998) observed cooperativity in the binding of bicarbonate to the enzyme and postulated a bicarbonate-induced conformational change to explain the effects of this ion on the sensitivity of the enzyme to its allosteric effectors.

Possible Physiological Role of Allosteric Regulation

Despite the low intracellular concentration of fMg^{2+}, all the potential activity of the enzyme could be realized if the levels of tPEP were high enough to produce saturating concentrations of MgPEP. However, considering that the $S_{0.5}$(tPEP) under near physiological conditions is at least 10 mM, the levels of tPEP required for saturation of the enzyme would probably not be attainable in vivo. By increasing the affinity of the enzyme for MgPEP, any of the allosteric activators would allow saturation at much lower tPEP concentrations, particularly if the in vivo water activity resembles that in the presence of 20% glycerol. Because we assayed a tetrameric PEPC, the

observed effects of the allosteric activators or of glycerol in PEPC activity are due to activator- or glycerol-induced conformational changes that did not involve changes in the aggregation state of the enzyme, in agreement with the finding of homotropic and heterotropic effects in the tetrameric form of maize leaf PEPC (Tovar-Méndez et al., 1995; Mújica-Jiménez et al., 1998). With respect to glycerol, it is known that cosolutes that alter water activity can affect the affinities of some proteins for their ligands without affecting their state of aggregation (Coiombo et al., 1992; Rand et al., 1993).

The two allosteric sites are by no means redundant. Besides connecting the CO_2 assimilation pathway with the metabolic pathways of phosphorylated sugars and neutral amino acids, the ligand-bound allosteric sites affect the binding of the substrate MgPEP and inhibitor malate in quite different ways. These kinetic differences acquire special relevance under conditions close to those prevailing under illumination (Fig. 5) when the degree of activation of the enzyme brought about by Glc6P is much lower than that brought about by neutral amino acids. It is important to point out that the magnitude of the effects of both kind of activators on malate inhibition cannot be fully appreciated by measuring increases in I_{50} for malate caused by a fixed concentration of a given activator (Echevarría et al., 1994; Gao and Woo, 1996). To evaluate the full potential of the activators in the presence of the inhibitor, it is necessary to determine the maximum enzyme activity achieved at saturating concentration of the activator and at fixed, physiological concentration of the inhibitor.

Among the phosphorylated sugars which bind to the allosteric Glc6P site, Glc6P is the strongest activator (Doncaster and Leegood, 1987; Bandarian et al., 1992; Mújica-Jiménez et al., 1998, Tovar-Méndez and Muñoz-Clares, unpublished results). Because of this, it is to be expected that saturation of the site by another phosporylated sugar will not result in higher PEPC activity than that determined at saturating Glc6P. The Glc6P site could be important during the night or at

the onset of illumination, before the build up of malate that takes place during the first hour after illumination (Rodriguez-Sotres et al., 1987). Once the levels of malate are high, saturation of the Glc6P site would give only a marginal advantage. It is interesting that increasing the concentrations of Glc6P above 20 mM would not result in further increases in PEPC activity. In fact, inhibition results. However, it is not likely that inhibition would occur in vivo because concentrations of triose phosphate and Glc6P estimated to exist under conditions of illumination do not exceed this level (Stitt and Heldt, 1985).

Our results indicate that the allosteric site for neutral amino acids is crucial for achieving appreciable levels of PEPC activity under near physiological conditions. Different from the phosphorylated sugars, the neutral amino acids do not inhibit PEPC at high concentrations. Therefore, they will produce further increases in the activity even though their levels were increased above 100 mM. Under conditions of illumination the concentration in mesophyll cytosol of two ligands of this site, Gly and Ser, are low (Weiner and Heldt, 1992) but the concentrations of Ala are high, 30-40 mM (Leegood, 1985; Weiner and Heldt, 1992), enough to arise PEPC activity significantly, even in the presence of high concentrations of malate, as shown in Fig. 5. Thus, Ala may be the principal ligand of this allosteric site, and the degree of saturation of PEPC by this amino acid will be high enough to arise its activity significantly, even in the presence of high concentrations of malate.

We estimated that the limiting PEPC activity attainable in vivo, if saturation by MgPEP takes place, would be less than 50% of the maximum activity attainable in vitro under optimum conditions of bicarbonate concentration. Laisk and Edwards (1997), on the basis of very different experiments, proposed that the activity of C_4 PEPC during steady state conditions of photosynthesis is 25% of the maximum enzyme capacity. The high level of PEPC protein in the cytoplasm of C_4-mesophyll cells of plants (Uedan and Sugiyama, 1976; Hague and Sims, 1980)

might be an adaptation for sustaining the steady state rate of flux through the photosynthetic CO_2 assimilation pathway despite the limitations imposed by the PEPC kinetic properties and the conditions of its environment.

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gure 1. Kinetics of saturation of nonphosphorylated maize-leaf PEPC by tPEP at 10 mM carbonate and at 0.4 (○) or 10 mM (●) fMg^{2+} in the absence (A) and presence of 10 mM Glc6P 3), 10 mM Gly (C) or 5 mM malate (D). In the concentration range of tPEP used in these experiments (0.028 to 22.4 mM), MgPEP concentrations ranged from 0.0017 to 1.36 mM at 0.4 mM fMg^{2+} and from 0.0173 to 13.84 mM at 10 mM fMg^{2+}. fPEP concentrations (trianionic form) ranged from 0.0236 to 18.91 mM at 0.4 mM fMg^{2+}, and from 0.0096 to 7.69 mM at 10 mM fMg^{2+}. The points are the experimental data. The lines are the result of the best fit of the experimental data to equation 1, 2 or 3 as appropriate. The shaded area corresponds to the estimated physiological range of tPEP concentration.

Figure 2. Kinetics of saturation of nonphosphorylated maize-leaf PEPC by Glc6P (A and C) in the absence (●,■) and presence of 50 mM Gly (□) and by Gly (B and D) in the absence (●,■) and presence (□) of 10 mM Glc6P. The concentrations of fMg^{2+} in the assays were 0.4 (■,□) or 10 mM (●), and tPEP was 0.1 mM (A and B) or 3 mM (C and D). The points are the experimental data. The lines are the result of the best fit of the experimental data to equation 4 or 5 as appropriate. Within parenthesis are given the $A_{0.5}$ values estimated for each data set.

Figure 3. Kinetics of saturation of nonphosphorylated maize-leaf PEPC by malate at 0.4 (●,■) or 10 mM (○,□) fMg^{2+} and 0.1 mM (A) or 3 mM (B) tPEP. The inset shows the saturation by malate at 0.4 mM fMg^{2+} and 0.1 mM tPEP in a small scale. The points are the experimental data. the lines are the result of the best fit of the experimental data to equation 6 or 7 as appropriate. In Table II are given the corresponding concentrations of fPEP and MgPEP. Within parenthesis are given the $A_{0.5}$ values estimated for each data set.

Figure 4. Kinetics of of saturation of nonphosphorylated (■) and phosphorylated (□) maize-leaf PEPC by PEP (A), Gly (B), Glc6P (C) and malate (D) at 0.4 mM fMg^{2+} and 0.1 mM bicarbonate. Assays were carried out under the standard conditions described in Materials and Methods. In B, C and D the tPEP concentration was 3 mM.

Figure 5. Kinetics of saturation of phosphorylated maize-leaf PEPC by Glc6P (■), Ala (□) or Gly in the absence (●) and presence (○) of 10 mM Glc6P and in the absence (A) or presence (B) of 20% (v:v) glycerol. The insets show the saturation by Glc6P in a small scale.. Assays were carried out in the presence of 20 mM malate at 3 mM tPEP, 0.1 mM bicarbonate, and 0.4 mM fMg^{2+}. The points are the experimental data. The lines are the result of the best fit of the experimental data to equation 4 or 5 as appropriate.

Figure 6. Effects of activators on the specific activity of nonphosphorylated (dark bars) and phosphorylated (white bars) maize-leaf PEPC in the absence (A and B) and presence of 20 mM malate (C and D). Assays were performed at 0.1 (A and C) or 3 mM (B and D) tPEP, 0.4 mM fMg^{2+} and 0.1 mM bicarbonate in the absence or presence of 10 mM Glc6P or 10 mM Gly as indicated. The enzyme activity at each condition, as a percentage of the maximum activity achieved at saturating concentrations of MgPEP and bicarbonate, is given above each bar within parenthesis.

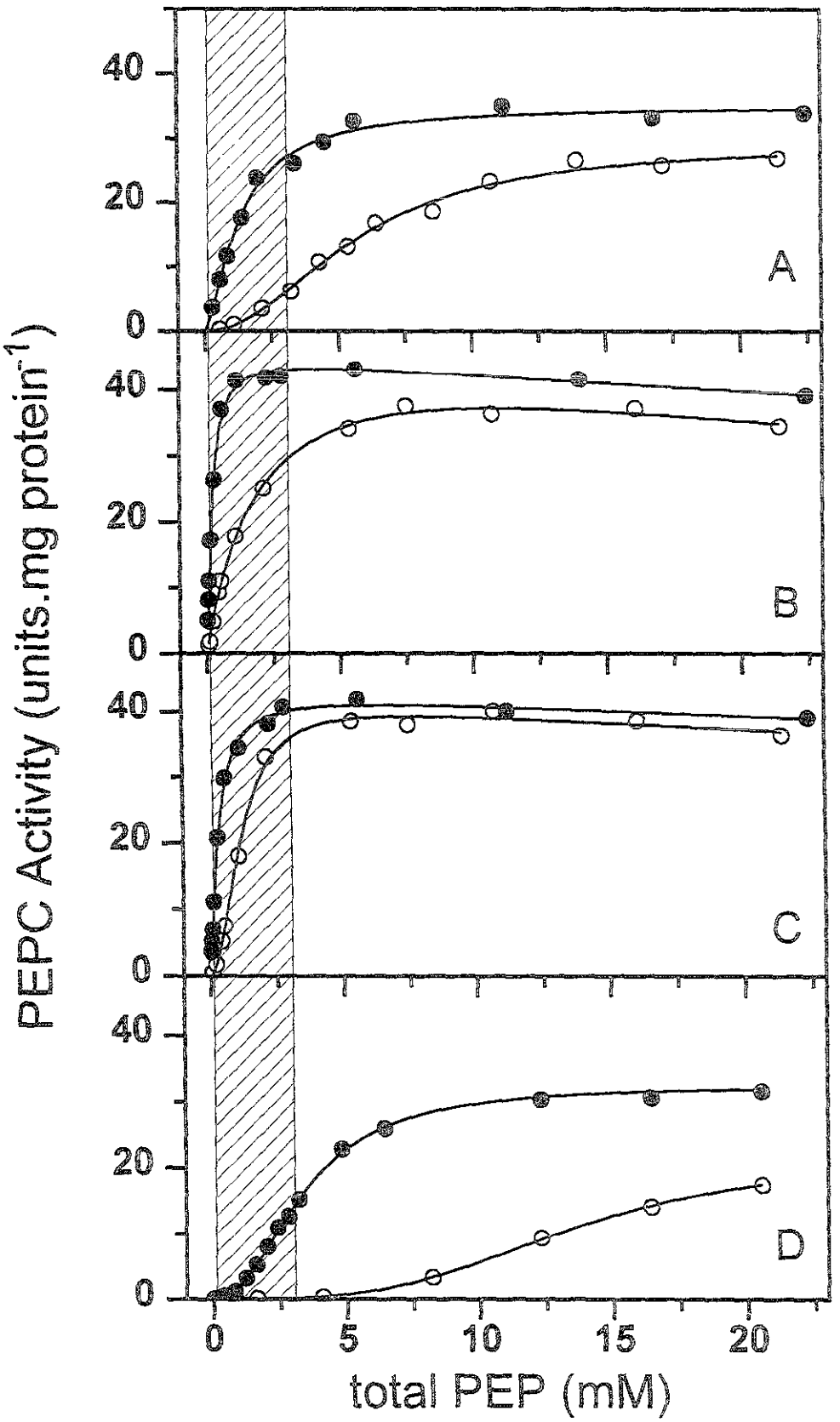

Figure 1. Tovar-Méndez *et al.*

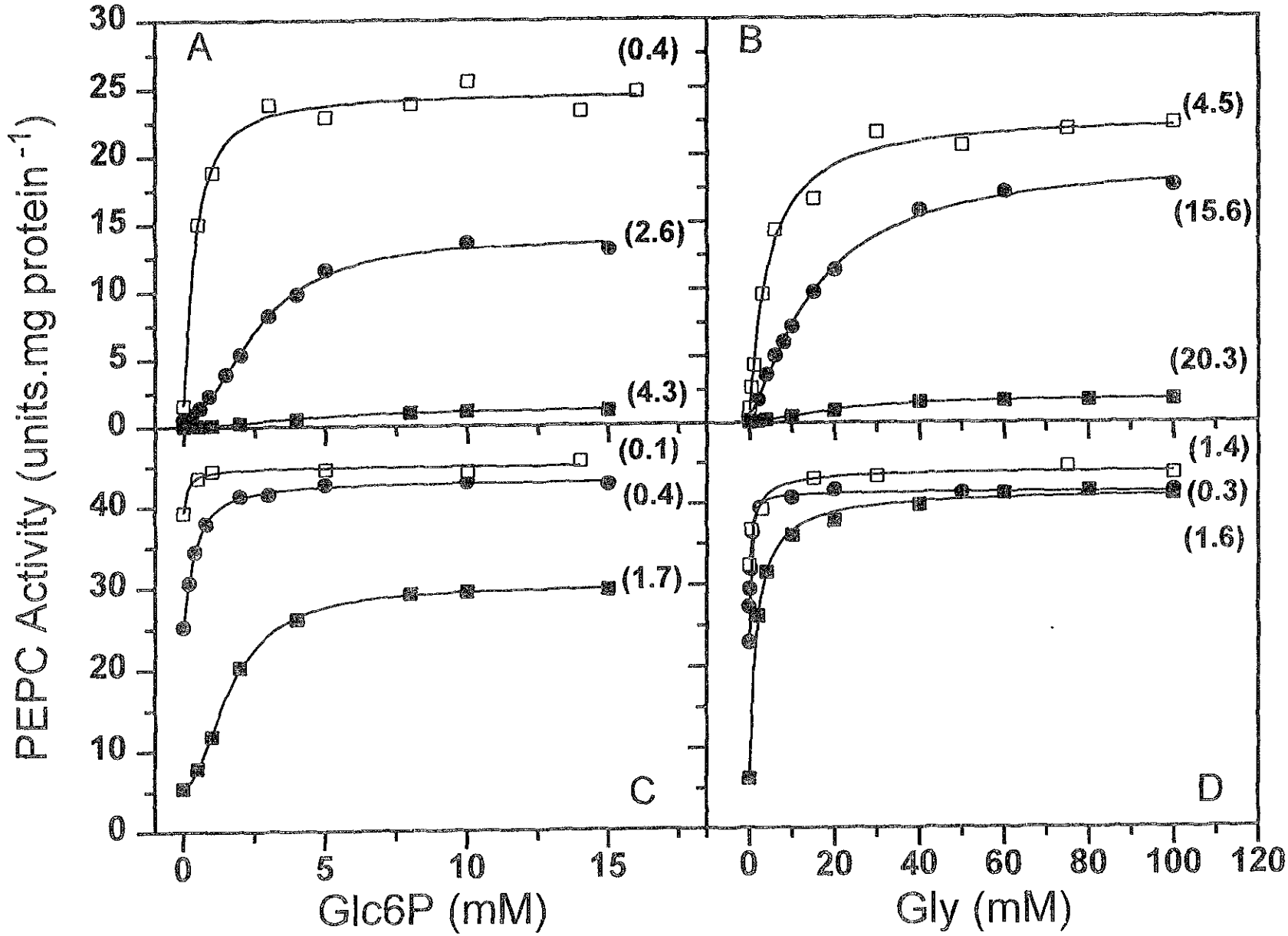

Figure 2. Tovar-Méndez *et al.*

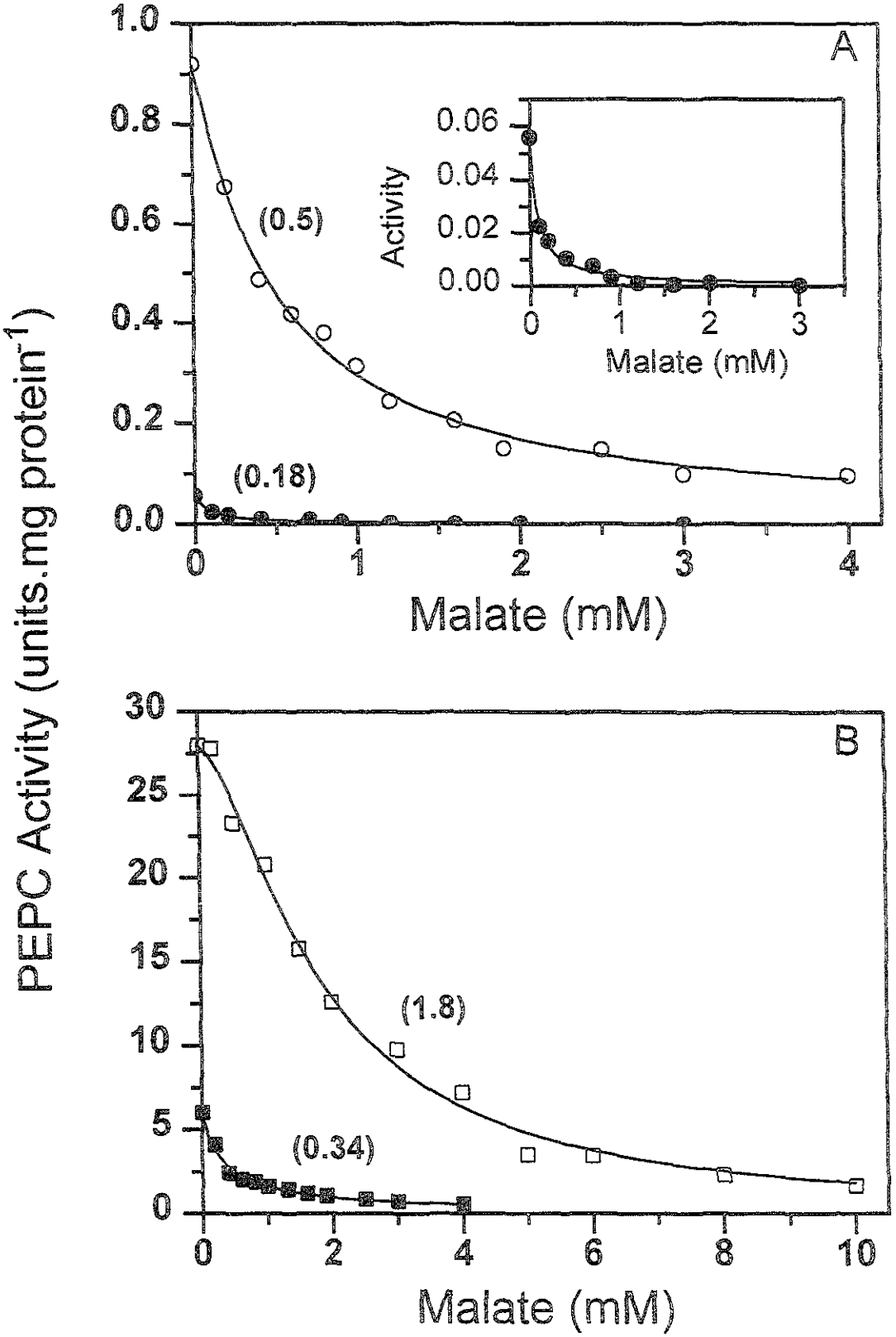

Figure 3. Tovar-Méndez *et al.*

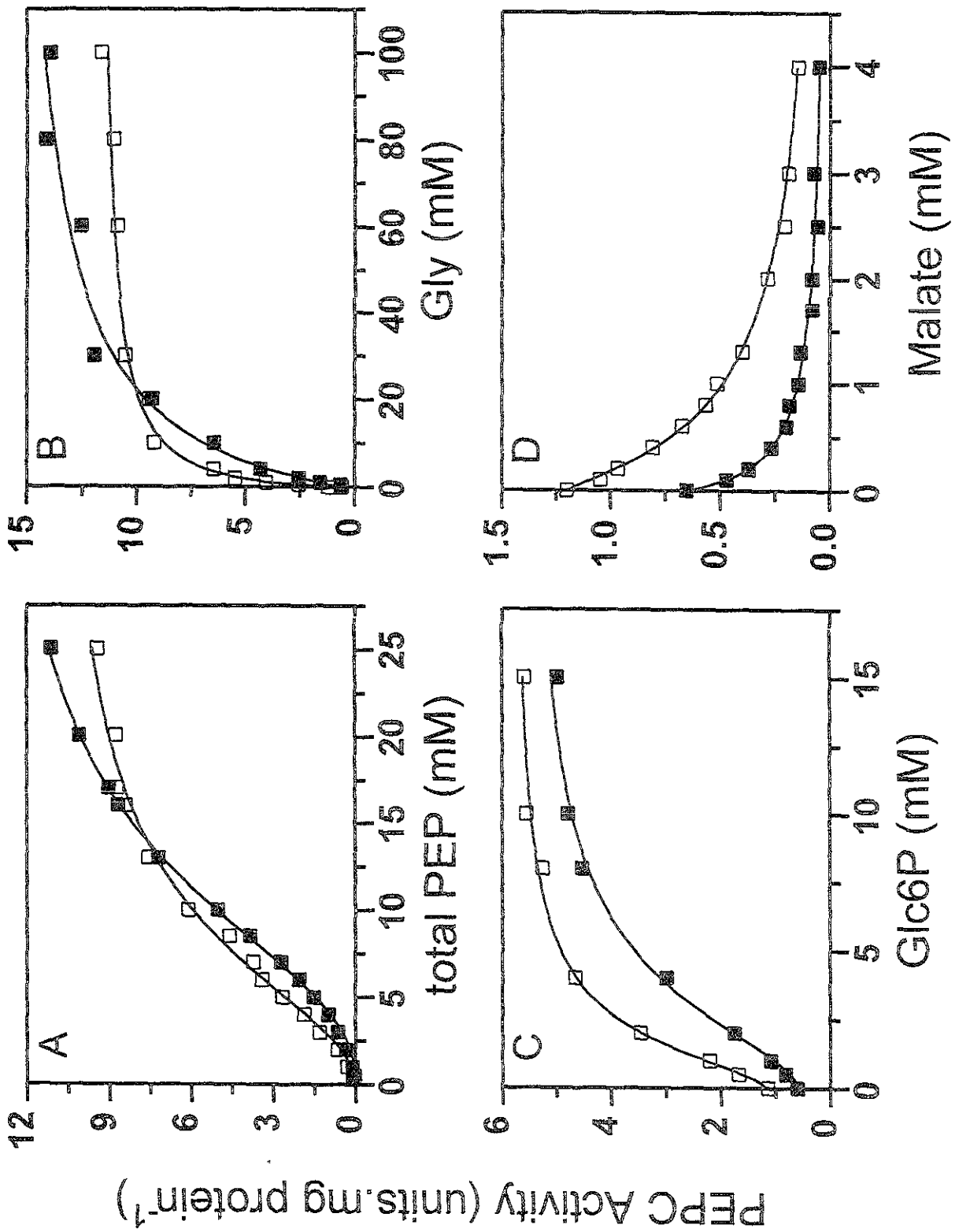

Figure 4. Tovar-Méndez *et al.*

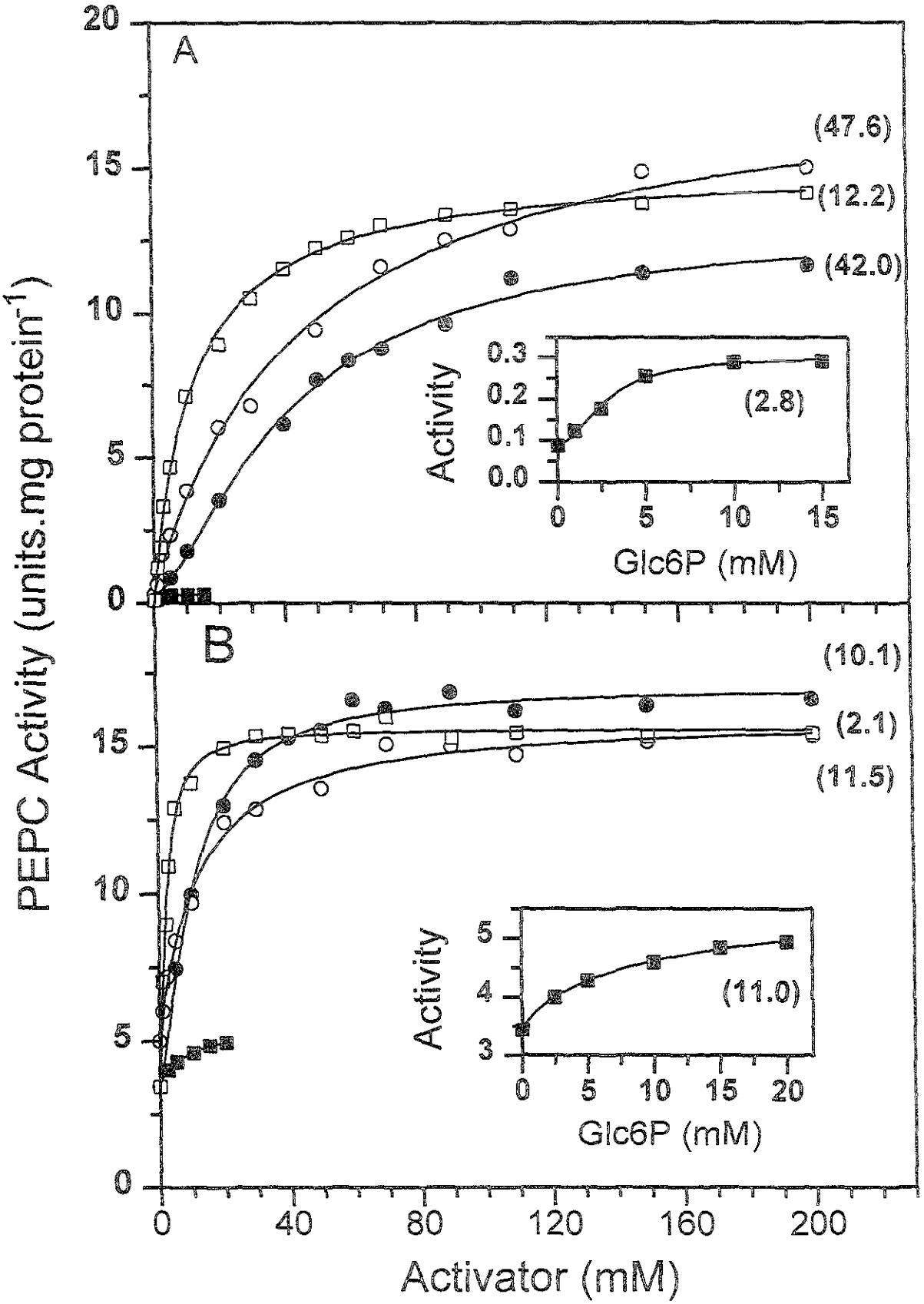

Figure 5. Tovar-Méndez *et al.*

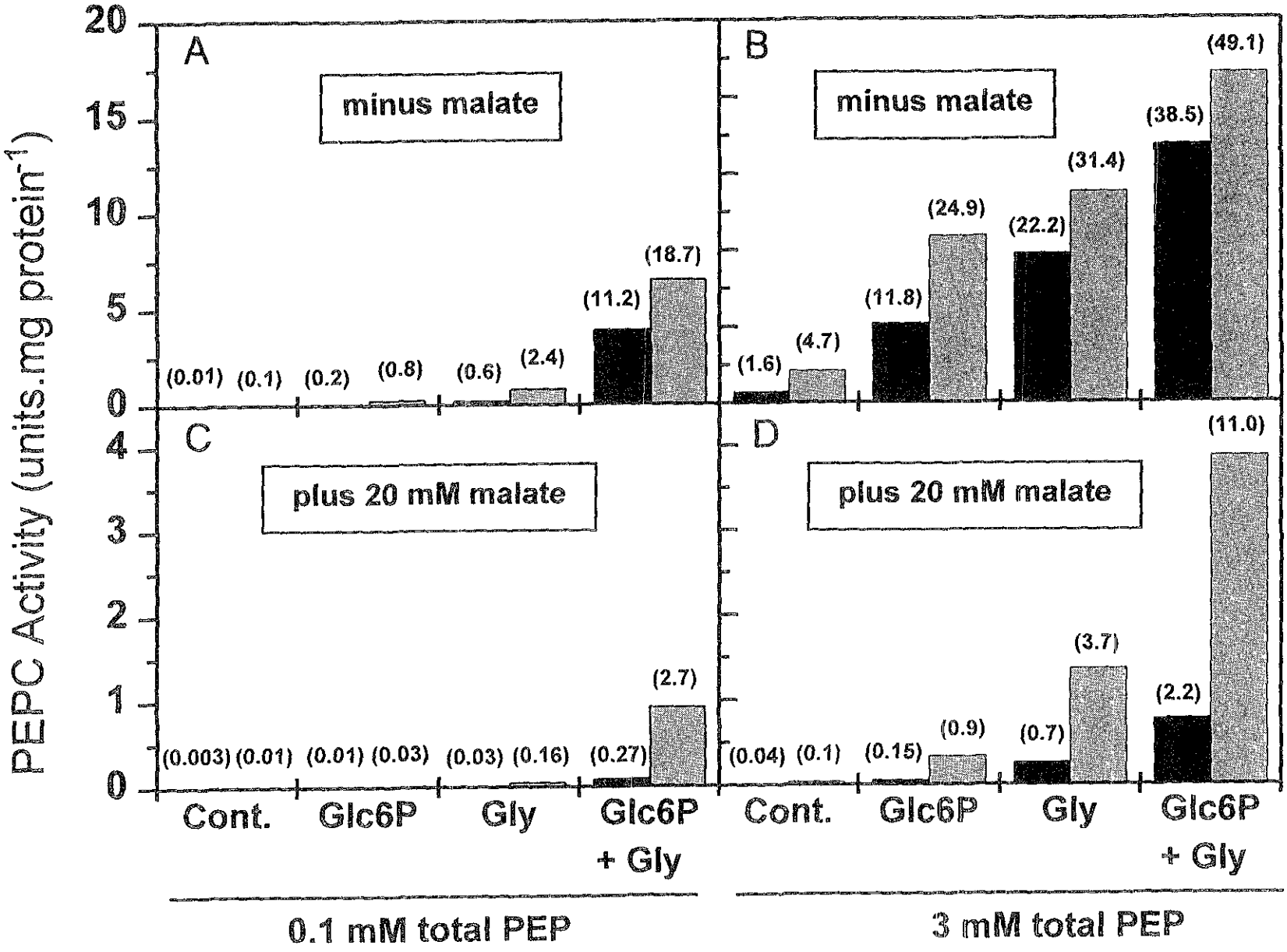

Figure 6. Tovar-Méndez *et al.*

Table I. *Effect of activators on the $I_{0.5}$ for malate of the nonphosphorylated and phosphorylated forms of maize leaf PEPC at pH 7.3, 0.4 mM fMg^{2+}, and 10 mM bicarbonate*

Values ± SD are given in mM and were estimated by the best fit to equation 6.

Enzyme form	tPEP	Control	+ 10 mM Glc6P	+ 10 mM Gly	+ 10 mM Glc6P + 10 mM Gly
	mM				
Nonphosphorylated PEPC					
	0.1	0.17 ± 0.01	0.23 ± 0.04	0.47 ± 0.05	1.74 ± 0.08
	3.0	0.34 ± 0.04	0.77 ± 0.04	4.78 ± 0.23	5.82 ± 0.24
Phosphorylated PEPC					
	0.1	0.37 ± 0.02	0.49 ± 0.03	0.67 ± 0.04	3.61 ± 0.03
	3.0	1.53 ± 0.16	5.38 ± 0.16	17.1 ± 0.6	34.9 ± 2.0

Table II. *Apparent kinetic parameters[a] of the nonphosphorylated and phosphorylated forms of maize leaf PEPC at pH 7.3, 0.4 mM fMg^{2+}, and 0.1 mM bicarbonate*

Values ± SD were estimated by the best fit to equations 2, 5, and 6 for saturation by tPEP, by Glc6P or Gly, and by malate, respectively.

Parameter	Nonphosphorylated	Phosphorylated
Saturation by tPEP		
V_{max} (units mg protein^{-1})	13.4 ± 0.3	11.0 ± 0.5
$S_{0.5}$ (mM)	12.3 ± 0.3	9.1 ± 0.6
n	2.1 ± 0.1	2.0 ± 0.2
Saturation by Glc6P[a]		
va_{max} (units mg protein^{-1})	5.5 ± 0.2	5.8 ± 0.1
$A_{0.5}$ (mM)	3.9 ± 0.3	2.0 ± 0.1
n	1.7 ± 0.1	1.6 ± 0.1
Saturation by Gly[a]		
va_{max} (units mg protein^{-1})	16.1 ± 0.5	11.5 ± 0.2
$A_{0.5}$ (mM)	14.5 ± 1.6	3.1 ± 0.3
n	1.0 ± 0.1	0.9 ± 0.1
Saturation by malate[a]		
I_{50} (mM)	0.27 ± 0.01	0.72 ± 0.017

[a] The apparent kinetic parameters were determined at 3 mM tPEP.

7. DISCUSIÓN GENERAL

Dado que los resultados de este trabajo se presentaron y discutieron por separado, a continuación se hace una discusión integrada de todos ellos.

A) La PEPC tetramérica de hoja de maíz, posee las propiedades alostéricas descritas para la enzima en estudios cinéticos de velocidad inicial

Los estudios de unión indican claramente que la forma tetramérica de la enzima presenta las propiedades alostéricas homotrópicas y heterotrópicas observadas en estudios cinéticos de estado estacionario [Coombs *et al.*, 1973; Nishikido & Takanashi, 1973; Huber & Edwards, 1975; Uedan & Sugiyama, 1976], y que concuerda con que los resultados en nuestros experimentos cinéticos de velocidad inicial son atribuibles a la forma tetramérica de la PEPC, dada la lenta disociación del tetrámero de la PEPC en el medio de reacción [sección 6.1.1]. El hecho de que el tetrámero de la PEPC posea las propiedades alostéricas descritas para la enzima, es de gran relevancia, pués si la regulación alostérica de la enzima se basara en un proceso de asociación-disociación, existiría la posibilidad de que no ocurriera *in vivo* dada la elevada concentración de PEPC (37.5 μM) que se ha estimado existe en el citoplasma de las células del mesófilo en hojas de plantas C_4 [Jiao & Chollet, 1991].

Lo anterior argumenta en contra de la relevancia de la regulación *in vivo* por cambios en el estado de agregación y nos permite proponer que la disociación de la enzima no tiene significancia fisiológica.

A este respecto, poco después de la aparición de nuestro trabajo [Tovar-Méndez *et al.*, 1995, en sección 6.1.2.1.], el significado no fisiológico del proceso de asociación-disociación fue sugerido en la revisión hecha por Chollet *et al.* (1996), considerando como evidencia que las formas fosforilada y no fosforilada de la PEPC de hojas de plantas C_4 y CAM, tienen el mismo estado de agregación y, sin embargo, presentan diferente sensibilidad a inhibición por malato [McNaughton *et al.*, 1989; Weigen & Hincha, 1992].

B) Papel de fMg^{2+}, fPEP y MgPEP en el mecanismo cinético de la fosfoenolpiruvato carboxilasa de hoja de maíz

En la literatura de la PEPC existe la controversia respecto a que especies reconoce el sitio activo: algunas evidencias indican que el complejo MgPEP es el verdadero substrato de la enzima [Mukerji, 1977; Wedding *et al.*, 1989; Rodríguez-Sotres & Muñoz-Clares, 1990]. Sin embargo, también se ha reportado que son las especies libres de magnesio y de PEP las que se unen a la enzima [Janc *et al.*, 1992]. Por esta discrepancia y porque todos estos estudios fueron hechos con enzima truncada del extremo amino terminal, nos planteamos caracterizar cinéticamente la saturación de la PEPC con magnesio y con PEP, para interpretar adecuadamente el papel del magnesio y del PEP como substratos y como posibles reguladores de la actividad de la enzima. Con el fin de conocer cual es el substrato de la enzima *in vivo*, la saturación fue analizada en un intervalo de concentraciones de magnesio y de PEP cercanas a las fisiológicas. El análisis de los datos [Artículo 1] nos permitió concluir que, en ausencia de activadores, el substrato de la enzima es el complejo MgPEP y que, la presencia de concentraciones saturantes de Glc6p o de Gly, el sitio activo de la enzima puede unir a las especies libres, pero con muy baja afinidad; siendo el substrato preferencial el complejo MgPEP.

Janc *et al.* (1992) concluyeron que las especies libres son las que se unen a la enzima y no el complejo MgPEP, esta discrepancia con nuestros resultados probablemente sea consecuencia de que ellos en sus experimentos utilizaron las concentraciones totales sin considerar que el ión magnesio y PEP forman el complejo MgPEP. Esto porque, los gráficos de dobles recíprocos de datos obtenidos en experimentos como los hechos por Janc *et al.* (1992) pueden conducir a errores de interpretación, como es concluir que la enzima reconoce a las especies libres y no al complejo MgSubstrato [Giachetti & Vanni, 1991].

Evidencias indirectas sugerían que fPEP puede ser un activador de la PEPC [Jenkins *et al.*, 1986; Rusting *et al.*, 1988; Rodríguez-Sotres & Muñoz-Clares, 1990; Rusting *et al.*, 1991]. Nuestros resultados demuestran que fPEP se une al sitio alostérico activador para Glc6P [sección 6.1.2.2.; Artículo 1] y que activa por su unión

al complejo enzima-MgPEP [Artículo 1], así la activación por fPEP parece ser debida al mecanismo cinético y no a un cambio conformacional inducido por su unión. A este respecto, nuestros resultados sugieren que la cooperatividad en la saturación de la enzima por MgPEP tiene dos componentes, uno debido a la cooperatividad de unión de MgPEP al sitio activo y otro debido a la activación por fPEP.

C) La regulación de la PEPC de hoja de maíz

La significancia fisiológica de un efecto regulatorio observado *in vitro* es siempre dudoso, particularmente cuando las condiciones *in vivo* (pH, concentración de substratos, nivel de efectores, etc) no están establecidos inequívocamente. Sin embargo, datos de esta naturaleza permiten la construcción de modelos tentativos concernientes a la situación prevaleciente en las células.

Con vistas a estimar la relevancia de la regulación de la PEPC por metabolitos y por fosforilación, realizamos los experimentos mostrados en el Artículo 2, donde se estudió la cinética de las formas de la enzima prevalecientes en la noche (no fosforilada) y en el día (fosforilada) considerando las concentraciones reportadas de fMg^{2+} [Yazaki *et al.*, 1988], de bicarbonato [Jenkins *et al.*, 1989], de malato [Stitt & Heldt, 1985a] y de protones [Raghavendra *et al.*, 1993; Yin *et al.*, 1993] presentes en el citoplasma de las células del mesófilo de hoja maíz durante los períodos de luz, así como la concentración de PEP y los cambios de este metabolito inducidos por luz en hojas de plantas de maíz [Leegood, 1985; Stitt & Heldt, 1985; Leegood & von Caemmerer, 1988].

Fosforilación de la PEPC

Nuestros resultados demuestran que la fosforilación de la PEPC aumenta su afinidad por el complejo MgPEP (y por las especies libres) y no afecta significativamente su *Vmax* sugiriendo que la constante catalítica de la enzima no se modifica como consecuencia de su fosforilación [resultados en Artículos 1 y 2]. Estos resultados están de acuerdo con que la PEPC extraída de hojas de maíz expuestas a la luz tienen mayor afinidad por PEP que la enzima extraída de hojas mantenidas en obscuridad, y con que la *Vmax* no se afecta por la exposición de las hojas a la luz

[Huber *et al.*, 1986; Doncaster & Leegood, 1987], ya que la fosforilación de la PEPC es inducida por luz de hojas de plantas C_4 [Jiao *et al.*, 1991; Jiao & Chollet, 1991; Duff & Chollet, 1995]. También, explican que la actividad de la enzima medida a concentraciones saturantes de substratos no cambia con la fosforilación de la PEPC [Nimmo *et al.*, 1987]. Aunque otros autores reportaron que la fosforilación de la PEPC de hojas de sorgo aumenta ambos, la afinidad de la enzima por PEP y la *Vmax* [Duff *et al.*, 1995].

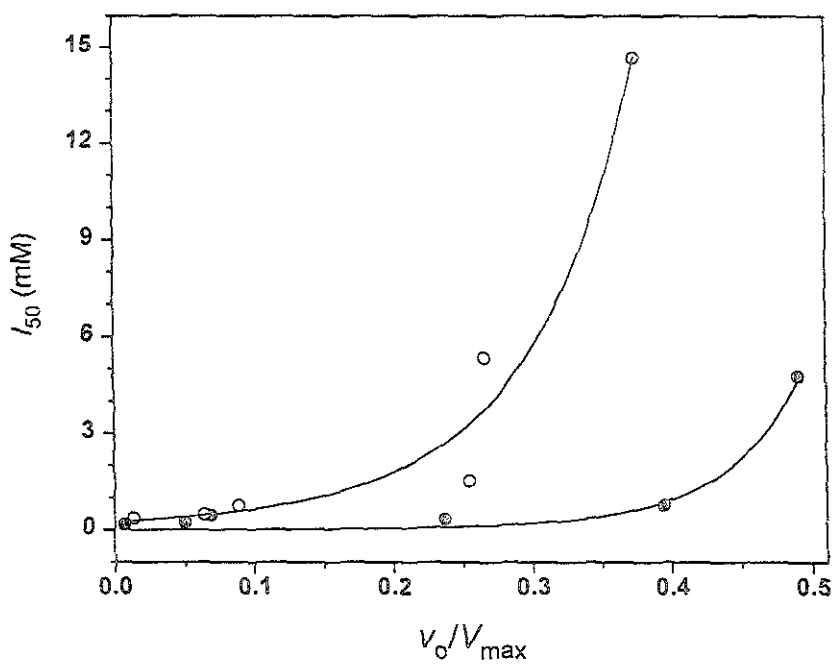

Figura 7.1. Representación gráfica de la relación entre el I_{50} para malato y la fracción de saturación de la PEPC por MgPEP (expresada como $v_o/Vmax$). Los datos son de la PEPC fosforilada (○) y de la PEPC no fosforilada (●), del Artículo 2. Las líneas son el ajuste de los datos a la ecuación 14 del Artículo 2.

La fosforilación de la PEPC disminuye la afinidad (medida como I_{50}) de la enzima por malato [Nimmo *et al.*, 1987; Echevarria *et al.*, 1994; Duff *et al.*, 1995; resultados en Artículo 2]. Este efecto de la fosforilación puede ser consecuencia del aumento de

la fracción de saturación de la enzima por MgPEP, resultante de que la enzima fosforilada tiene mayor afinidad por MgPEP. Ya que, como se indica en el Artículo 2, el I_{50} es una medida aparente de la afinidad de la enzima por malato que depende del grado de saturación de la enzima por MgPEP. Sin embargo, la relación entre el I_{50} para malato y la fracción de saturación de la enzima por MgPEP (expresada como $v_o/Vmax$), es diferente entre la PEPC fosforilada y la PEPC no fosforilada [Figura 7.1., página 38], lo cual indica que efectivamente la fosforilación disminuye la afinidad de la PEPC por malato y que la afinidad aparente de la PEPC (fosforilada o no fosforilada) por malato depende del grado de saturación de la enzima por MgPEP.

La $A_{0.5}$ para los activadores Glc6P y Gly no se ve afectada por la fosforilación de la PEPC a concentraciones saturantes de bicarbonato [Artículo 1 y 2], sin embargo, nosotros observamos una disminución del $A_{0.5}$ para Glc6P y para Gly (de 2 y 4.5 veces, respectivamente) a concentración subsaturante de bicarbonato [Tabla I en Artículo 2]. No tenemos una posible explicación para este fenómeno. Una observación interesante es el hecho de que el $A_{0.5}$ por Glc6P y el $A_{0.5}$ por Gly determinado a fMg^{2+} 0.4 mM y a PEP total 3 mM, aumenta 2 y 9 veces respectivamente, como consecuencia de la disminución de la concentración de bicarbonato de 10 a 0.1 mM [Figura 2 y Tabla I en Artículo 2]. Por lo cual, es posible que bajo condiciones subsaturantes de bicarbonato la fosforilación pueda afectar de manera indirecta, la afinidad aparente de la enzima por Glc6P y por Gly. A este respecto, en la PEPC de hojas de sorgo, el $A_{0.5}$ para Glc6P [Duff *et al.*, 1995; Bakrim *et al.*, 1998] y para Gly [Bakrim *et al.*, 1998] determinada a bicarbonato 1 mM, disminuye ligeramente como consecuencia de la fosforilación de la PEPC.

Finalmente, se ha propuesto que el proceso de fosforilación-defosforilación es el punto central en la regulación de la PEPC de plantas C_4 [Jiao & Chollet, 1991; Chollet *et al.*, 1996] el cual propone que la forma no fosforilada es menos activa, más sensible a inhibición por malato y menos sensible a activación por Glc6P, que la forma fosforilada. Sin embargo, nuestros resultados nos permiten concluir que la fosforilación regula a la PEPC principalmente aumentando su afinidad por MgPEP y disminuyendo su afinidad por malato. Así, nuestros resultados advierten que el

modelo propuesto por el grupo de Chollet [Jiao & Chollet, 1991; Chollet *et al.*, 1996] no es del todo correcto e incompleto. Aunque claramente la fosforilación es parte importante de la regulación positiva de la enzima, como lo demuestran los resultados en el Artículo 2 y el hecho de que el estado de fosforilación de la PEPC influencia la fotosíntesis *in vivo* de sorgo y de maíz [Bakrim *et al.*, 1993].

Activación por hexosas y triosas fosfato.

Se ha descrito que Glc6P tiene un efecto mixto sobre la saturación de la PEPC de hoja de maíz por PEP (a concentración fija del ión magnesio) y que elimina la cooperatividad de dicha saturación [Coombs *et al.*, 1973; Uedan & Sugiyama, 1976]. La activación de la enzima por Glc6P produce un ligero aumento en la*Vmax* [Coombs *et al.*, 1973; Uedan & Sugiyama, 1976; datos en la presente tesis]. En cuanto al efecto de Glc6P sobre la afinidad de la enzima por el substrato, nosotros lo estudiamos con mayor detalle. Así, encontramos que concentraciones saturantes de Glc6P disminuye fuertemente la $S_{0.5}$ de la enzima por MgPEP y favorece la unión de fPEP al sitio activo de la enzima libre (unión que no se observa en ausencia de Glc6P en el intervalo de concentración de fPEP usado) [Artículo 1]. Además, Glc6P elimina la cooperatividad de la saturación por substrato (MgPEP y fPEP) del sitio activo de la enzima y elimina la activación por fPEP (como consecuencia de que se unen al mismo sitio), lo que se refleja en la pérdida de cooperatividad en la saturación de la enzima por fPEP [datos en Artículo 1] o por PEP total a concentración fija de fMg^{2+} [Artículo 2] ó a concentración fija de Mg^{2+} total [Coombs *et al.*, 1973; Uedan & Sugiyama, 1976].

A fMg^{2+} 0.4 mM el aumento del I_{50} para malato inducido por Glc6P 10 mM es muy pequeño tanto en la PEPC no fosforilada como en la PEPC fosforilada [Tabla I en Artículo 2]; a altas concentraciones del ión magnesio (10 mM), Glc6P 3 mM no afecta en I_{50} de la enzima extraida de plantas de maíz expuestas a la luz (presumiblemente fosforilada) es nulo [Gao & Woo, 1996], aunque en PEPC de sorgo fosforilada*in vitro* Glc6P 15 mM aumentó aproximadamente 5 veces el I_{50} para malato (determinado a 5 mM del ión magnesio) [Bakrim *et al.*, 1998]. Lo anterior sugiere que el efecto de

Glc6P sobre el I_{50} para malato depende de la concentración de substrato (MgPEP) y de la concentración de Glc6P. Por lo que, una aproximación a la relevancia fisiológica del efecto antagonista de Glc6P sobre la inhibición de la enzima por malato es el experimento mostrado en la Figura 5 [Artículo 2], donde se muestra que cuando la PEPC fosforilada se satura con Glc6P en presencia de concentraciones fisiológicas de malato (20 mM), a concentraciones fisiológicas de substratos (MgPEP y bicarbonato), de fMg^{2+} y de protones prevalecientes durante los períodos de luz, la inhibición de la enzima por malato no se elimina. Éste resultado sugiere que la unión de malato y de Glc6P a la PEPC no es mutuamente excluyente. A este respecto, Glc6P se une a la enzima de forma muy similar en ausencia y en presencia del inhibidor malato, como lo indican nuestros hallazgos de que el $L_{0.5}$ y el número de Hill de la cinética de unión de Glc6P a su sitio alostérico [Tabla 1 en memorias en extenso] y que el $A_{0.5}$ y el número de Hill de la cinética de estado estacionario de la activación por Glc6P de la PEPC [Tabla I y Figura 5 del Artículo 2] no se afectan considerablemente por la presencia de malato. Sin embargo, la protección máxima ejercida por Glc6P frente a la insensibilización a activación de la PEPC por Glc6P [Tabla I en memorias en extenso] y la actividad de la enzima saturada con Glc6P [Tabla 1 y Figura 5 del Articulo 2] es mucho menor en presencia de malato. Lo cual indica que la enzima puede unir simultáneamente a malato y a Glc6P y que el complejo enzima-malato-Glc6P no une a MgPEP, comportándose como una enzima inhibida.

Es posible especular que el papel del sitio alostérico activador para Glc6P (al que se unen triosas fosfato, hexosas fosfato y fPEP) puede ser relevante para la activación de la enzima bajo condiciones en las que la concentración de malato sea baja. Como es el caso del periodo de obscuridad y/o del inicio del periodo de luz [Rodríguez-Sotres *et al.*, 1987].

Activación de la PEPC por aminoácidos neutros

Se ha descrito que Gly tiene un efecto mixto sobre la saturación de la PEPC de hoja de maíz por PEP (a concentración fija del ión magnesio) [Nishikido & Takanashi,

1973; Uedan & Sugiyama, 1976; Stiborová & Leblová, 1985] y que no elimina la cooperatividad de dicha saturación [Uedan & Sugiyama, 1976; Gillinta & Grover, 1995]. Nosotros comprobamos que la activación de la enzima por una concentración saturante Gly produce un ligero aumento en la *Vmax* [Artículo 1]. Como en el caso de Glc6P, el efecto de Gly sobre la afinidad de la enzima por el substrato, fue estudiado con mayor detalle. Así, encontramos que concentraciones saturantes de Gly disminuye fuertemente la $S_{0.5}$ de la enzima por MgPEP y favorece la unión de fMg^{2+} y en menor grado de fPEP al sitio activo de la enzima libre (unión que no se observa en ausencia de Gly en el intervalo de concentración de fMg^{2+} y de fPEP usado) [Artículo 1]. Además, Gly elimina la cooperatividad de la saturación por MgPEP y por fMg^{2+}, del sitio activo de la enzima, y no elimina la activación por fPEP, provocando que en presencia de concentración saturante de Gly se presente cooperatividad positiva aparente en la saturación de la enzima por fPEP [datos en Artículo 1] o por PEP total [datos en Artículo 2] a concentración fija de fMg^{2+}, o por PEP total a concentración fija de Mg^{2+} total [Uedan & Sugiyama, 1976; Gillinta & Grover, 1995].

La activación por aminoácidos neutros no fue considerada por mucho tiempo como un factor importante de la regulación de ésta enzima. Probablemente esto sea debido a un antiguo reporte donde se indica que sólo la enzima PEPC de plantas C_4 monocotiledóneas es activada por aminoácidos neutros como Gly [Nishikido & Takanashi, 1973]. Sin embargo, estos experimentos fueron realizados a pH 8.5 en presencia de 0.6 mM de MgPEP (PEP 1 mM y magnesio 10 mM), condiciones en las que Gly activa marginalmente la PEPC de hoja de maíz [Tabla 4 en Artículo 2]. Además, reportes recientes [Gao & Woo, 1996; Bakrim *et al.*, 1998] han planteado la necesidad de considerar la activación de la PEPC por aminoácidos neutros, en vista del marcado aumento del I_{50} para malato inducido por la presencia de aminoácidos neutros como Gly o Ala. Nuestros resultados [Figura 5 en Artículo 2] resaltan la relevancia de la activación por aminoácidos neutros, ya que en condiciones cercanas a las fisiológicas de substratos (MgPEP y bicarbonato), de fPEP, de fMg^{2+} y de protones presentes durante los períodos de luz, la inhibición de la PEPC fosforilada por concentraciones fisiológicas de malato (20 mM) puede ser contrarrestada

ımente si se considera la activación por aminoácidos neutros. Probablemente Ala el aminoácido activador principal de la PEPC considerando la elevada ;entración de Ala (30-40 mM) que se ha estimado está presente en las células del ófilo de maíz durante el período de luz [Leegood, 1985; Stitt and Heldt, 1985].

actividad de la PEPC es limitada por la concentración intracelular de iones gnesio, de bicarbonato y de PEP

_a concentración tan baja de fMg^{2+} (0.4 mM) presente *in vivo* [Yazaki *et al.*, 1988] ermina que sólo una pequeña proporción del PEP presente en la célula del ısófilo se encuentre complejado con magnesio, por lo cual, la concentración del bstrato (MgPEP) de la PEPC es mucho menor a la concentración de PEP racelular. Otra limitante es la concentración de bicarbonato como lo muestra la tividad de la PEPC cuando se determina a una concentración cercana a la evaleciente *in vivo*, ya que es aproximadamente la mitad de la actividad eterminada a concentraciones de bicarbonato saturante [Artículo 2].

La concentración de PEP es otra de las limitantes. En las hojas de maíz durante el ıeríodo de obscuridad la concentración intracelular de PEP es baja (de 0.1 a 0.5 mM) / aumenta cuando las hojas se exponen a la luz por la exposición a la luz (de 2.5 a 4.5 mM) [Leegood, 1985; Stitt & Heldt, 1985; Leegood & von Caemmerer, 1988]. Este cambio en la concentración de PEP es esencial para que la actividad PEPC aumente ya que sin este cambio, aún considerando la activación de la enzima por fosforilación, por Glc6P y por Gly, la actividad de la PEPC es muy pequeña [Figura 5 y 6 en Artículo 2]. Sugiriendo que el incremento de PEP es fundamental para el aumento de la actividad de la PEPC y en última instancia para la fijación de CO_2 por las hojas de maíz.

Un modelo tentativo de la regulación de la PEPC de hojas de maíz

Dada la baja concentración intracelular de PEP estimada en hojas de maíz durante períodos de obscuridad [Leegood, 1985; Stitt & Heldt, 1985], la PEPC permanece casi inactiva [Figura 6 en Artículo 2]. Cuando las hojas de maíz se exponen a al luz, se presenta incrementos en la concentración intracelular de PEP, de azúcares fosfato,

de aminoácidos neutros, y de malato [Williams & Kennedy, 1978; Leegood & Furbank, 1984; Stitt & Heldt, 1985; Rodríguez-Sotres *et al.*, 1987], por lo cual es posible que en los primeros tiempos de la exposición de las hojas de maíz a la luz, la actividad de la enzima aumente principalmente porque el incremento en la concentración de PEP, azúcares fosfato y aminoácidos neutros, contrarrestan la inhibición de la PEPC producido por el aumento de la concentración de malato y, conforme aumentan los niveles de malato, aumenta la importancia de la activación por aminoácidos neutros. El el máximo de la fosforilación de la PEPC se alcanza aproximadamente a las dos horas de que la planta de maíz [McNaughton *et al.*, 1991] o de sorgo [Pierre *et al.*, 1992] se expone a la luz, mientras que el cambio en los niveles de PEP, azúcares fosfato y de aminoácidos neutros en las células de maíz, se dá en los primeros minutos de exposición a la luz de la planta de maíz [Williams & Kennedy, 1978; Leegood & Furbank, 1984]. Por lo cual, durante la exposición prolongada de las plantas de maíz a la luz, la inhibición de la PEPC por malato es contrarrestada por el efecto sinérgico de la fosforilación de la PEPC y por la importante activación de la PEPC por aminoácidos neutros (como se muestra en la Figura 5 en Articulo 2), principalmente Ala cuya concentración citosólica en células del mesófilo durante el período de luz es alta (30-40 mM) [Leegood, 1985; Weiner & Heldt, 1992].

Un aspecto importante de resaltar es que aunque Glc6P favorece la activación de la enzima por Gly en presencia de malato 20 mM en condiciones de luz (Figura 5 en Artículo 2), este efecto no se presentó con altas concentraciones de glicerol (20 % v/v); por lo cual, si consideramos que la presencia de glicerol simula el ambiente citosólico de la célula, este resultado sugeriría que en esas condiciones Glc6P, y presumiblemente triosas fosfato, hexosas fosfato y fPEP, no favorecen el efecto de los aminoácidos neutros.

8. CONCLUSIONES

A continuación se presentan las principales conclusiones sobre el mecanismo cinético y la regulación alostérica de la PEPC de hoja de maíz derivadas de los resultados obtenidos en el presente trabajo.

1. El mecanismo de regulación alostérica de la PEPC no se basa en la interconversión dímero-tetrámero de la enzima. Nuestros resultados sugieren que la enzima posee varios estados conformacionales inducidos por sus diferentes ligandos.

2. En ausencia de activadores, el complejo MgPEP es el substrato de la PEPC; y es el substrato preferido en la presencia de dichos efectores.

3. PEP libre se une al sitio alostérico para Glc6P cuando el sitio activo tiene unido a MgPEP.

4. La fosforilación de la PEPC aumenta la afinidad de la PEPC por MgPEP, por fMg^{2+} (en presencia de activadores) y por fPEP (en presencia de activadores) y disminuye la afinidad de la PEPC por el inhibidor malato.

5. La PEPC presenta una actividad muy baja en las condiciones de concentración de substratos y de protones presumiblemente cercanas a las prevalecientes durante el período de obscuridad, y es altamente sensible a los cambios en los niveles de PEP, de sus efectores alostéricos y de su estado de fosforilación inducidos por la luz.

6. El incremento en la concentración intracelular de PEP en la célula del mesófilo de maíz, es esencial para que la actividad PEPC aumente en las condiciones prevalecientes durante el período de luz.

7. En las condiciones presumiblemente prevalecientes en las células del mesófilo de maíz durante el período de luz, la inhibición de la PEPC por malato puede ser contrarrestada efectivamente por la activación cinérgica de la enzima por aminoácidos neutros y por la fosforilación de la PEPC.

9. PERSPECTIVAS

En este trabajo se presentan algunos aspectos importantes de la cinética y de la regulación de la PEPC de hoja de maíz, que deben ser considerados en estudios posteriores. Estudios como son el caracterizar el mecanismo cinético de activación de la enzima por azúcares fosfato y por aminoácidos neutros, y el mecanismo cinético de la inhibición de la enzima por ácidos dicarboxílicos. Además de caracterizar la interacción cinética de los diferentes efectores de la enzima, y determinar el efecto de la fosforilación de la enzima sobre dicha interacción, con vistas a reforzar el papel de la fosforilación en la regulación de la enzima planteados en este trabajo. Así, en conjunto, estos experimentos ayudarán a entender el comportamiento de la enzima.

Por otra parte, un aspecto por confirmar es la importancia fisiológica de la activación de la enzima por aminoácidos neutros y establecer la posible relevancia fisiológica de la activación de la enzima por azúcares fosfato. A este respecto, la técnica reportada por Dever *et al.* (1995) para generar plantas mutantes no transgénicos de *Amaranthus edulis* (una planta C_4) que carecen de la PEPC de hoja, puede ser una estrategia para generar un vehículo ideal para la transformación y expresión de mutantes de la PEPC de plantas C_4, no activables por aminoácidos neutros y/o por azúcares fosfatos, que permitiría evaluar la importancia fisiológica de la regulación de la enzima por estos activadores.

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