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TESIS DOCTORAL

ENZIMAS REGULADORAAS DEL METABOLISMO DIAZOTRÓFICO Y DEL POTENCIAL OSMÓTICO EN CIANOBACTERIAS

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UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

DOCTORADO EN CIENCIAS DEL MAR
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ENZIMAS REGULADORAS DEL METABOLISMO DIAZOTROFICO Y DEL POTENCIAL OSMOTICO EN CIANOBACTERIAS

Tesis Doctoral presentada por Dña. Antera Martel Quintana
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Los Directores La Doctorando

Las Palmas de Gran Canaria, a 10 de Junio 1993

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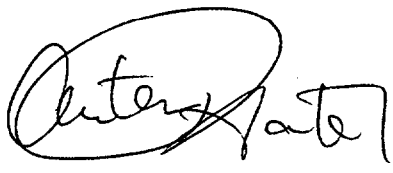
Hacen constar:

Que la Lcda. en Ciencias del Mar Dña. Antera Martel Quintana ha realizado el presente trabajo como Memoria de Tesis Doctoral, en el Departamento de Fisiología Botánica de la Universidad de Uppsala y en el Instituto de Algología Aplicada de la Universidad de Las Palmas de Gran Canaria, bajo su dirección, y se presenta con su V°B°.

Las Palmas de Gran Canaria, Junio de 1993.

ENZIMAS REGULADORAS DEL METABOLISMO DIAZOTROFICO Y DEL POTENCIAL OSMOTICO EN CIANOBACTERIAS

Memoria que presenta, la Lcda. en Ciencias del Mar, Antera Martel Quintana para aspirar al grado de Doctor.



Las Palmas de Gran Canaria, Junio de 1993.

A mis Padres

...ese experto futuro que inventamos
nosotros y el azar cada vez
más nosotros y menos el azar.
M. Benedetti

PREFACIO

Esta tesis es un resumen de los siguientes artículos, y algunos resultados no publicados.

I Martel A, Jansson E, García-Reina G & Lindblad P (1993) Ornithine cycle in *Nostoc* PCC 73102. Arginase, OCT and arginine deiminase, and the effects of addition of external arginine, ornithine, or citrulline. *Archives of Microbiology* 159: 506-511.

II Jansson E, Martel A & Lindblad P (1993) Ornithine Cycle in *Nostoc* PCC 73102: Stimulation of In Vitro Ornithine Carbamoyl Transferase by Addition of Arginine. *Current Microbiology* 26: 75-78.

III Martel A, Yu S, García-Reina G, Lindblad P & Pedersén M (1992) Osmotic-adjustement in the cyanobacterium *Spirulina platensis*: Presence of an α-glucosidase. *Plant Physiology and Biochemistry* 30: 573-578.

IV Martel A, Yu S, García-Reina G, Pedersén M & Lindblad P (1993) Occurrence and immunolocalization of an α-glucan phosphorylase in the cyanobacterium *Spirulina platensis*. *Planta* (remitido).

V Martel A, Yu S, Codd GA & Lindblad P (1993) α-Glucan phosphorylase in the unicellular cyanobacteria *Synechocystis* PCC 6803, *Synechoccocus* PCC 6301, and *Synechoccocus* PCC 7942 (en preparación).

Este trabajo ha sido llevado a cabo por Antera Martel Quintana en el Departamento de Fisiología Botánica de la Universidad de Uppsala (Suecia), y el Instituto de Algología Aplicada de la Universidad de Las Palmas de Gran Canaria (España), en base al convenio de institucional de colaboración establecido entre ambas universidades.

INDICE

ABREVIACIONES

ARN	Acido ribonucleico
CLA	Clorofila *a*
DAMO	Diacetilmonoxima
GS	Glutamina sintetasa
INP	α-Isonitrosofenona
GOGAT	Glutamato sintasa
OCT	Ornitín-carbamil-fosfato
PAGE	Electroforésis en geles de poliacrilamida
PS	Peso seco
SDS	Sodio Dodecil Sulfato

1.- INTRODUCCION

1.1.- Ciclo de la ornitina y metabolismo heterotrófico en cianobacterias

Las cianobacterias forman asociaciones simbióticas con algas, hongos (líquenes), briofitos, pteridofitos (*Azolla*), plantas vasculares (Cicadáceas y la angiosperma *Gunnera*), animales (esponjas marinas), protistas no-fotosintéticos y bacterias (Stewart et al. 1983, Smith & Douglas 1987, Rai 1990).

La mayoría de las cianobacterias simbiontes (cianobiontes) son fijadoras de nitrógeno, por lo que se considera que su principal papel en la relación simbiótica es el de proporcionar nitrógeno al huésped (Rai 1990, Rowell & Kerby 1991). La forma en que el nitrógeno fijado es transferido desde el cianobionte puede variar, aunque parece ser amoniaco en la mayoría de los casos estudiados (Stewart & Rowell 1977, Peters et al. 1985). En el cianobionte, los bajos niveles de actividad glutamina sintetasa (GS), la principal enzima asimiladora de amoniaco (Stewart et al. 1975, Wolk et al. 1979, Guerrero & Lara 1987, Ohmori & Omori 1990, Sprent & Sprent 1990), hacen que el amoniaco producido durante el proceso de fijación de N_2 sea excretado (Stewart & Rowell 1977, Rai et al. 1980, Orr & Haselkorn 1982, Stewart et al. 1983, Meeks et al. 1985). En el cianobionte, la distribución de la GS es uniforme entre las células vegetativas y los heterocistes (Hällbom et al. 1986). Por el contrario, en las cianobacterias de vida libre los heterocistes contiene doble cantidad de GS que las células vegetativas (Rai et al. 1990).

Las cianobacterias en simbiosis con las Cicadáceas se encuentran alojadas en las raíces de la planta formando una zona cilíndrica diferenciada (Fig. 1). Al contrario que en otras asociaciones simbióticas, los cianobiontes presentan altas actividades GS-GOGAT, comparables con las encontradas en cianobacterias fijadoras de nitrógeno de vida libre (Lindblad & Bergman 1986, 1990). La cantidad relativa de GS en los heterocístes es del 30 al 40% superior que en las células vegetativas (Lindblad & Bergman 1986), lo que resulta lógico, ya que los heteocistes son las células especializadas para

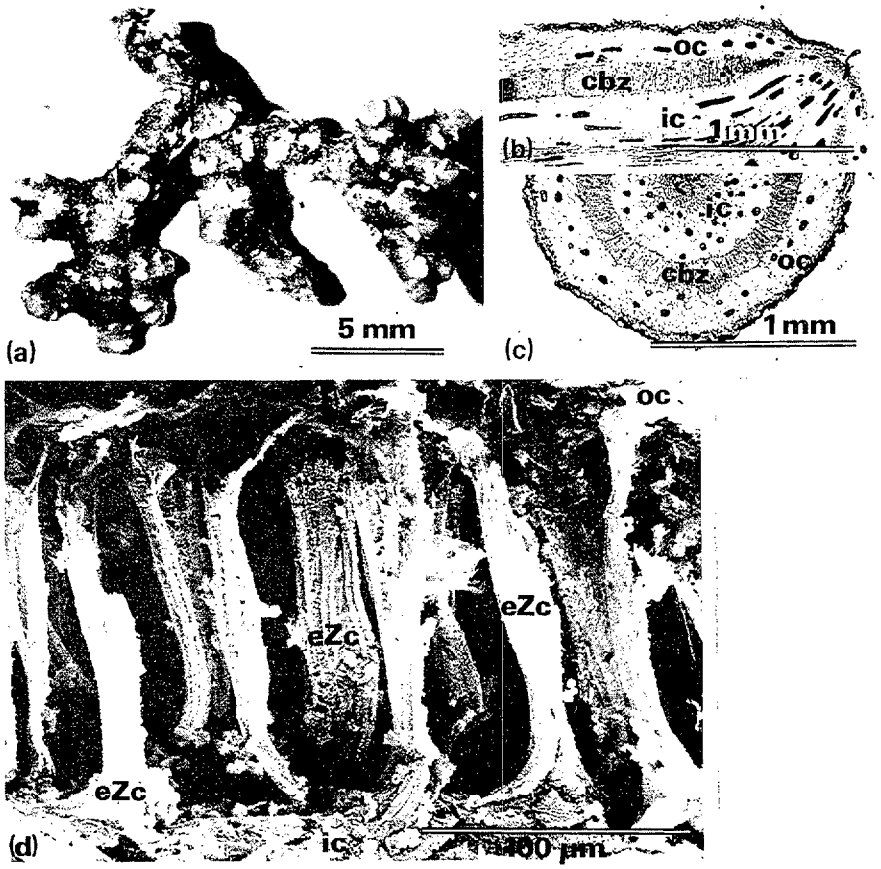


Figura 1. *Raices de la cicadácea Zamia skinneri. (a) Raices. (b) Sección transversal mostrando la zona de cianobacterias (cbz). (c) Micrografía al microscopio de barrido de la zona de cianobacterias; eZc, células elongadas de Zamia; ic, capa interna; oc, capa externa. (Lindblad et al. 1985).*

realizar la fijación de nitrógeno. El hecho de que posean un sistema GS-GOGAT funcional sugiere que el compuesto de nitrógeno transferido desde el cianobionte a la planta es un producto de la asimilación de amoniaco (Lindblad & Bergman 1986, Perraju et al. 1986, Lindblad et al. 1987).

Empleando ^{15}N se ha observado que el nitrógeno fijado es rápidamente transferido desde el cianobionte a la Cicadácea (Pate et al. 1988), siendo citrulina y glutamina los principales compuestos transferidos en el xilema desde las raíces al resto de la planta (Pate et al. 1988). Por otra

parte, experimentos similares sumunistrando $^{14}CO_2$ mostraron que el ^{14}C era incorporado en el ácido aspártico y en el grupo carbamil-fosfato de la citrulina, encontrados en las raíces y el xilema (Pate et al. 1986). Asimismo, se observó que los cianobiontes directamente aislados de *Macrozamia riedli* y el tejido no infectado de la planta sintetizaban citrulina cuando se les proveía con $^{14}CO_2$ y ornitina (Lindblad et al. 1991). Por lo tanto, tanto la cianobacteria como la planta son capaces de producir citrulina. La localización exacta de la conversión del N_2 fijado en citrulina está por determinar.

En las cianobacterias la citrulina se forma a través del ciclo de la ornitina. En este ciclo, el amoniaco producido en la fijación la N_2 es incorporado en forma de compuestos aminados y el exceso de nitrógeno es almacenado como cianoficina (un copolimero de aspartato y arginina en proporción 1:1) (Simon 1976, Allen 1988). La citrulina se forma por reacción de la ornitina y el carbamil-fosfato, catalizada por la enzima ornitín-carbamil-transferasa (OCT) (Fig. 2). La citrulina es finalmente convertida en argininosuccinato y arginina por las enzimas argininosuccinato sintetasa y argininosucinato-liasa, respectivamente (Fig. 5) (Holm-Hasen & Brown 1963, Hood et al. 1969, Lehninger 1982, Carr 1983, Schubert and Boland 1990, Ohmiri & Ohmori 1990). El carbamil-fosfato es sintetetizado por la enzima carbamil-fosfato-sintetasa empleando como sustratos NH_3 o glutamina, CO_2/HCO^{3-} y ATP (Fig. 2).

Estudios preliminares con cianobacterias de vida libre han demostrado la presencia de una enzima OCT de 80 kD y/o una enzima carbamil fosfato sintetasa en los extractos crudos de estos organismos (Bogges & Naylor 1975, Lawrie 1979, Carr 1983, Chen et al. 1978, Lindblad 1992). Ambos enzimas están localizados tanto en los heterocistes como en las células vegetativas de la cianobacteria fijadora de nitrógeno *Nostoc* PCC 73102 (Lindblad 1989, 1992).

En las cianobacterias simbiontes parte del nitrógeno fijado es transferido al huésped no siendo almacenado en foma de cianoficina. Hasta

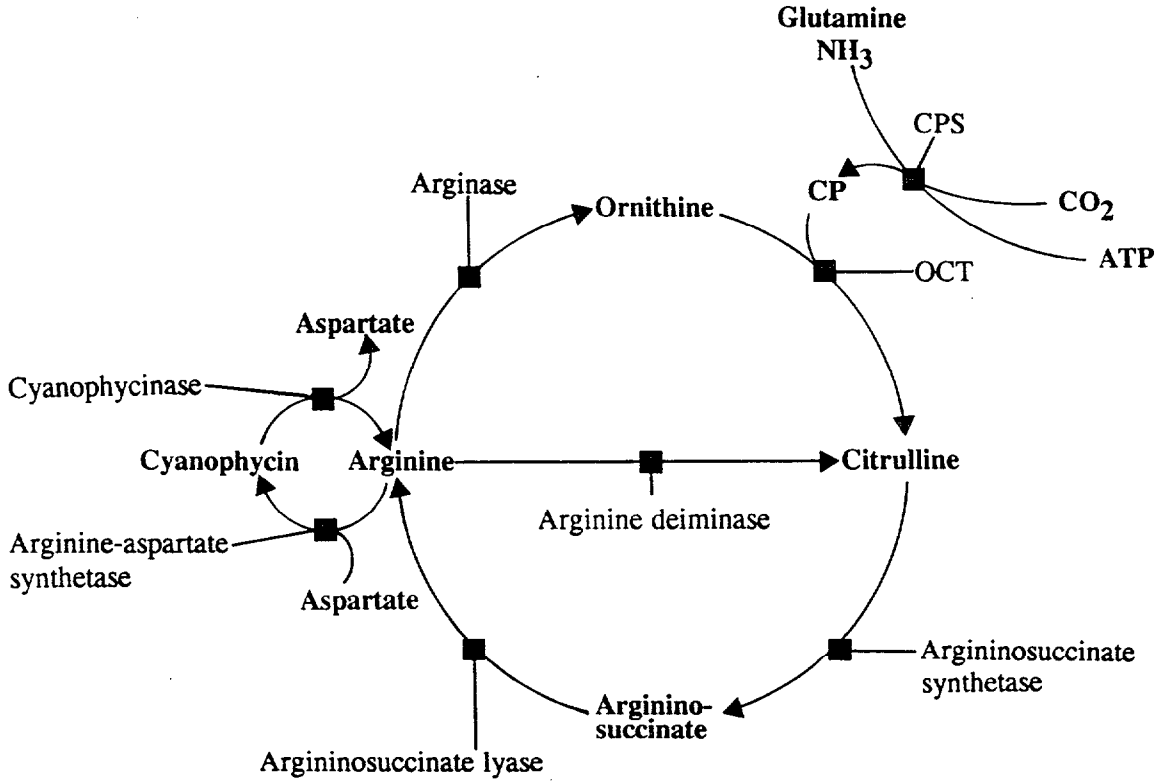


Figura 2. Representación esquemática del ciclo de la ornitina en las cianobacterias capaces de formar gránulos de cianoficina. CP: carbamil-fosfato, CPS: carbamil-fosfato-sintetasa, OCT: ornitín-carbamil-transferasa (Martel et al. 1983 [1]).

ahora han sido pocos los estudios realizados en base a obtener un mejor conocimiento de los mecanismos de regluación de la síntesis y degradación de este compuesto de reserva.

La cianoficina es metabolizada en los compuestos aminados arginina y aspartato (Gupta & Carr 1981 a, b; Carr 1983, Mackerras et al. 1990 a, b). La arginasa y la arginina deaminasa son las principales enzimas relacionados con este catabolismo. Arginasa cataliza la transformación de arginina para dar ornitina y urea, mientras la arginina deaminasa cataliza la conversión directa

de arginina en citrulina (Fig. 5) (Carr & Hood 1971, Weathers et al. 1978, Carr 1983, Lehninger 1982, Schubert & Boland 1990). En la cianobacteria filamentosa *Oscillatoria chalybea* el catabolismo de la cianoficina se realiza por mediación de la arginina (Bednarz & Schmid 1991). Estudios sobre la regulación de la biosínteisis y degradación de arginina en *Anabaena variabilis* mostraron que la actividad OCT no se alteró en presencia de arginina, mientras que las actividades arginasa y arginia deaminasa presentaron un incremento del 20%. Weathers et al. (1978) no detectaron efecto alguno del sutrato sobre la actividad arginasa en la cianobacteria unicelular *Aphanocapsa* PCC 6308. Por el contrario, en *Oscillatoria chalybea* la adición de arginina, ornitina o citrulina al medio de cultivo indujo un aumento significativo de la actividad arginasa (Bednarz & Schmid 1991).

En general, el ciclo de la ornitina ha sido poco estudiado en cianobacterias, por lo que tampoco se dispone de una metodología estandarizada que permita la cuantificación de los procesos de regulación.

En los estudios realizados hasta el momento con *Nostoc* PCC 73102, una cianobacteria de vida libre originariamente aislada de la Cicadácea *Macrozamia*, resultó interesante observar que en las células cultivadas en luz, la adición de azúcares en combinación con ornitina estimuló la síntesis de citrulina *in vivo* (Lindblad 1992), asimismo la tasa de crecimiento y la actividad nitrogenasa fueron estimuladas en presencia de los azúcares glucosa y fructosa en células cultivadas tanto en luz como en oscuridad (Lindblad 1992).

El metabolismo heterotrófico de las cianobacterias es especialmente importante en ciertos ambientes (Smith 1983), tales como en la asociación simbiótica con las raíces de las Cicadáceas (Lindblad & Bergman 1986, Lindblad et al. 1991), ya que parece existir una estrecha relación entre el metabolismo heterotrófico y la fijación de nitrógeno en las cianobacterias (Fogg 1959, Fay 1965, Rippka & Waterbury 1977, Ernst et al. 1984, Privalle 1984). El carbono es proporcionado por la planta y/o por la fijación de CO_2

en oscuridad (Lindblad & Bergman 1991, Haselkorn & Bukeima 1992). No existe información sobre la forma de carbono que es transferido, por lo que la identificación de estos compuestos requiere de mayor estudio.

Varios autores (Giesy 1964, Chao & Bowen 1971, Wolk 1973, Weber & Wöber 1975, Lehmann & Wöber 1976, Ernst et al. 1984) han demostrado la acumulación de glucógeno en cianobacterias de los géneros *Nostoc*, *Anabaena*, *Oscillatoria* y *Anacystis*, al cultivarlas en medios sin nitrógeno pero conteniendo diversos azúcares. Esta capacidad heterotrófica ha sido relacionada con la presencia de un sistema de transporte activo de azúcares (Rozen et al. 1986, Zhang et al. 1989, Schmetterer 1990). Las células de vida libre de *Nostoc* ATCC 29150 cultivadas en la oscuridad con la adición de fructosa al medio de crecimiento mostraron un modelo bifásico de asimilación de fructosa en el que la inducción de un sistema de alta afinidad por la fructosa se produjo en menos de 48 horas (Schmetterer & Flores 1987). Sin embargo, en *Nostoc* Mac, una cianobacteria aislada de la asociación simbiótica con *Macrozamia*, así como en la cianobacteria de vida libre *Anabaena variabilis*, el sistema de transporte de glucosa parece ser constitutivo (Pelroy et al. 1972, Fernandez Valiente et al. 1992).

En las cianobacteria fijadoras de nitrógeno, los azúcares son oxidados principalmente en la ruta de las pensosas fosfato (Fig. 4) (Wolk 1968, Smith 1982, Karni et al. 1984, Haselkorn & Bukeima 1992) para obtener compuestos reductores que junto con el N_2, bajos niveles de O_2 y gran cantidad de ATP son los requerimientos necesarios para la producción de NH_3 (Fay 1983, Lindblad & Guerrero 1983) (Fig. 4). Por lo tanto, administrando diversos azúcares a las cianobacterias de vida libre se puede estimular la actividad nitrogenasa (Fay 1965, Tredici et al. 1988) e incluso el desarrollo de heterocistes (Ladha & Kumar 1977). En este sentido queda por determinar si los azúcares también regulan la producción de compuestos aminados mediante el ciclo de la ornitina y cuáles son los mecanismos.

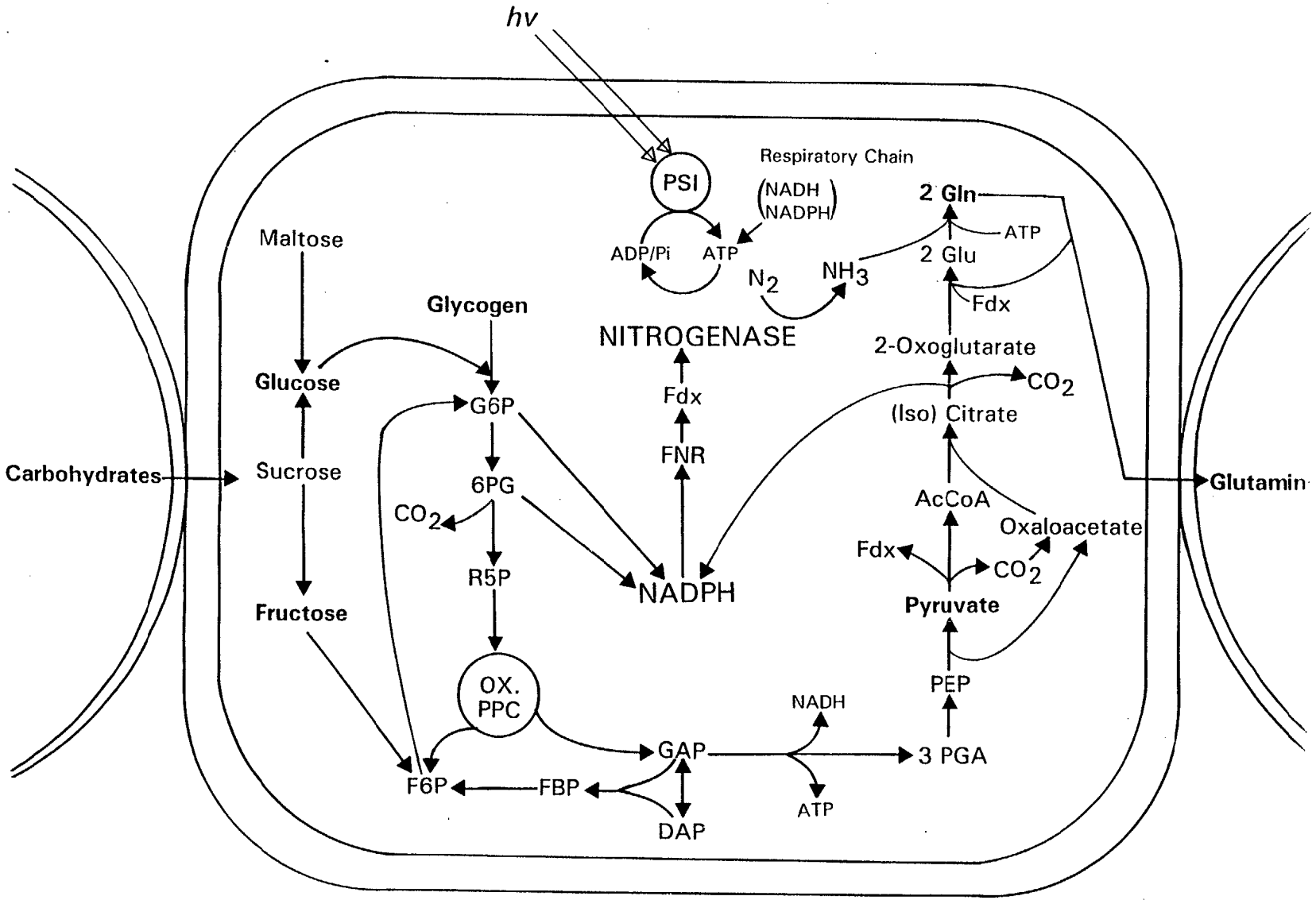


Figura 3. *Metabolismo del heterociste y donación de electrones a la nitrogenasa. Abreviaturas: G6P: glucosa-6-fosfato; 6PG: 6-fosfogluconato; R5P: ribulosa-5-fosfato, F6P: fructosa-6-fosfato; FBP: fructosa 1,6-bifosfato; GAP: gliceraldehido 3-fosfato; DAP: dihidroxiacetona fosfato; 3PGA: 3-fosfoglicerato; PEP: fosfoenol piruvato; AcCoA: acetil coenzimaA; Glu: glutamato; Gln: glutamina; Fdx: ferredoxina; FNR: ferredoxina-NADP oxidoreductasa; ox. PPC: ciclo de las pentosas fosfato; PSI: fotosistema I.*

1.2.- Osmoregulación en *Spirulina*

El género *Spirulina* se caracteriza por su un alto contenido en proteínas, vitaminas, minerales y ácidos grasos poli-insaturados (Jassby 1988), propiedades que le confieren un elevado interés como fuente alimenticia alternativa (Ciferri 1983, Fay 1983, Richmond 1986). Actualmente, se cultiva a nivel industrial para la producción de piensos, consumo humano, y para la extracción de pigmentos y vitaminas (Durand-Chastel 1980, Knutsen & Metting 1991).

El cultivo de *Spirulina* a gran escala se ve dificultado, desde el punto de vista económico, por el elevado coste de los nutrientes para preparar el medio de cultivo artificial (Materassi et al. 1984). La adaptación al cultivo en agua de mar de *Spirulina* spp. se ha convertido en una interesante alternativa, no sólo por la reducción de gastos en la preparación de los medios sino porque además permitiría extender estos cultivos a zonas que reunen condiciones ambientales idóneas (altas tasas de irradiación y temperaturas de moderada a altas) pero en las que la escasez de agua dulce constituye un factor limitante para el desarrollo de estos sistemas (Materassi et al. 1984, Knutsen & Metting 1991). Aunque existen especies marinas, son las especies de agua salobre *Spirulina maxima* y *S. platensis* las de mayor interés industrial.

Los experimentos encaminados al cultivo de *Spirulina maxima* en agua de mar (Materassi et al. 1984) han puesto de manifiesto que la transferencia al medio salino origina un fuerte estrés fisiológico, evidenciado por una disminución en la síntesis de proteinas, un aumento en el contenido de carbohidratos de la biomasa, una fuerte reducción del contenido en ficobiliproteinas y una fragmentación masiva de los tricomas (Materassi et al. 1984, Tredici et al. 1986, 1987). También se ha observado que la exposición de *S. platensis* a altas salinidades va acompañada de una gran demanda de energía (Vonshak et al. 1988) unido a una drástica inhibición de la fotosíntesis, la respiración y el creciemiento. Tras un periodo de latencia se

establece una baja tasa de crecimiento, correlacionada inversamente con la concentración NaCl en el medio de cultivo.

Además de los efectos tóxicos específicos de las sales presentes en el agua de mar, es necesario entender el(los) mecanismo(s) de adaptación osmótica de *Spirulina* a elevadas salinidades.

Vonshak et al. (1988) observaron que las células de *Spirulina platensis* sometidas a diferentes concentraciones de NaCl experimentan un aumento exponencial del contenido total en azúcares, lo que indica que los carbohidratos son los principales agentes osmoreguladores de *S. platensis*. Posteriormente, se demostró que el glucosil-glicerol [O-α-D-glucopiranosil-(1→2)-glicerol] es el principal osmótico, y que se acumula de forma directamente proporcional a la salinidad del medio (Warr et al. 1985 a, b, 1987). Cuando las células de *S. platensis* se sometieron a un choque hipersalino, la acumulación de glucosil-glicerol fue acompañada por una disminución del contenido en glucógeno (Warr et al. 1985 a). Un choque hipo-osmótico causó la diminución del contenido en glucosil-glicerol y un aumento en los niveles de glucógeno. Esto indica que la interconversión de glucosil-glicerol/glucógeno puede ser parcialmente responsable de los cambios en el contenido de glucosil-glicerol en *S. platensis* (Warr et al. 1985 a).

El glucosil-glicerol es un heteróxido compuesto de nueve átomos de carbono (Kollman et al. 1979), con una estructura análoga al floridoside (galactosil-glicerol) de las Rodofitas (Reed et al. 1984). En las algas rojas, la concentración de floridoside (galactosil-α-(1→2)-glicerol) aumenta a altas presiones osmóticas (Kauss et al. 1978, Kirst & Bisson 1979, Reed et al. 1980, Reed 1985). El floridoside es degradado por la enzima α-galactosidasa cuya actividad, regulada a nivel de síntesis, aumentó cuando el alga se transfirió desde un medio hipersalino a otro hiposalino, y disminuyó en el proceso inverso (Yu & Pedersén 1990 a, b).

La microalga *Pteriochroomonas malhamensis*, un flagelado de agua dulce que carece de pared celular, responde a la presión osmótica ajustando

su contenido en isofloridoside [O-α-D-galactopiranosil-(1→1)-glicerol], un derivado del polisacárido de reserva, crisolaminarina. Mientras las células estuvieron en condiciones osmóticas estables, la enzima isofloridoside-fosfato-sintasa existió como proenzima inactivo. Sin embargo, cuando la presión osmótica externa aumentó, la sintasa condujo la reacción hacia la formación de isofloridoside; cuando esta disminuyó, la reacción se invirtió favoreciendo la formación del crisolaminarina (Kauss & Thomson 1982).

En cianobacterias y en particular en *Spirulina* no se ha descrito la existencia de enzimas reguladoras del proceso osmótico, como podría ser el caso de la glucosidasa en la interconversión de glucosil-glicerol/glucógeno. Otra enzima que puede estar relacionada con este proceso osmótico en cianobacterias es la α-glucano fosforilasa (EC 2.4.1.1) que participa en la degradación y síntesis del glucógeno (Fredrick 1971, Shively 1988). En algas rojas esta enzima tiene un peso molecular estimado entre 200 y 243 kD y se encuentra localizada alrededor de los gránulos de almidón florideo en el citosol de *Gracilaria chilensis* y *G. tenuistipitata*, y en el pirenoide del alga verde *Enteromorpha intestinalis* (Yu et al. 1993).

1.3.- Objetivos

Los dos objetivos generales de esta tesis ha sido el estudio de los mecánismos de regulación del ciclo de la ornitina en cianobacterias fijadoras de nitrógeno y del proceso de regulación osmótica en *Spirulina platensis*. Los objetivos específicos fueron:

1.- Evaluar el efecto a largo plazo de la adición de azúcares sobre la actividad nitrogenasa.

2.- Desarrollar la metodología para determinar la existencia y cuantificar la actividad de ornitín-carbamil-transferasa, arginasa y arginina deaminasa en cianobacterias fijadoras de nitrógeno.

3.- Evaluar el efecto de la adición de compuestos aminados y azúcares sobre la actividad ornitín-carbamil-transferasa, arginasa y arginina deaminasa en cianobacterias fijadoras de nitrógeno.

4.- Desarrollar un método para cuantificar la actividad α-glucosidasa en cianobacterias.

5.- Determinar la presencia, caracterización y localización de las enzimas α-glucosidasa y α-glucano fosforilasa en cianobacterias, asi como su importancia en el proceso de regulación osmótica de *Spirulina platensis*.

2.- MATERIAL Y METODOS

2.1.- Especies y condiciones de cultivo

2.1.1.- *Nostoc* PCC 73102

Nostoc sp. PCC 73102, una cianobacteria fijadora de nitrógeno originariamente aislada de la Cicadácea *Macrozamia* (Fig. 4), se obtuvo de la Colección de Cultivos Pasteur (PCC), Paris, Francia (Rippka et al. 1979). *Nostoc* PCC 73102, la estirpe referencia para el género *Nostoc* en la clasificación de la PCC, es un fotoheterótrofo facultativo capaz de utilizar glucosa, fructosa y/o ribosa como fuente(s) de carbono (Rippka et al. 1979). Los cultivos axénicos se mantuvieron en medio de cultivo $BG11_0$ (medio BG_{11} sin la adición de $NaNO_3$; Stanier et al. 1971), a 25°C, en matraces Eherlen-meyer de 100 ó 125 ml, en agitación continua.

Las condiciones experimentales de cultivo par desarrollar los objetivos 1 y 3 se detallan en los artículos [I] y [II].

2.1.2.- *Spirulina platensis*

Spirulina platensis (Nordstedt) forma Geitler (Fig. 5), originariamente aislada de un lago alcalino de la República del Chad, se obtuvo de la colección Sammlung von Algenkulturen, Göttigen Universität, Alemania. Los cultivos se mantuvieron en medio Zarrouk (modificado de Zarrouk 1966) (Tabla 1), en agitación, a 25°C e irradiación continua a 220 μmol m^{-2} s^{-1} [THORN Polylux 4000, y OSRAM Warmton Warm White (400-700 nm] [III-V].

Las células se cultivaron experimentalmente según se describe en [III].

Para determinar el efecto del NaCl sobre la actividad α-glucano-fosforilasa las células de *Spirulina* se cultivaron en medio Zarrouk en luz o en oscuridad, sin y con la adición de 1, 2 ó 4% de NaCl (el medio Zarrouk contiene 0.1% de NaCl). Después de 0, 3 y 4 días de cultivo se tomaron muestras para determinar la actividad *in vitro* de la enzima α-glucano-fosforilasa y el contenido total de glucógeno.

2.1.3.- Cianobacterias unicelulares

Las cianobacterias unicelulares *Synechococcus* PCC 6301 (*Anacystis nidulans*), *Synechococcus* PCC 7942 (*Synechococcus* R2) y *Synechocystis* PCC 6803 se cultivaron en medio BG11 líquido (Rippka et al. 1979) (Tabla 3), en agitación, a 25°C e irradiación continua de 80 μmol m^{-2} s^{-1}.

2.2.- Cuantificación de la actividad enzimática

2.2.1.- Actividad nitrogenasa *in vivo*

La actividad nitrogenasa (EC 1.7.99.2)en *Nostoc* PCC 73102 se determinó *in vivo* empleando el método de reducción de acetileno (Lindblad & Bergman 1986, Lindblad 1992, Lindblad & Guerrero 1993). Los datos se expresaron en función del contenido en clorofila *a* y del tiempo, según se describe en el artículo [I].

2.2.2.- Ensayos de actividad enzimática *in vitro*

Las actividades *in vitro* se determinaron mediante los métodos colorimétricos que se describen a continuación.

Ornitín-carbamil-transferasa

La actividad OCT (EC 2.1.3.3)en las células de *Nostoc* PCC se cuantificó empleando un método modificado del descrito por Boyde & Rahmatullah (1980), basado en la detección colorimétrica de citrulina con el compuesto diacetilmonoxima (DAMO), según se describe en los artículos [I] y [II]. Las principales modificaciones del método original para aumentar la sensibilidad en la detección de citrulina (Boyde & Rahmatullah 1980) incluyeron: *(i)* la disminución del volumen de la mezcla cromogénica, y *(ii)* el aumento del tiempo de incubación.

Tabla 1. Medio de crecimiento Zarrouk para *Spirulina*, modificado de Zarrouk (1966).

Compuesto	Cantidad	Solución Gaffron* (gl^{-1} agua destilada)	
$NaHCO_3$	13.61	A.- $Na_2Wo_4.2H_2$	0.365
Na_2CO_{36}	4.03	$(NH4)_6Mo_7O_{24}.4H_2O$	0.906
K_2HPO_4	0.50	KBr	1.190
$NaNO_3$	2.50	KI	0.830
K_2SO_4	1.00	$ZnSO_4.7H_2O$	2.870
NaCl	1.00	$Cd(NO_3)_2.4H_2O$	1.340
$MgSO_4.7H_2O$	0.20	$Co(NO_3)_2.6H_2O$	1.460
$CaCl_2.2H_2O$	0.04	$CuSO_4.5H_2O$	1.250
$FeSO4{\cdot}7H_2O$	0.01	$NiSO_4.7H_2O$	1.317
EDTA	0.08	B.- $Cr(NO_3){\cdot}9H_2O$	0.407
Solución Gaffron	0.8ml	$VOSO_4.5H_2O$	0.119
H_2O destilada	1.00 l	$KAl(SO_4)_2.12H_2O$	2.370
		C.- H_3BO_4	3.100
		$MnSO_4.H_2O$	2.496

* se mezclan 10 ml de A y B con 100 ml de C.

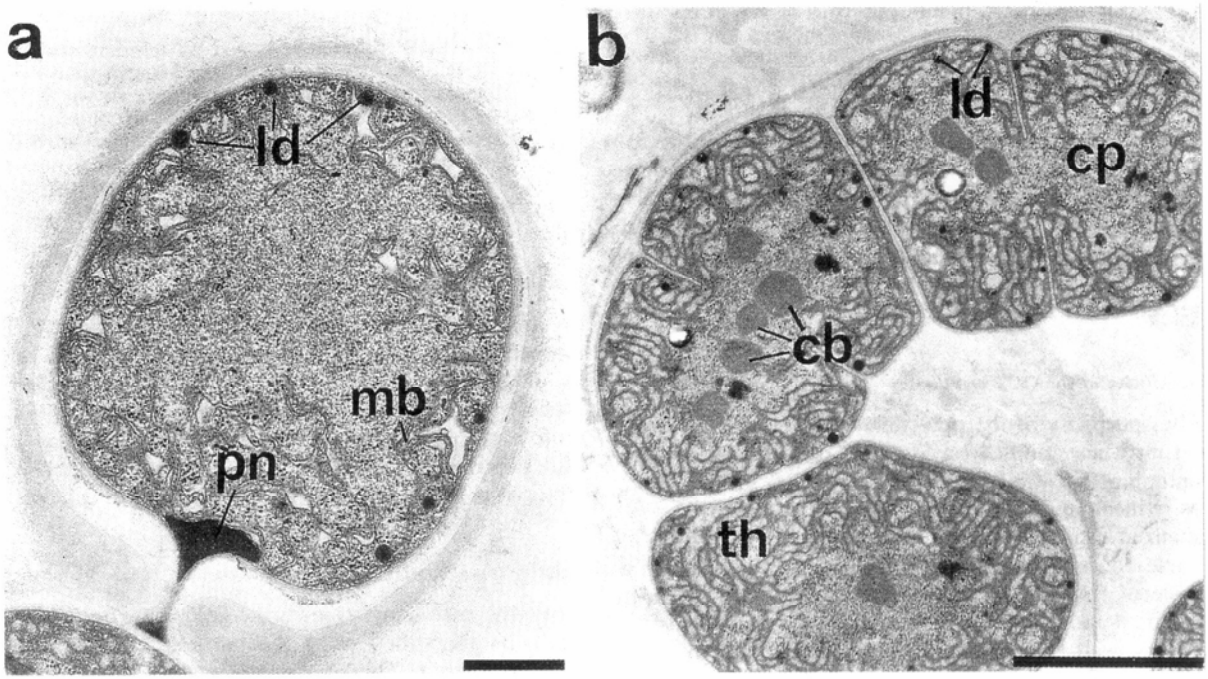


Figura 4. *Fotografías al microscopio de transmisión electrónica de Nostoc PCC 73102 creciendo en medio BG11$_0$. (A) Heterociste. Obsérvese el nódulo polar (pn), las membranas (mb) y las gotas lipídicas (ld). (B) Una parte del filamento consistente en varias células vegetativas. El centroplasma (cp), donde se localizan los carboxisomas (cb), rodeados de los tilacoides (th). Barras = 1 μm.*

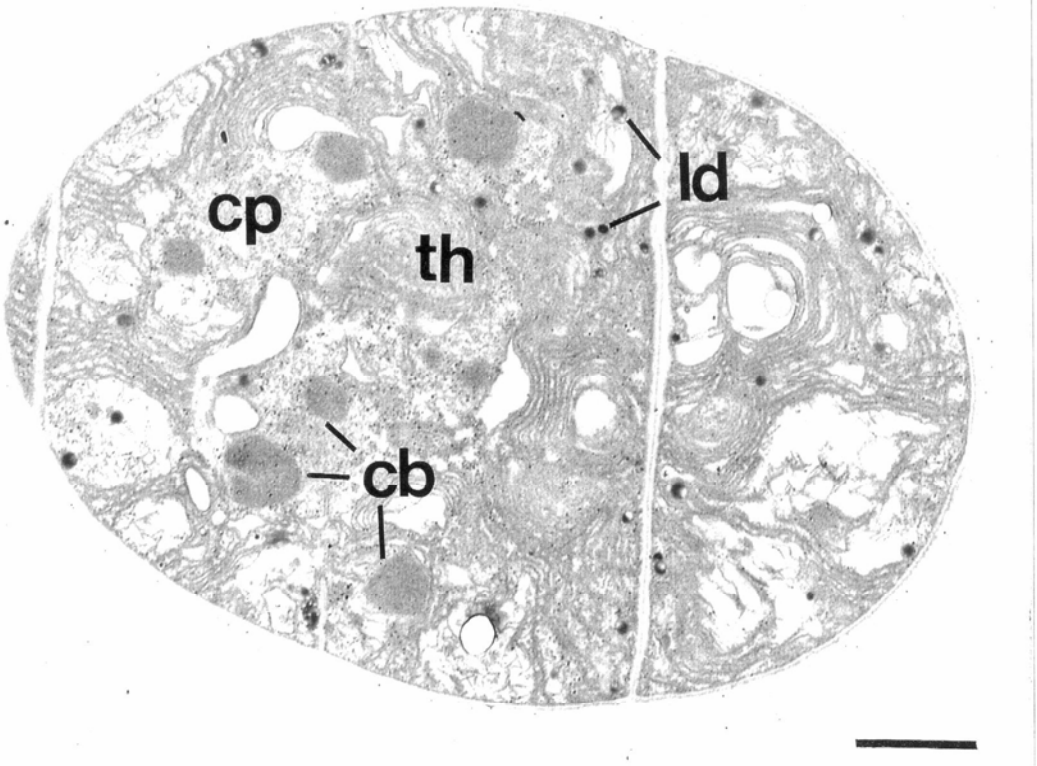


Figura 5. *Fotografía al microscópio electrónico de Spirulina platensis. Obsérvese los carboxisomas (cb), las membranas tilacoidales (th), las gotas lipídicas (ld) y el citoplasma (cp).*

Arginasa y arginina deaminasa

La actividad arginasa en las células de *Nostoc* PCC 73102 se cuantificó emplenado una modificación del método de Archibald (1944), basado en la detección colorimétrica de urea con α-isonitrosofenona (INP), como se describe en el artículo [I]. Las principales modificaciones sobre el método original (Archibald 1944) incluyeron el aumento *(i)* del tiempo de centrifugación requerido para la obtención de los extractos crudos, y *(ii)* de la concentración óptima de arginina en la mezcla reactiva.

La actividad arginina deaminasa se cuantificó combinando los métodos desarrollados para medir las actividades OCT y arginasa en cianobacterias, según se detalla en el material y métodos del artículo [I].

α-D-glucosidasa

Para desarrollar el método que permita cuantificar la actividad α-D-glucosidasa (EC 3.2.1.20) en cianobacterias (objetivo 4) se ensayaron diferentes sustratos : trealosa, maltosa, metil-α-D-glucoside y *p*-nitrofenil-α-D-glucopiranoside. A su vez, se utilizaron diferentes tampones, pH, tiempos de incubación y se analizó la relación entre el contenido total de proteínas del extracto crudo y la actividad enzimática.

La preparación del extracto crudo y el método analítico para cuantificar la actividad α-glucosidasa en las células de *Spirulina platensis* se describe en el apartado de material y métodos del artículo [III].

Efecto de inhibidores

El efecto de los inhibidores de la transcripción de ARN (estreptomicina y metil-purina), o de la síntesis de proteínas (cloramfenicol) sobre la actividad enzimática de α-glucosidasa en las células de *Spirulina platensis* sometidas a un choque hipo-osmótico se describe en el artículo [III].

α-Glucano-fosforilasa

La actividad *in vitro* de la enzima α-glucano-fosforilasa (EC 2.4.1.1) se determinó a partir de la fosforólisis de los α-1,4-glucanos. La glucosa-1-fosfato producida se cuantificó empleando una reacción enzimática acoplada con fosfoglucomutasa y glucosa-6-fosfato deshidrogenasa (Yu and Pedersén 1991 a).

Las células de *Spirulina platensis* se recolectaron mediante centrifugación (13000 xg, 10 min) y se resuspendieron en un tampón de extracción [Bicina-NaOH (50 mM) (pH 8.25) conteniendo β-mercaptoetanol (0.1%)]. La suspensión celular se homogeneizó por ultrasonidos (MSE, Ultrasonic Power Unit # 12-63, Reino Unido) durante 3 x 1 min en hielo y se centrifugó (13,000 xg, 10 min) para recolectar el sobrenadante.

La actividad α-glucano-fosforilasa se determinó incubando 100 μl del extracto crudo con 300 μl de tampón P [ácido cítrico-NaOH (67 mM) (pH 6.5), KH_2PO_4 (27 mM), NaF (13 mM), NaN_3 (0.1 %) y Dextrina 20 (1.33 %)]. La reacción se llevó a cabo a 30°C durante 30 min y se detuvo incubando los tubos a 100°C durante 1 min. Los controles se prepararon añadiendo el extracto crudo al final de la reacción, justo antes de calentarlas a 100°C. La mezcla de la reacción se llevó a un volumen final de 1 ml añadiendo fosfoglucomutasa (0.8 U)/glucosa-6-fosfato deshidrogenasa (1 U), NADP (0.6 μM), glucosa-1,6-bifosfato (0.02 μM), y el tampón Tris-HCl (160 mM) (pH 7.5) conteniendo $MgCl_2$ (20 mM). Después de incubar la muestra a 30°C durante 10 min, se midió la absorbancia a 304 nm. La actividad específica de la α-glucano-fosforilasa se expresó como μmol de glucosa-1-fosfato producido min^{-1} mg^{-1} proteína.

Cuantificación de proteínas

El contenido en proteínas de los extractos crudos de *Nostoc* PCC 73102 y *Spirulina platensis* se calculó con el método descrito por Peterson (1983), empleando albúmina bovina como estándar.

2.3.- Cuantificación de glucógeno

El contenido en glucógeno de las células de *Spirulina platensis* se cuantificó empleando el método descrito por Warr et al. (1984). El glucógeno, extraído a 100°C con KOH concentrado, se precipitó con etanol. Posteriormente se trató con antrona y se determinó colorimétricamente como glucosa.

2.3.1.- Cuantificación del peso seco

Para calcular el peso seco *Spirulina platensis* se centrifugaron 10 ml de una suspensión celular. El pelet y se incubó 24 h a 80°C sobre trozos de papel de aluminio previamente secados.

2.4.- Técnicas electroforéticas

2.4.1.- PAGE-nativa

La electroforesis del extracto crudo sobre geles de poliacrilamida (PAGE-nativa) se empleó para analizar los perfiles totales de proteinas, la tinción *in vitro* de la actividad enzimática y los análisis de inmunotransferencia Western en *Nostoc* PCC 73102 [II], *Spirulina platensis* [IV], y de las cianobacterias unicelulares *Synechocystis* PCC 6803, *Synechoccocus* PCC 6301, *y Synechoccocus* PCC 6301 [V].

2.4.2.- Métodos de tinción

Ornitín-carbamil-transferasa

La tinción la actividad OCT en las células de *Nostoc* se realizó empleando el método de Farkas et al. (1987) según el protocolo descrito en el artículo [II]. Para desartar la posible interferencia de la enzima aspartato-carbamil-transferasa (EC 2.1.3.2)(otro enzima que teóricamente puede dar la misma respuesta que OCT), se reemplazó la ornitina por ácido aspártico, como se describe en el artículo [II].

Para evaluar la influencia del pH en la presencia de OCT en las

cianobacterias, se cultivó *Nostoc* en oscuridad en un medio a diferentes pH (6.0, 7.0, 8.0 y 9.0), según se describe en el artículo [II].

α-Glucano-fosforilasa

La tinción de la actividad glucano-fosforilasa en *Spirulina platensis*, y las cianobacterias unicelulares *Synechocystis* PCC 6803, *Synechoccocus* PCC 6301, y *Synechoccocus* PCC 6301, se llevó a cabo mediante el procedimiento descrito en los artículos [IV] y [V]

2.4.3.- PAGE-SDS

Los extractos crudos de *Nostoc* [II], *Spirulina platensis* [IV], las cianobacterias unicelulares *Synechocystis* PCC 6803, *Synechoccocus* PCC 6301, y *Synechoccocus* PCC 6301 [V] se destanuralizaron añadiendo un tampón de solubilización [Tris-HCl (10 mM) (pH 8.0), conteniendo EDTA (1 mM), β-mercaptoetanol (5%), SDS (2.5%) y Triton X-100 (5%)] en una proporción de 1:1 e incubándolos durante 5 min a 100°C. Las PAGE-SDS se ensayaron sobre geles con un gradiente de 10-15% en un equipo PhastSystem (Pharmacia Ltd., Suecia) (Lindblad & Sellstedt 1991).

2.4.4.- Análisis de inmunotransferencia Western

Los análisis de inmunotransferencia Western para la determinación de la especificidad de los anticuerpos contra la OCT purificada de *Pisum sativum* en los polipéptidos de *Nostoc*, y de la especificidad de los anticuerpos contra la α-glucano-fosforilasa (purificada de *Gracilaria chilensis)* en las proteínas y polipéptidos de *Spirulina platensis*, y en los polipéptidos de las cianobacterias unicelulares se describen en los artículos [II], [IV] y [V], respectivamente.

2.5.- Ultraestructura y localización celular de los antígenos

2.5.1.- Caracterización ultraestructural

Las células se prepararon para la caracterización estructural y posterior visualización en un microscopio de transmisión electrónica (Philips CM10, operando a 60 kV),siguiendo el método descrito por Lindblad et al. (1985).

2.5.2.- Microscopía inmunoelectrónica

La aplicación de esta técnica permitió determinar, *(i)* qué células contiene un antígeno específico, *(ii)* dónde se localizó el antígeno en la célula, y *(iii)* la cantidad relativa de antígeno en los diferentes tipos de células. Como marcadores se emplearon partículas coloidales de oro.

Marcadores inmunológicos

La fijación, la inclusión, la obtención de los cortes y el marcado inmunológico de las células de *Spirulina* [IV] y de las cianobacterias unicelulares [V] se realizó seguiendo el método descrito por Lindblad & Sellstedt (1991), usando antisuero policlonal de conejo dirigido contra la α-glucano-fosforilasa purificada de *Gracilaria chilensis* [tiempo de incubación 3h ; dilución 1:400 ; Yu & Pedersén 1991 a] y un segundo anticuerpo [IgG de cabra contra el suero de conejo, dilución 1:20, tiempo de incubación 1 h; BioCell, Cardiff, Reino Unido] marcado con partículas coloidales de oro de 5nm, como se detalla en los artículos [IV] y [V].

Recuento de partículas

La cuantificación relativa de las partículas de oro ligadas a la α-glucano fosforilasa en las células de *S. platensis* se realizó empleando el analizador de partículas de un proceasador de imágenes (Quantel, Newbury, Reino Unido), como se describe en [IV].

Estimación de volúmenes

Las áreas de las células de *Spirulina* y de los carboxisomas se estimaron

mediante un procesador de imagen [IV]. El volumen celular y el volumen de los carboxisomas se calcularon asumiendo que las células de *Spirulina* eran cilíndricas y los carboxisomas, cúbicos. El número y los volúmenes individuales y totales de los carboxisomas se determinaron en diez células diferentes.

2.6.- Tasa de crecimiento

La tasa de crecimiento de *Spirulina platensis* cultivada en medio Zarrouk sin (control) o con la adición de 2 y 4% de NaCl se expresó el función del contenido en clorofila (μg Clorofila ml^{-1}).

3.- RESULTADOS

3.1.- Efecto de la adición de azúcares sobre la actividad nitrogenasa en *Nostoc* PCC 73102 [I]

En la Figura 2 en [I] se muestra los resultados de actividad nitrogenasa de *Nostoc* PCC 73102.

Las células cultivadas en oscuridad exhibieron sólo un 6 % de la actividad nitrogenasa detectada en las células creciendo en luz [18.1 ($\pm$2.0) y 1.1 4($\pm$0.3) nmol C_2H_4 mg^{-1} (Clorofila *a*) h^{-1} (medias $\pm$ DS, n=4), respectivamente]. Sin embargo, la adición de glucosa y fructosa en oscuridad igualó los niveles de actividad nitrogenasa obtenidos en luz (Fig. 2a en [I]). La capacidad de los azúcares para estimular altas tasas de actividad nitrogenasa se mantuvo tras 12 días de incubación en oscuridad antes de la adición de azúcares (Fig. 2b en [I]).

3.2.- Cuantificación de la actividad ornitín-carabamil-transferasa [I, II]

La sensibilidad del método para cuantificar la actividad OCT aumentó al disminuir el volumen del reactivo cromogénico desde 3 ml a 600 μl y mezclándolo con 200 μl de muestra (70 μl de extracto, 30 μl de la mezcla reactiva, y 100 μl de ácido tricloroacético). La máxima intensidad de color se alcanzó después de 30 min de incubación a 100°C (Fig. 6).

El efecto de la adición de azúcares y compuestos aminados sobre la actividad OCT se muestra en la Figura 2b y la Tabla 1 del artículo [I], respectivamente.

Las células de *Nostoc* incubadas en luz y en oscuridad sin o con la adición de azúcares estimularon la actividad OCT, observándose los mayores niveles en oscuridad (Fig. 3c en [I]).

La adición de ornitina y arginina en oscuridad indujeron las mayores actividades OCT (12 y 7 veces la actividad observada en las células creciendo en luz, respectivamente) (Tabla 3, Tabla 1 en [I]).

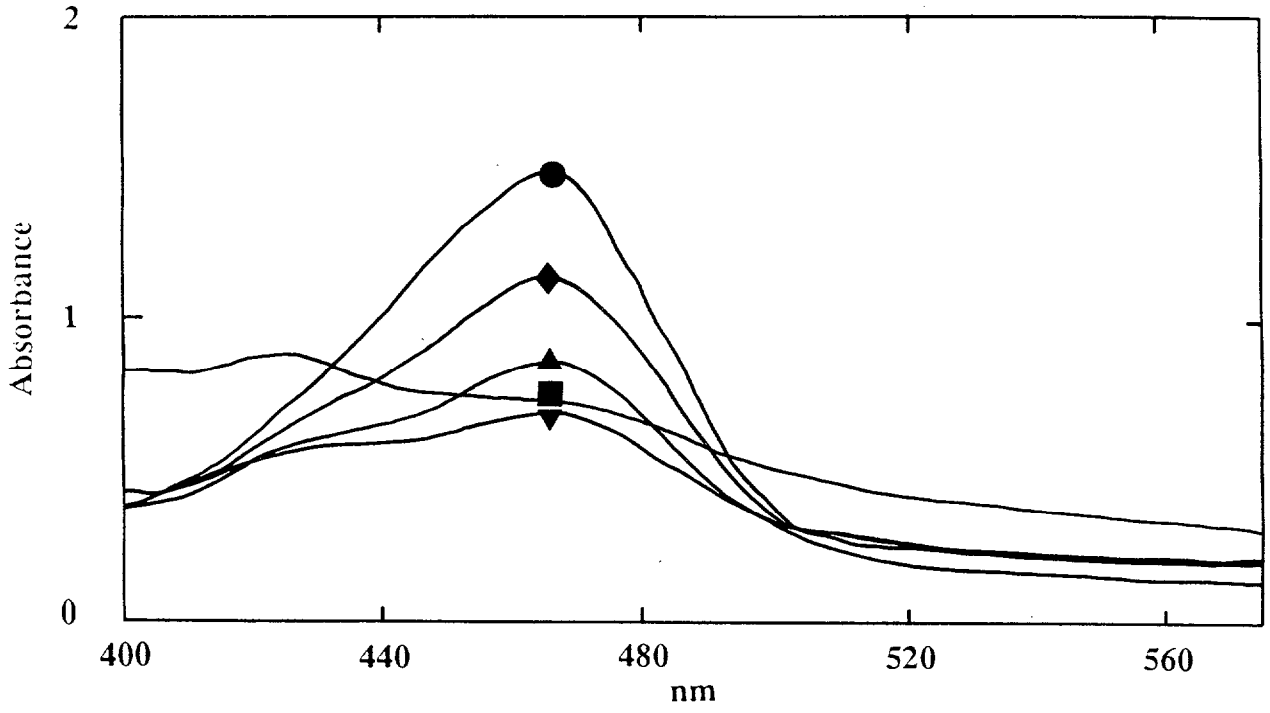


Figura 6. *Variación de la sensibilidad en la determinación de citrulina en función del tiempo de incubación con DAMO. Una solución conteniendo citrulina (1 mM) se incubó a 100°C 5 (■), 10 (▼), 15 (▲), 20 (◆) y 30 (•) min junto con 400 µl de ácido férrico y 200 µl de la solución DAMO, antes de medir la absorbancia a 464.*

El método para la tinción de OCT (Farkas et al. 1978) fue específico para esta enzima. La aspartato-carbamil-transferasa no mostró actividad cuando en el método se sustituyó ornitina por su sustrato (aspartato).

En los cultivos de *Nostoc* a diferente pH únicamente se observó una OCT activa *in vitro* con un peso molecular de 80 kD. Tras 26 horas de inducción (oscuridad + arginina) se detectó una segunda OCT de 118 kD aproximadamente (Fig. 1 en [II]).

Los análisis de inmunotransferencia Western del extracto crudo de *Nostoc* con los anticuerpos dirigidos contra la proteína nativa o desnaturalizada de la OCT purificada de *Pisum sativum*, reconocieron ambas

enzimas (80 y 118 kD) (Fig. 2 en [II]). Con el extracto desnaturalizado, únicamente se detectó una banda, con un peso molecular de 40 kD aproximadamente.

3.3.- Cuantificación de la actividad arginasa [I]

El aumento del tiempo de centrifugación de los extractos a 1 h (14000 xg) (Fig. 7), y el aumento de la concentración de arginina desde 30 a 60 mM en la reacción, fueron las modificaciones que aumentaron la sensibilidad del método (Archibald 1944) para medir la actividad arginasa *in vitro*.

El efecto producido por los azúcares y los compuestos aminados sobre la actividad arginasa y arginina deaminasa se expresan en la Figura 2 y la Tabla 1 del artículo [I], respectivamente.

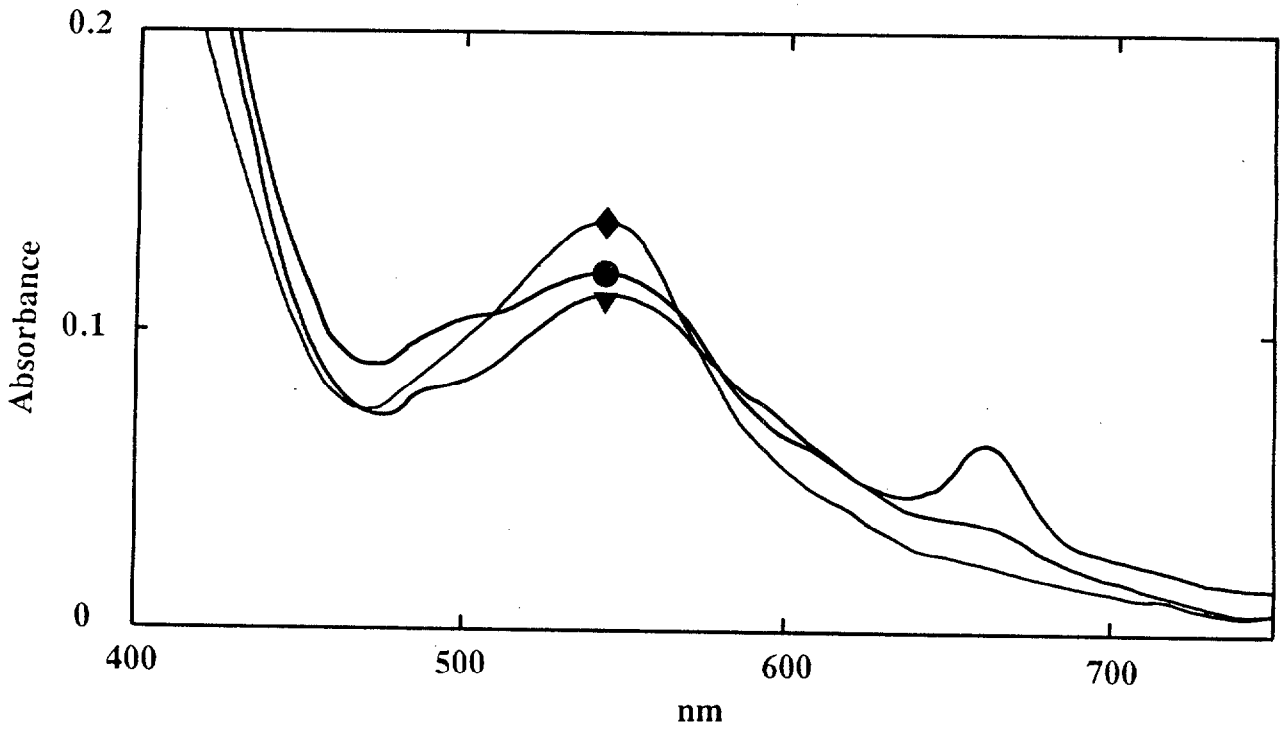


Figura 7. Variación de la sensibilidad en la detección de urea en función del tiempo de centrifugación de los extractos (40 min, ▼ ; 60 min, ◆ ; 75 min, ●).

3.4.- Efecto de la adición de azúcares y compuestos aminados sobre la actividad OCT, arginasa y arginina deaminasa [I]

La actividad arginasa aumentó significativamente en las células que crecieron en presencia de arginina u ornitina. La mayor actividad se observó en las células cultivadas en oscuridad con la adición de arginina u ornitina (comparado con los cultivos en luz mostraron un aumento 71 y 23 veces superior, respectivamente) (Tabla 3, Tabla 1 en [I]). La adición de citrulina al medio causó menores efectos sobre la actividad arginasa.

Resultados similares se obtuvieron con arginina deaminasa. Las mayores actividades se alcanzaron en las células cultivadas en oscuridad con la adición de arginina (Tabla 3, Tabla 1 en [I]).

Tabla 3. Actividades de arginasa, ornitín-carbamil-transferasa y arginina deaminasa en *Nostoc* PCC73102, (media ± DS). [a] Arg: arginina; Orn: orniti-na; Cit: citrulina. [b] % de la actividad comparada con las células cultivadas en luz sin la adición externa de arginina, ornitina o citrulina al medio de cultivo.

Cultivo	Arginasa	OCT	Arginina deaminasa
	(nmol producto formado μg prot^{-1} min^{-1})		
Luz	69 ± 5 (100)[b]	64 ± (100)[b]	158 ± 2 (100)[b]
Luz + Arg[a]	533 ± 127 (772)	136 ± 23 (212)	1009 ± 53 (693)
Luz + Orn[a]	797 ± 257 (1155)	196 ± 56 (306)	1576 ± 144 (997)
Luz + Cit[a]	131 ± 3 (190)	26 ± 21 (41)	242 ± 8 (153)
Oscuridad	189 ± 4 (287)	140 ± 9 (219)	490 ± 7 (310)
Oscuridad + Arg	4913 ± 92 (7120)	450 ± 19 (703)	5913 ± 18 (3742)
Oscuridad + Orn	1578 ± 18 (2287)	780 ± 15 (1219)	1862 ± 13 (1178)
Oscuridad + Cit	270 ± 167 (391)	122 ± 3 (191)	318 ± 9 (201)

El cultivo en oscuridad (células con baja actividad nitrogenasa) (Fig. 2a en [I], estimuló la actividad arginasa (Fig. 3a en [I]). Las células que crecieron tanto en luz como en oscuridad con la adición de azúcareş (células con alta actividad nitrogenasa) (Fig. 2a en [I]), mostraron bajos niveles de actividad arginasa (Fig. 3a en [I]). Efectos similares se observaron en la actividad arginina deaminasa (Fig. 3c en [I]).

3.5. Cuantificación de la actividad α-D-glucosidasa [III]

La enzima α-glucosidasa mostró actividad únicamente con el sustrato *p*-nitrofenil-α-D-glucopiranoside. Las mayor actividad glucosidasa se detectó cuando el extracto de *Spirulina platensis* se preparó en tampón fosfato (NaH_2PO_4 -NaOH (50 mM)). Ensayos anteriores usando Bicina-NaOH (50 mM) (pH 8.2) no mostraron niveles de actividad detectables (datos no publicados). El extracto se pudo mantener a $+4^o$ durante 24 horas sin que perdiera actividad. El pH óptimo para la actividad glucosidasa fue 7.4 (Fig. 1a en [III]).

La Fig. 1b [III] muestra la actividad glucosidasa en función del tiempo de incubación a 30^oC y pH 7.4. Usando un tampón fosfato con un pH 7.4 y un tiempo de incubación de 30 min, se examinó la relación entre las proteínas totales frente a la actividad enzimática. Con valores menores de diez μg, o menos de proteínas no se detectaron niveles de actividad glucosidasa. Sin embargo, se observó una respuesta lineal cuando se emplearon concentraciones totales de proteínas entre 10 y 40 μg (Fig. 3c en [III]).

3.6.- Efecto de la concentración de NaCl sobre α-glucosidasa en *Spirulina platensis* [III]

El efecto del choque hipo-osmótico sobre la actividad glucosidasa en los extractos de *Spirulina platensis* se muestran en la Figura 2 del artículo [III]. Las células que crecieron en condiciones de hipersalinidad [medio Zarrouk (0.1% NaCl) conteniendo 1 ó 4% de NaCl] mostraron niveles detectables de

actividad glucosidasa. Cuando las células cultivadas con 4% de NaCl se transfirieron a condiciones de hiposalinidad [medio Zarrouk (0.1 % NaCl)] la actividad glucosidasa, aumentó, alcanzando el máximo nivel a las 10 horas de choque hipo-osmótico. Después de este periodo la actividad disminuyó hasta alcanzar su nivel inicial 25 horas más tarde (Fig.2 en [III]). Una respuesta similar se observó en los cultivos que se transfirieron desde 1 % de NaCl. La actividad glucosidasa aumentó a lo largo de 10 h de choque hiposmótico para posteriormente disminuir y alcanzar su nivel inicial después de 20 h (Fig. 2 en [III]). La actividad glucosidasa en los cultivos transferidos desde 4% de NaCl fue significativamente superior a la observada en las células transferidas desde 1% de NaCl.

Los resultados presentados en la Tabla 1 del artículo [III], muestran que el aumento de la actividad glucosidasa inducido por un choque hiposmótico fue inhibido tanto por los inhibidores de la síntesis de proteínas (cloramfenicol y estreptomicina) como de ARN (metil-purina).

3.7.- Localización y caracterización de la α-glucano-fosforilasa en *Spirulina platensis* y cianobacterias unicelulares [IV, V]

Los anticuerpos dirigidos contra la α-glucano-fosforilasa purificada del alga roja *Gracilaria chilensis* (Yu & Pedersén 1991 a, b), reconocieron una proteína y un polipéptido en *Spirulina platensis* con un peso molecular aproximado de 220 y 42 kD (Fig. 2 en [IV]), respectivamente. La tinción de la actividad glucano-fosforilasa en los extractos crudos de *S. platensis* mostró una única banda con un peso molecular de 219 ± 19 kD (media ± DS, n=3) (Fig. 1 en [IV]).

La glucano-fosforilasa también se detectó en los extractos crudos de las cianobacterias unicelulares *Synechocystis* PCC 6803, *Synechoccocus* PCC 6301, y *Synechoccocus* PCC 7942. Los anticuerpos policlonales dirigidos contra la glucano-fosforilasa de *Gracilaria chilensis* reconocierón una única subunidad. Los pesos moleculares fueron 71, 58 y 57 para *Synechocystis* PCC 6803,

Synechoccocus PCC 7942 *y Synechoccocus* PCC 6301, respectivamente (Fig. 2 en [V]).

La tinción (Fig. 1 en [V]) mostró dos formas de la enzima glucano-fosforilasa en *Synechocystis* PCC 6803 (96 kD y 123 kD) y *Synechoccocus* PCC 7942 (234 kD y 248 kD). Sin embargo, en los extractos de *Synechoccocus* PCC 6301 se observaron tres formas con pesos moleculares de 92 kD, 224 kD y 251 kD, aproximadamente.

La enzima α-glucano fosforilasa se localizó en el área tilacoidal y en los carboxisomas de las células de *Spirulina platensis* (Fig. 3 en [IV]). El marcado asociado a los carboxisomas se estimó en 690 ± 105 (media ± DS) partículas de oro μm^{-2} comparado con 36 (± 14) en el resto de la célula (19 veces más glucano-fosforilasa por área celular en los carboxisomas que el citoplasma) (Tabla 1 en [IV]). Sin embargo, recalculando el número relativo de partículas por volumen se observó que sólo un tercio de la glucano-fosforilasa estaba confinada en los carboxisomas.

Synechocystis PCC 6803, *Synechoccocus* PCC 6301 *y Synechoccocus* PCC presentaron un marcado en todas las células. La intensidad del marcado fue mayor en los carboxisomas de *Synechoccocus* PCC 6301 y *Synechoccocus* PCC 7942 (Fig. 4 en [V]). Sin embargo, en *Synechocystis* PCC 6803 la fosforilasa se localizó sólo en la zona tilacoidal, y no en los carboxisomas.

3.8.- Efecto del NaCl sobre la α-glucano fosforilasa en *Spirulina*

En la Figura 8 se muestran el efecto de diferentes concentraciones de NaCl sobre el contenido total de glucógeno en las células en tratamientos en luz y en oscuridad.

En la Figura 9 se presentan la variación de la actividad glucano-fosforilasa en función de la concentración de NaCl en el medio de cultivo tanto en luz como en oscuridad.

Las células cultivadas en luz mostraron un aumento del contenido en

glucógeno (Fig. 8a), que fue inhibido por la adición de NaCl al medio de cultivo. El contenido total de glucógeno disminuyó en oscuridad sin o con la adición de NaCl. El contenido intracelular de glucógeno disminuyó con el aumento de la concentración de NaCl en el medio (Fig. 8b).

La actividad glucano-fosforilasa fue estimulada por la adición de NaCl en oscuridad (Fig. 9b). Sin embargo, en luz el aumeto de la concentración de NaCl causo una disminución de la actividad (Fig. 9a).

3.9. Efecto del NaCl sobre la tasa de crecimiento en *Spirulina*

La adición de NaCl al medio de cultivo (Fig. 10) dio lugar a una inhibición del crecimiento que se reanudó 24 h más tarde. Las tasas de crecimiento obtenidas se correlacinarón inversamente con la concentración de NaCl en el medio, observándose una disminución del 50% respecto al control (medio Zarrouk) en los cultivos con 4% de NaCl.

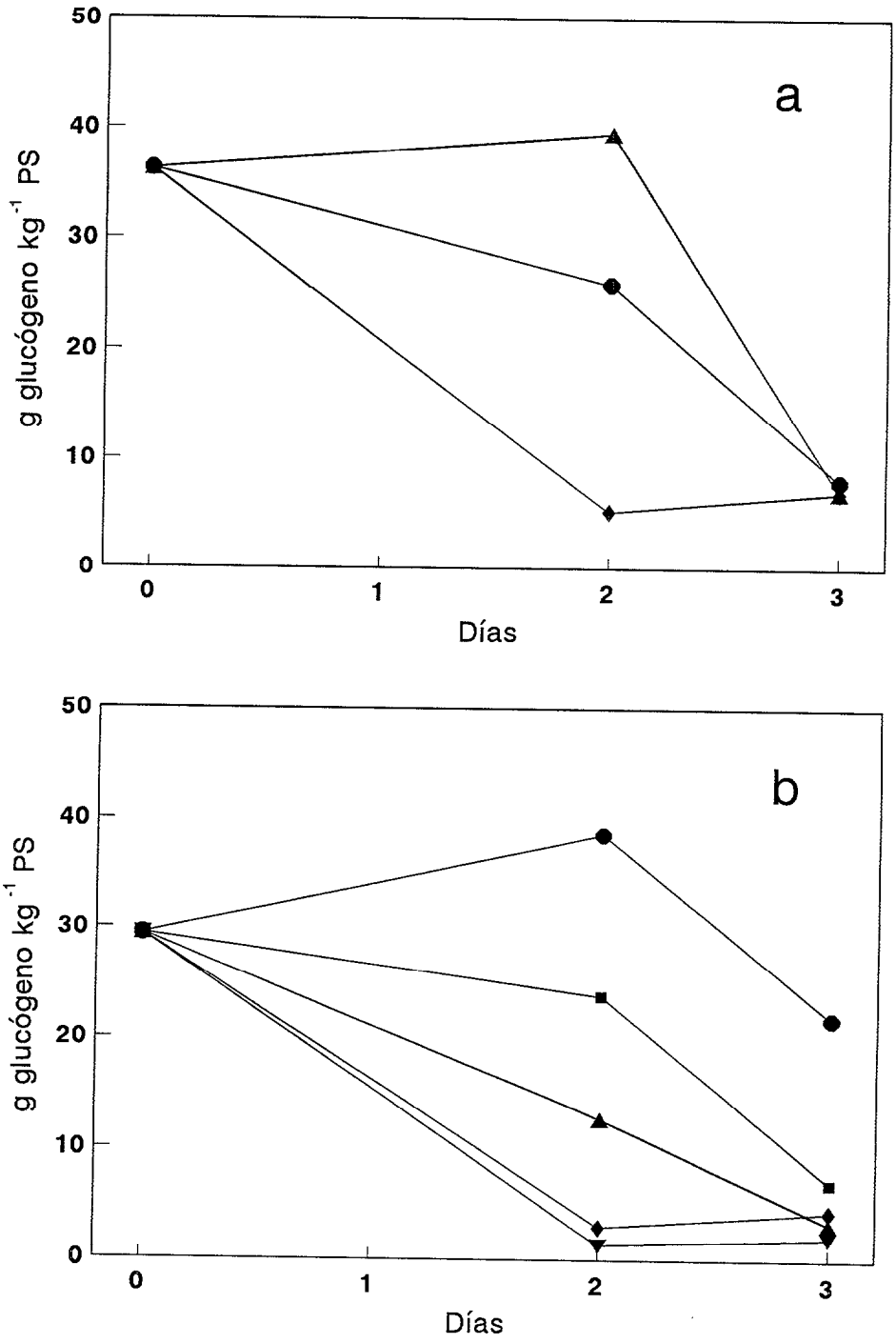


Figura 8. *Efecto del NaCl sobre el contenido en glucógeno de Spirulina platensis. (a) A tiempo cero, las células creciendo en luz se transfirieron desde medio Zarrouk (0.1 % NaCl) a medio Zarrouk conteniendo 1% NaCl (▲), 2% NaCl (●) ó 4 % (◆). (b) Las células de Spirulina se cultivaron en luz (●) o en oscuridad sin (■; control) o con la adición de 1% NaCl (▲), 2% NaCl (▼) ó 4 % (◆). Después de tres días de cultivo se cuantificó el contenido total en glucógeno.*

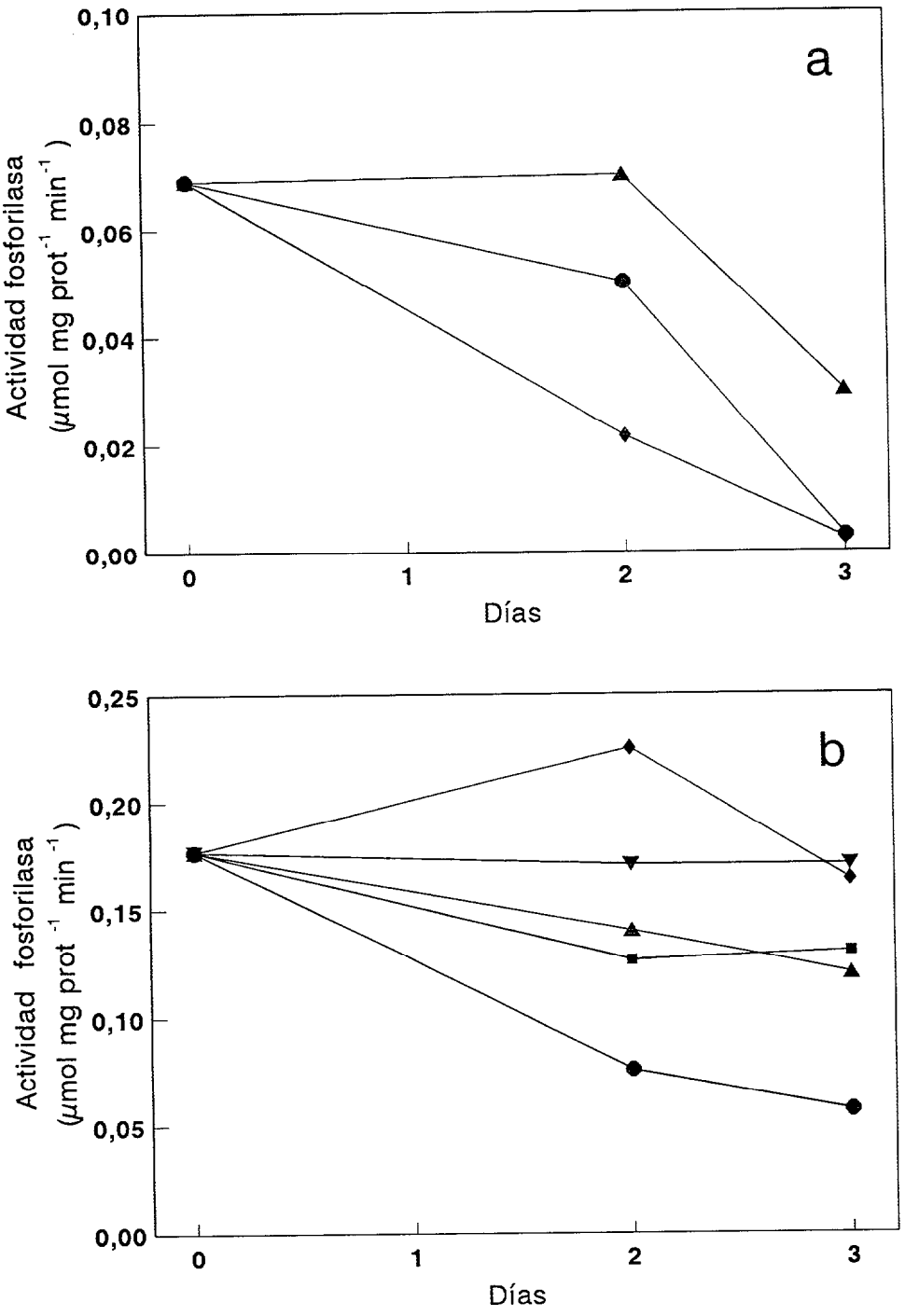


Figura 9. *Efecto del NaCl sobre la actividad α-glucano-fosforilasa en los extractos crudos de Spirulina platensis. (a) A tiempo cero, las células creciendo en luz se transfirieron desde medio Zarrouk (0.1 % NaCl) a medio Zarrouk con 1% NaCl (▲), 2% NaCl (●) ó 4 % (◆). (b) Las células de Spirulina se cultivaron en luz (●) o en oscuridad sin (■; control) o con la adición de 1% NaCl (▲), 2% NaCl (▼) ó 4 % (◆). Después de tres días de cultivo se cuantificó la actividad α-glucano-fosforilasa.*

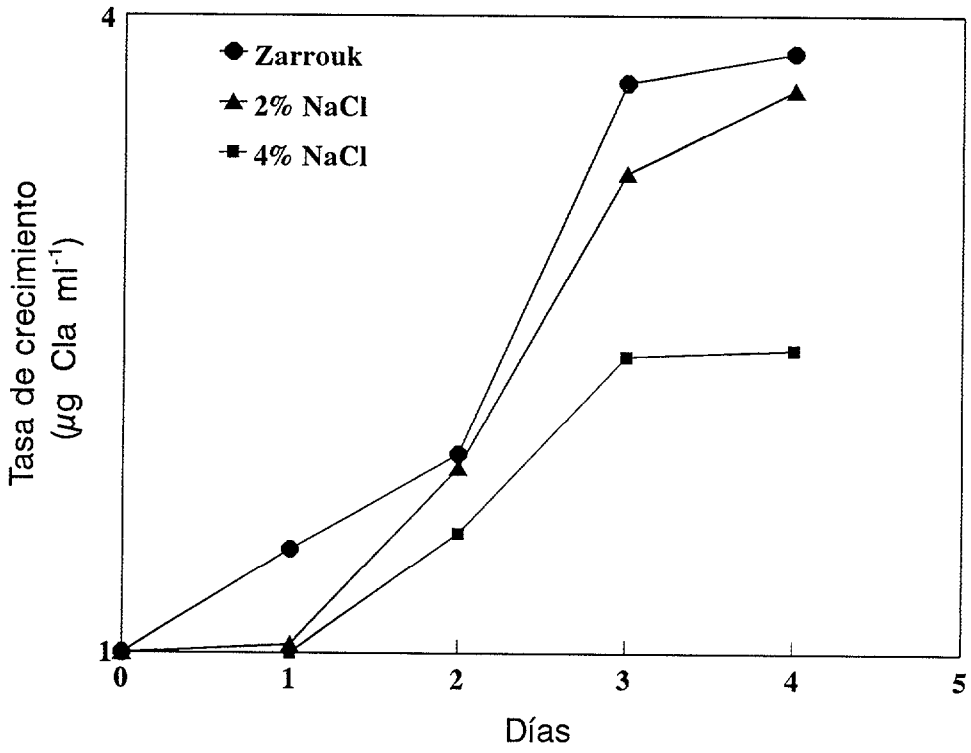


Figura 10. *Efecto del ClNa sobre la tasa de crecimiento de Spirulina platensis. El eje de ordenadas se representa en escala logarítmica.*

4.- DISCUSION

4.1.- Efecto de la adición de azúcares y compuestos aminados sobre la actividad arginasa, OCT, arginina deaminasa y nitrogenasa

Las modificaciones realizadas sobre los métodos descritos para la determinación de OCT (Boyde & Rahmatullah 1980) y arginasa (Archibald 1944) en plasma sanguíneo permitieron obtener una buena resolución y la cuantificación de estos enzimas en los extractos de la cianobacteria fijadora de nitrógeno *Nostoc* PCC 73102. Igualmente, el método desarrollado para analizar arginina deaminasa fue bastante efectivo y permitió, por primera vez, cuantificar la presencia y actividad de dicha enzima en cianobacterias. Como era de esperar la actividad de estas tres enzimas fue estimulada por sus respectivos sustratos (Tabla 3, Tabla 1 en [I]), sin embargo ornitina, producto final de la reacción catalizada por arginasa, indujo aumentos significantivos en la actividades arginasa y arginina deaminasa (Tabla 3, Tabla 1 en [I]). La ornitina, durante el tiempo de cultivo, fue rápidamente convertida en arginina/cianoficina sirviendo de sustrato a las enzimas arginasa y arginina deaminasa, lo que concuerda con los resultados de Bednarz & Schmid (1991) en *Oscillatoria chalybea*, en los que se detectó un aumento de actividad arginasa por la adición tanto de arginina como de ornitina. Por el contrario, en *Anabaena variabilis* (Hood & Carr 1971) y *Aphanocapsa* PCC 6308 (Weathers et al. 1978), la adición de arginina no alteró las actividades enzimáticas.

La ausencia de cambios significativos en la actividades OCT, arginasa y arginina deaminasa al añadir citrulina al medio de cultivo (Tabla 3, Tabla 1 en [I]) indica que este compuesto aminado no es metabolizado tan rápidamente, bien por ausencia de un sistema de transporte que permita su asimilación por las células o por la existencia de un sistema de acoplamiento entre las enzimas OCT y argininosuccinato sintetasa que sólo utilice la citrulina formada a través del ciclo de la ornitina. Estos resultados, sin embargo, contrastan con los obtenidos con *Oscillatoria chalybea* en los que la

presencia de citrulina estimuló la actividad arginasa (Bednarz & Schmid 1991), lo que podría indicar la existencia de regulaciones específicas de estos enzimas entre cianobacterias fijadoras y no fijadoras de nitrógeno.

En consonancia con lo descrito por otros autores (Bottomley & Stewart 1977, Sprent & Sprent 1990, Steinberg & Meeks 1991, Haselkorn & Buikema 1992), la asimilación y metabolización de glucosa y fructosa en oscuridad (Fig. 2 en [I]) está relacionada con elevadas actividades nitrogenasa, incluso en tratamientos a largo plazo en oscuridad antes de añadir los azúcares (Fig. 2 en [I]). Esto permite a la cianobacteria disponer de una fuente continua de nitrógeno, que utiliza para sus funciones metabólicas y cuyo exceso almacena en forma de cianoficina, reprimiendo la actividad de arginasa y arginina deaminasa (Fig 3 en [I]). Sin embargo, cuando las células se cultivaron en oscuridad sin la adición de azúcares, la cantidad de ATP producido (principalmente por fosforilación oxidativa; Bottomley et al. 1977, Tel-Or et al. 1977, Houchins 1975, Haselkorn & Bukeima 1992) no fue suficiente para desarrollar altas actividades nitrogrenasa (Fig. 1 en [I]) y por tanto su aporte fue insuficiente para cubrir las demandas metabólicas de las células. Consecuentemente, las reservas internas de nitrógeno (cianoficina) fueron mobilizadas y, por inducción de las enzimas catabólicas arginasa y arginina deaminasa (Fig. 3 en [I]), la arginina es convertida en una forma de nitrógeno utilizable. Estos resultados concuerdan con los de Lindblad (1992), que demostraron la disminución de la actividad nitrogenasa de *Nostoc* PCC 73102 por la adición de arginina, ornitina o citrulina. Nuestros resultados indican que, al igual que en *Anabaena cylindrica* (Makerras et al. 1990), la cianoficina tiene un papel dinámico en el metabolismo del nitrógeno asi como una función temporal de almacenamiento.

La adición de arginina al medio de cultivo no sólo indujo altas actividades OCT *in vitro* sino también la aparición de una segunda OCT de 118 kD. Este no es un efecto producido por el pH (a todos los pH analizados sólo se observó una única OCT de 80 kD) (datos no publicados) como ocurre

en la bacteria *Aeromonas formicans*, donde OCT es un monómero a pH 6.0, y un trímero a pH 8.0 (Tricot et al. 1989), sino la respuesta al aminoácido añadido (arginina) (Figs. 1, 2 en [II]). A diferencia de la OCT inducida en bacterias por la adición externa de arginina (Lehninger 1982, Tricot 1989), la segunda OCT inducida en *Nostoc* PCC 73102 es de menor peso molecular (Fig. 1 en [II]). El reconocimiento de enzimas de 80 y 118 kD por los anticuerpos de la OCT purificada de *Pisum sativum* demuestra que ambas son OCT y que son homólogas en la composición de sus subunidades (sólo se detectó una banda de 40 kD en el extracto desnaturalizado) (Fig. 2 en [II]). Previamente se había sugerido que la enzima de 80 kD era un dímero con dos subunidades idénticas (Lindblad 1992), por lo que la enzima de 118 kD debe ser un trímero con subunidades idénticas.

4.2 Osmoregulación

4.2.1. Cuantificación del efecto del NaCl sobre la actividad α-glucosidasa en *Spirulina*

Se describe por primera vez la existencia de la enzima glucosidasa en la cianobacterias. El perfil de pH utilizado para la cuantificación de la actividad glucosidasa mostró un máximo a 7.4,lo que contrasta con las glucosidasas de plantas vasculares, en las que el rango óptimo de pH es ácido (pH 4-5) (Dey & Campillo 1984). Sin embargo, al igual que las glucosidasas de plantas vasculares (Dey & Campillo 1984), no fue necesario ningún cofactor para determinar la actividad catalítica de la glucosidasa en *Spirulina platensis*.

La reserva intracelular de glucosil-glicerol en *Spirulina platensis* aumenta a altas presiones osmóticas y en respuesta a un choque hipo-osmótico el glucosil-glicerol puede ser convertido en glucógeno (Warr et al. 1985 a). Las mayores actividades glucosidasa se detectaron al transferir las células desde 4 % NaCl a medio Zarrouk comparado con aquellos transferidos desde 1 % (Fig. 2 en [III]), lo que indica la existencia de un proceso de regulación osmótica por medio de la mobilización de las reservas

de glucosil-glicerol en las células. Resultados similares se han descrito en las macroalgas *Gracilaria tenuistipitata* y *G. sordida*, al transferirlas desde un medio de 5% a 1% de salinidad, proceso en el cual la actividad de α-galactosidasa aumentó durante 24 horas para luego permanecer constante (Yu & Pedersén 1990 a). Por el contrario, la actividad glucosidasa en las células de *S. platensis* disminuyó rápidamente al cabo de 10 horas, alcanzando un nivel similar al inicial a las 25 horas (Fig 2 en [III]).

La inhibición de la α-glucosidasa por la adición de inhibidores de la síntesis de ARN y proteínas (Tabla 1 en [III]) demuestra que la regulación de esta enzima tiene lugar a nivel de transcripción y que no se trata de una forma pre-existente que puede ser activada o inactivada, como es el caso de la enzima isofloridoside fosfato sintasa, que interviene en la regulación osmótica de *Poteriochroomonas malhamensis* (Kauss et al. 1978, Kauss & Thomson 1982). Por otra parte, la galactosidasa en *P. mahalmensis*, al igual que en las algas rojas *G. tenuistipitata* y *G. sordida*, es regulada a nivel de síntesis (Kreuzer & Krauss 1980, Yu & Pedersén 1990 a). La actividad galactosidasa en estas Rodofitas disminuyó cuando se añadieron tanto inhibidores de la síntesis ARN como de proteínas (Yu & Pedersén 1990 a).

Estos datos deben ser tratados con precaución ya que los inhibidores utilizados no son específicos y podrían afectar a la síntesis de proteínas de otras rutas metabólicas.

4.2.2. Presencia, caracterización y localización de la α-glucano-fosforilasa en *Spirulina* y cianobacterias unicelulares

La enzima glucano-fosforilasa se encuentra localizada en la zona tilacoidal de las células de *Spirulina platensis* (Fig. 3 en [IV]) y de las cianobacterias unicelulares *Synechocystis* PCC 6803, *Synechoccocus* PCC 7942 y *Synechoccocus* PCC 6301 (Fig. 4 en [V]), donde se depositan los gránulos de glucógeno (almidón cianofíceo). Sin embargo, en *S. platensis* asi como en las dos cianobacterias unicelulares del género *Synechococcus*, la glucano-fosforilasa

también se localizó en los carboxisomas. Esta es la primera vez que se describe la presencia de una enzima que no sea la RuBisCO (Ribulosa Bifosfato Carboxilasa/Oxidasa) en los carboxisomas. Los carboxisomas de cianobacterias son estructuras poliédricas compuestas principalmente de proteínas, contieniendo entre 7 y 15 polipéptidos de los cuales, hasta este momento, sólo se habían identificado las dos subunidades de la enzima RuBisCO (Biederman & Westphal 1979, Lanaras & Codd 1981, Price et al. 1992). También en las plantas vasculares, al menos dos fomas de la enzima fosforilasa están presentes, una en el cloroplasto, donde se encuentra el almidón, y otra en el citosol (Gebrandy & Verleur 1971, Richardson & Matheson 1977, Steup & Melkonian 1981, Shimamura et al. 1982). Recientemente la glucano-fosforilasa se ha localizado alrededor de almidón florideo en el citosol de las algas rojas *Gracilaria chilensis* y *G. tenuistipitata* y en el pirenoide del alga verde *Eteromorpha intestinalis* (Yu et al. 1993).

Existe la posibilidad de que una forma no-activa de la glucano-fosforilasa esté localizada en los carboxisomas, mientras la forma activa se encuentra en el citoplasma; la técnica de marcado inmunológico empleada únicamente detecta anticuerpos reconocibles, los cuales pueden estar en forma activa o inactiva. Esta interpretación también asume que existe una correlación lineal entre el número de partículas de oro y la cantidad de antígeno.

La multiplicidad de formas de la enzima glucano-fosforilasa encontradas entre las distintas cianobacterias (una forma en *Spirulina platensis*, dos formas en *Synechocystis* y *Synechoccocus* 7942, y tres formas en *Synechoccocus* 6301) (Fig. 1B en [IV] y Fig. 1 en [V]) es una respuesta bioquímica a la heterogeneidad de la molécula de glucano y/o a su estructura (Beck & Zeigler 1983) ya que la composición del almidón o el glucógeno varía no sólo con la transición entre la forma particulada (granular) y la forma soluble, sino también como resultado de la alteración en la proporción entre las fracciones amilasa y amilopectina (Beck & Zeigler 1983). Por lo tanto,

cabía esperar diferencias entre la cianobacteria filamentosa *S. platensis* que acumula almidón cianofíceo (altamente ramificado y con enlaces α-1→4 predominantemente (Meeuse 1962)) y las cianobacterias cocoides, presumiblemente primitivas dentro de su clase, que acumulan glicoproteinas como producto final del proceso fotosintético. Los estudios sobre la biosítesis de los polisacáridos en cianobacterias (Fredrick 1959) revelan que a medida que se progresa en la escala evolutiva, hay un disminución gradual de los niveles de las enzimas ramificadoras (los enzimas que controlan la formación de amilopectina), con lo que las cianobacterias más primitivas (las cianobacterias unicelulares) contendrían menor cantidad de polisacáridos ramificados (Fredrick 1959).

Las glucano-fosforilasas detectadas en las diferentes cianobacterias tienen como característica común que solo presentan una subunidad con pesos moleculares que varían entre las distintas cianobacterias (Fig. 2 en [IV] y Fig. 2 en [V]), pero que están por debajo de los observados en la subunidad de almidón-fosforilasa en algas rojas (Yu & Pedersén 1991 a, b) y en la glucógeno-fosforilasa en animales (Titani et al. 1977).

Por lo tanto, las diferencias en la presencia y localización subcelular de la glucano-fosforilasa entre las diferentes cianobacterias (Figs. 1,2,3 en [IV], Figs. 1, 2, 4 en [V]) deben estar relacionadas con la heterogeneidad de las moléculas y la estructura de los gránulos de glucógeno entre estos organismos procariotas. Las diferencias en la localización de la glucano fosforilasa en las cianobacterias puede constituir un mecanismo de regulación de la actividad enzimática.

4.3. Efecto del NaCl sobre la α-glucano-fosforilasa en *Spirulina*

La disminución del contenido en glucógeno en *Spirulina platensis* tras un choque hipo-osmótico (Figs. 8 y 9) coincide con un aumento en la actividad glucano-fosforilasa, lo que demuestra la función de esta enzima en el proceso de regulación osmótica.

Se sabe que la glucano-fosforilasa cataliza la fosforilación reversible de los glucanos α-1,4 en las plantas vasculares y que, por el contrario, la glucógeno-fosforilasa sólo funciona como enzima degradativa en animales (Fukui 1983). El papel de la glucano-fosforilasa en *Spirulina platensis* parece variar con las condiciones de cultivo, degradando el glucógeno en la oscuridad (degradación acentuada por la adición de NaCl) y sintetizándolo en la luz (Figs 8, 9).

En general, la exposición de *Spirulina platensis* a altas concentraciones de NaCl provoca una inhibición del crecimineto (Fig. 10) debido a la represión de las actividades fotosintética y respiratoria (Vonshak 1988). En esta primera etapa de adaptación al estrés salino se produce una degradación del glucógeno (Fig. 8) en la que participan las enzimas α-glucano-fosforilasa y α-glucosidasa, lo que se traduce en un aumento del osmótico glucosil-glicerol (Warr et al. 1985). En una segunda etapa, las células reanudan el crecimiento (Fig. 10). Las altas tasas respiratorias observadas por Vonshak et al. (1988) en cultivos de *Spirulina* a los que se añadió NaCl, podrían ser causadas por la diminución de la tasa de crecimiento (Fig. 10). Al mismo, tiempo el glucógeno continuaría siendo degradado para suministrar la energía necesaria para mantener el crecimiento de las células de *S. platensis* sometidas a estrés salino.

5.- CONCLUSIONES

1.- La adición externa de carbono (glucosa y fructosa) a las células cultivadas en oscuridad induce niveles de actividad nitrogenasa *in vivo* similares, o incluso mayores, a las de células creciendo en luz. La capacidad de los azúcares para estimular la actividad nitrogenasa pemanece tras 12 días de incubación en oscuridad.

2.- Las modificaciones de los métodos originales para cuantificar OCT y arginasa en plama sanguíneo permitieron determinar la presencia de estas enzimas y cuantificar sus actividad en la cianobacteria fijadora de nitrógeno *Nostoc* PCC 73102. Es posible cuantificar la actividad arginina deaminasa combinando los métodos desarrolldos para medir las actividades ornitín-carbamil-transferasa y arginasa.

3.- Las enzimas arginasa, ornitín-carbamil-transferasa y arginina deaminasa están presentes en la cianobacteria filamentosa fijadora de nitrógeno *Nostoc* PCC 73102. Sus actividades están reguladas por arginina, ornitina y citrulina. Las actividades arginasa y arginina deaminasa aumenta significativamente al combinar el cultivo en oscuridad con la adición de arginina u ornitina. Se sugiere la existencia de regulaciones específicas de los enzimas ornitín-carbamil-transferasa, arginasa y arginina deaminasa entre las cianobacterias fijadoras y no fijadoras de nitrógeno.

4.- Además de la enzima OCT de 80 kD descrita previamente, la adición de arginina al medio de cultivo induce una segunda OCT con un peso molecular aproximado de 118 kD en *Nostoc* PCC 73102 y que está compuesta por tres subunidades idénticas de 40 kD.

5.- El pH óptimo para analizar α-glucosidasa en la cianobacteria *Spirulina platensis* es de 7.4 y no es necesario ningún cofactor para cuantificar su

actividad.

6.- La enzima α-galactosidasa está presente en la cianobacteria filamentosa *Spirulina platensis*, está relacionada con el proceso de regulación osmótica y su actividad es regulada a nivel de transcripción.

7.- La enzima α-glucano-fosforilasa está presente en la cianobacteria filamentosa *Spirulina platensis* y en las cianobacterias unicelulares *Synechocystis* PCC 6803, *Synechoccocus* PCC 6301 y *Synechoccocus* PCC 7942.

8.- En *Spirulina platensis* α-glucano-fosforilasa es una enzima con peso molecular aproximado de 220 kD compuesta cuatro subunidades idénticas de 47 kD y está localizada en el área tilacoidal y en los carboxisomas de *Spirulina platensis*. En las cianobacterias unicelulares *Synechocystis* PCC 6803, *Synechoccocus* PCC 6301 y *Synechoccocus* PCC 7942 la enzima α-glucano-fosforilasa presenta multiples formas. La enzima se localiza en el citoplasma y en los carboxisomas de *Synechoccocus* PCC 6301 y *Synechoccocus* PCC 7942. En *Synechocystis* PCC 6803 la enzima sólo se localiza en zona tilacoidal.

9.- Las diferencias en la presencia y localización subcelular de la glucano-fosforilasa entre las diferentes cianobacterias probablemente están relacionadas con la heterogeneidad de las moléculas y la estructura de los gránulos de glucógeno entre estos organismos procariotas.

10.- Existen evidencias para considerar que la enziama α-glucano-fosforilasa tiene el doble función como regulador osmótico en *Spirulina platensis* sintetizando glucógeno en luz y degradándolo en oscuridad.

Deseo expresar mi más sincera gratitud a todas las personas que han hecho posible esta tésis y en especial

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7.- ARTICULOS

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Ornithine cycle in *Nostoc* PCC 73102. Arginase, OCT and arginine deiminase, and the effects of addition of external arginine, ornithine, or citrulline

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Abstract. Arginase, ornithine carbamoyl transferase (OCT) and arginine deiminase activities were found in cell-free extracts of *Nostoc* PCC 73102, a free-living cyanobacterium originally isolated from the cycad *Macrozamia*. Addition of either arginine, ornithine or citrulline to the growth medium induced significant changes in their in vitro activities. Moreover, growth in darkness, compared to in light, induced higher in vitro activities. The in vitro activities of arginase and arginine deiminase, two catabolic enzymes primarily involved in the breakdown of arginine, increased substantially by a combination of growth in darkness and addition of either arginine, or ornithine, to the growth medium. The most significant effects on the in vitro OCT activities where observed in cells grown with the addition of ornithine. Cells grown in darkness exhibited about 6% of the in vivo nitrogenase activity observed in cells grown in light. However, addition of external carbon (glucose and fructose) to cells grown in darkness resulted in in vivo nitrogenase activity levels similar to, or even higher than, cells grown in light. Growth with high in vivo nitrogenase activity or in darkness with the addition of external carbon, resulted in repressed levels of in vitro arginase and arginine deiminase activities. It is suggested that nitrogen starvation induces a mobilization of the stored nitrogen, internal release of the amino compound arginine, and an induction of two catabolic enzymes arginase and arginine deiminase. A similar and even more pronunced induction can be observed by addition of external arginine to the growth medium

Key words: Arginase – Arginine deiminase – Cyanobacterium – Nitrogenase activity – *Nostoc* – Ornithine carbamoyl transferase

Many cyanobacteria are able to carry out biological nitrogen fixation, an energy requering process in which atmospheric N_2 is fixed into ammonia (Postgate 1987; Sprent and Sprent 1990; Fay 1992; Lindblad and Guerrero 1993). The ammonia is then incorporated into amino compounds, primarily through the enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT) (Stewart et al. 1975; Guerrero and Lara 1987; Ohmori and Ohmori 1990; Sprent and Sprent 1990). Excess of nitrogen might be stored as cyanophycin, a copolymer of the amino compounds aspartate and arginine in 1 : 1 ratio (Simon 1976; Allen 1988). Arginine is formed by combining ornithine and carbamoyl phosphate (CP) into citrulline by ornithine carbamoyl transferase (OCT) (Fig. 1), before the citrulline is further converted to argininosuccinate and arginine by argininosuccinate synthetase and argininosuccinate lyase, respectively (Fig. 1) (Holm-Hansen and Brown 1963; Hood et al. 1969; Lehninger 1982; Carr 1983; Schubert and Boland 1990; Ohmori and Ohmori 1990). CP is synthesized from NH_4^+ or glutamine, CO_2/HCO_3^- and ATP by carbamoyl phosphate synthetase (CPS) (Fig. 1). Earlier studies showed the presence of both CPS and OCT in the filamentous heterocystous cyanobacterium *Nostoc* PCC 73102 (Lindblad 1989, 1992; Jansson et al. 1993). Immunolocalization and transmission electron microscopy demonstrated a cellular localization to both the nitrogen-fixing heterocysts and the photosynthetic vegetative cells (Lindblad 1989, 1992). Native-polyacrylamide gel electrophoresis in combination with in situ activity stain revealed an in vitro active OCT with a molecular mass of approximately 80 kDa (Lindblad 1992). Moreover, addition of external arginine to the growth medium induced not only a higher in vitro OCT activity but also a second in vitro active OCT with a molecular mass of about 118 kDa (Jansson et al. 1993).

Cyanophycin is metabolized into the two amino compounds arginine and aspartate (Gupta and Carr 1981a, b; Carr 1983; Mackerras et al. 1990a, b). In the filamentous nonheterocystous cyanobacterium *Oscillatoria chalybea* this catabolism has been suggested to be via an arginine-mediated induction (Bednarz and Schmid 1991). Arginase and arginine deiminase are the two enzymes primarely involved in this catabilism. Arginase hydrolizes arginine to ornithine and urea, while arginine deiminase catalyze the direct

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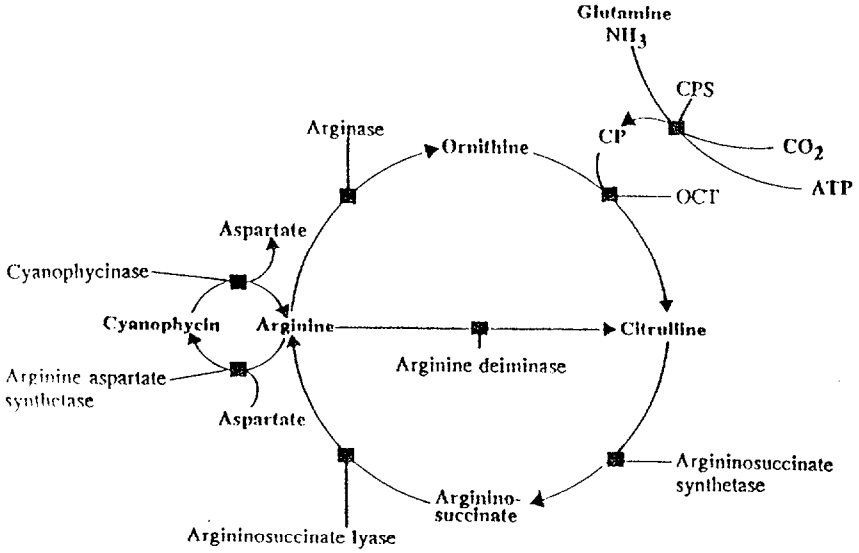

Fig. 1. Schematic presentation of the ornithine cycle in cyanobacteria capable of forming cyanophycin granules. *CP*, Carbamoyl phosphate; *CPS*, carbamoyl phosphate synthetase; *OCT*, ornithine carbamoyl transferase

conversion of arginine into citrulline (Fig. 1) (Hood and Carr 1971; Weathers et al. 1978; Carr 1983; Lehninger 1982; Schubert and Boland 1990).

The present work continues our characterization of the ornithine cycle in the cyanobacterium *Nostoc* PCC 73102. We demonstrate that certain amino compounds of the cycle are important in regulating the key enzymes arginase, OCT and arginine deiminase.

Material and methods

Organism and growth condition

Nostoc sp. strain PCC 73102 [American Type Culture Collection (ATCC) 29133], a free-living filamentous heterocystous cyanobacterium originally isolated from the cycad *Macrozamia*, was obtained from the Pasteur Culture Collection (PCC), Paris, France (Rippka et al. 1979). *Nostoc* PCC 73102, the reference strain for the genus *Nostoc* in the PCC classification, is a facultative photoheterotroph able to use glucose, fructose and/or ribose as carbon source(s) (Rippka et al. 1979). Axenic cultures were maintained in $BG11_0$-medium (Stanier et al. 1971), at 25 °C, using magnetic stirrers to continuously homogenize the cell suspensions. The experimental cells were transferred to, final volume, 30 ml $BG11_0$-N [$BG11_0$-medium + 10 mM Hepes-NaOH (pH 7.5)] in 100 ml E-flasks and placed shaking in light [Thorn Polylux 4000, and Osram Warmton Warm White (400–700 nm), providing 90 $\mu mol\text{-}m^{-2}s^{-1}$ at the surface of the flasks] or in darkness, without or with the addition of external amino compounds (5 mM of ornithine, citrulline or arginine) and/or carbon (60 mM glucose, 60 mM fructose, or a combination of 30mM glucose and 30mM fructose). Growth in darkness was achieved by wrapping the E-flasks with aluminium foil. Cells were analyzed for in vivo nitrogenase activity (below) before harvested (centrifugation, 3500 × *g*), and kept in liquid nitrogen until being used in the in vitro enzyme activity measurements (below).

Nitrogenase activity

In vivo nitrogenase (EC 1.7.99.2) activity was measured as acetylene dependent ethylene production (Lindblad and Bergman 1986; Lindblad 1992; Lindlad and Guerrero 1993), and expressed per chlorophyll *a* and time. The chlorphyll *a* content was calculated according to Harborne (1973), extracting the cells in 80% (v/v) acetone for 6 to 12 h in darkness before the absorbances at 663 and 645 nm were measured using a Hitachi U-2000 (Tokyo, Japan) spectrophotometer.

In vitro ornithine carbamoyl transferase (OCT) activity

OCT (EC 2.1.3.3) was measured according to a modified method of Boyde and Rahmatullah (1980), based on the colorimetric detection of citrulline using diacetyl monoxime (DAMO, systematic name: 2,3-butanedoine monoxime; see Jansson et al. 1993). The background level (= control) used was a sample without the addition of carbamoyl phosphate. Major modifications of the originally described method (Boyde and Rahmatullah 1980) included: (i) a decrease in relative amount of chromogenic reagent, and (ii) an increase in the incubation time, both in order to increase the sensitivity in detecting the amino compound citrulline.

In vitro arginase and arginine deiminase activity

Cells were broken by sonication (MSE, Ultrasonic Power Unit # 12-63;London,UK) in 10 mM potassium-phosphate buffer (pH 8.0), centrifuged (14.000 × *g*, 4 °C, 1 h) before the supernatants were used in the in vitro assays. The reaction mixture contained 200 mM EPPS [N-(2-hydroxyethyl) piperazine-N'-(3-propanesulfonic acid), pH 8.5], 60 mM L-arginine, and 3 mM $MnCl_2$ in a final volume of 600 µl. The reaction was carried out for 30 min at 30 °C. Then the mixture was deproteinized, and the reaction stopped, by addition of 20 µl of 2 N H_2SO_4, centrifuged for 5 min and the supernatants collected. A sample without the addition of arginine was used as a control (= background level). The acidified reaction mixture was used to determine in vitro arginase (EC 3.5.3.1) and in vitro arginine deiminase (EC 3.5.3.6) activities, respectively.

In vitro arginase activity was measured according to a modified method of Archibald (1944), based on the colorimetric detection of urea using α-isonitrosophenone (INP); 500 µl of the acidified mixture was combined with 536 µl of phosphate sulphuric acid solution (concentrated H_3PO_4, H_2SO_4 and dH_2O in a ratio of 3 : 1 : 1) and 43 µl of INP solution (4 g of INP in 100 ml of 100% ethanol). The samples were boiled at 100 °C for one hour, the tubes allowed to cool down to room temperature (approximately 15 min) before the absorbance at 540 nm was measured. The in vitro arginase activities were then calculated using a standard curve, linear between 0 and 1 µmol of urea. Major modifications of the previously described method (Archibald 1944) included determinations of: (i)

the centrifugation time required for obtaining cell-free extracts, and (ii) the optimal arginine concentration in the reaction mixture.

The in vitro arginine deiminase activity was determined by colorimetric detection of citrulline, combining 100 µl of the acidified reaction mixture with DAMO as described above for the in vitro OCT activity (see also Jansson et al. 1993).

Total amount of protein in the cell-free extracts were calculated using the method described by Peterson (1983), and bovine serum albumin to standardize the assay procedure.

Results

Optimizing the methods for measuring in vitro ornithine carbamoyl transferase (OCT) and arginase activities in Nostoc PCC 73102

By modifying the original method of Boyde and Rahmatullah (1980) it was possible to measure in vitro OCT activities in cell-free extracts of the cyanobacterium *Nostoc* PCC 73102. Highest sensitivity was achieved by decreasing the volume of the chromogenic reagent from 3 ml to 600 µl and mix it with 200 µl of the sample (70 µl cell-free extract, 30 µl reaction mixture, and 100 µl TCA). Moreover, maximum colour development occurred after 30 min of boiling in a water bath at 100 °C, and both shorter or longer boiling times diminished the intensity of the colour. Similarly, several modifications were required to increase the sensitivity of the published method (Archibald 1944) for measuring in vitro arginase activity: (i) a centrifugation time of the sonicated sample for at least 1 h at 14.000 × *g*, and (ii) the optimal colour development occurred at an arginine concentration of 60 mM during the incubation.

Effect of external amino compounds on in vitro arginase, OCT and arginine deiminase activities

Generally, darkness induced higher in vitro activities compared to cells grown in the light (Table 1). The in vitro arginase activity increased substantially in cells grown with the addition of either arginine or ornithine to the growth medium. The highest activity was observed in cells grown in darkness with the addition of arginine followed by cells grown in darkness with the addition of ornithine, 71× and 23× increase compared to cells grown in the light, respectively (Table 1). Addition of citrulline to the growth medium had only minor stimulatory effects on the in vitro arginase activities. Interestingly, very similar effects were observed when assaying in vitro arginine deiminase activities. Again, the highest activity was observed in cells grown in darkness with the addition of arginine followed by cells grown in darkness with the addition of ornithine (Table 1). Darkness with the addition of ornithine induced the highest in vitro OCT activity followed by darkness with the addition of arginine, 12× and 7× the activities observed in cells grown in the light, respectively (Table 1). Addition of citrulline had either no (darkness) or negative (light) effect on the in vitro OCT activities.

In vitro nitrogenase activities

Cells grown in darkness exhibited about 6% of the in vivo nitrogenase activity observed in cells grown in light [18.1 (± 2.0) and 1.1 (± 0.3) nmol $C_2H_4 \cdot mg^{-1}$ (Chl*a*) $\cdot h^{-1}$ (means ± SD, $n = 4$), respectively]. However, addition of external carbon (glucose and/or fructose) greatly stimulated the in vivo nitrogenase activity in darkness (Fig. 2a). When a combination of glucose and fructose was added to cells grown in darkness the levels of in vivo nitrogenase activities reached those obtained for cells grown in the light. This capacity of external carbon to support high in vivo nitrogenase activity in cells grown in darkness remained even after keeping the cells for up to 12 days in darkness before the external carbon was added (Fig. 2b).

Effect of external carbon (glucose and fructose) on in vitro arginase, OCT and arginine deiminase activities

Growth in darkness, i.e. cells having only low in vivo nitrogenase activity (Fig. 2), induced higher in vitro

Table 1. In vitro arginase, ornithine carbamoyl transferase (OCT) and arginine deiminase activities in *Nostoc* PCC 73102. The cells were grown in light or darkness without or with the addition of either external arginine, ornithine or citrulline in the growth medium for 4 days before being analyzed for in vitro enzyme activities. Means ± SD ($n = 3$)

Growth condition[a]	Arginase		OCT		Arginine deiminase	
	nmol product formed · µg^{-1}				(prot) · min^{-1}	
Light	69 ± 5	(100)[b]	64 ± 6[d]	(100)[b]	158 ± 2	(100)[b]
Light + Arg[c]	533 ± 127	(772)	136 ± 23[d]	(212)	1009 ± 53	(639)
Light + Orn[c]	797 ± 257	(1155)	196 ± 56	(306)	1576 ± 144	(997)
Light + Cit[c]	131 ± 3	(190)	26 ± 21	(41)	242 ± 8	(153)
Darkness	198 ± 4	(287)	140 ± 9[d]	(219)	490 ± 7	(310)
Darkness + Arg	4913 ± 92	(7120)	450 ± 19[d]	(703)	5913 ± 18	(3742)
Darkness + Orn	1578 ± 18	(2287)	780 ± 15	(1219)	1862 ± 13	(1178)
Darkness + Cit	270 ± 167	(391)	122 ± 3	(191)	318 ± 9	(201)

[a] Addition of arginine, ornithine or citrulline to light-grown cells reduced cell growth, measured as chlorophyll *a* content, with 6, 15, or 20%, respectively. However, similar additions to cells grown in darkness resulted in a 45, 15, or 16% increase, respectively

[b] % activity compared to cells grown in light without the addition of external arginine, ornithine or citrulline to the growth medium

[c] Arg, Arginine; Orn, Ornithine; Cit, Citrulline

[d] Data from Jansson et al. 1993

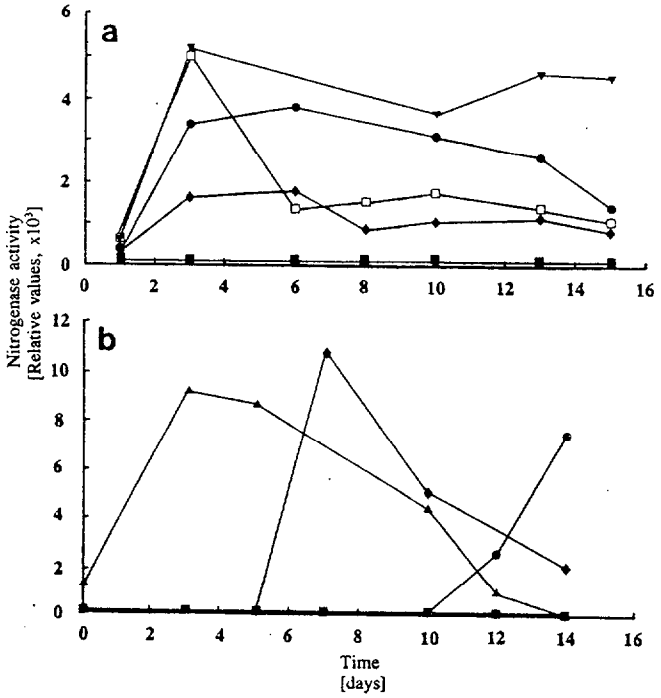

Fig. 2a, b. In vivo nitrogenase activity (acetylene reduction) by the free-living cyanobacterium *Nostoc* PCC 73102.*(a)* The cells were grown in light (□), or darkness without (■; = control, standardized to 100%) or with the addition(s) of external carbon [60mM glucose (◆),60mM fructose (●), a combination of30mM glucose and30mM fructose (▼) to the growth medium *(b)Nostoc* PCC 73102 grown in darkness without (■; = control, standardized to 100%) or with the addition of external carbon (a combination of30mM glucose and30mM fructose) to the growth medium. The external carbon sources were added after 0 (▲), 5 (◆), and 10 (●) days of incubation in darkness. Means [SD did not exceed ± 5% (n = 4)]

arginase activities (Fig. 3a). Cells grown in either light or in darkness with the addition of external carbon, i.e. cells having high in vivo nitrogenase activities (Fig. 2), showed decreased levels of in vitro arginase activities (Fig. 3a). Both light and darkness induced higher in vitro OCT activities, with the highest activities observed in cells grown in darkness without addition of external carbon (Fig. 3b). Interestingly, very similar effects lei were observed for in vitro arginine deiminase (Fig. 3c) as for arginase (Fig. 3a) activities.

Discussion

Arginase, ornithine carbamoyl transferase (OCT) and arginine deiminase were found to be present in cell-free extract of the filamentous heterocystous cyanobacterium *Nostoc* PCC 73102. Addition of either arginine, ornithine, or citrulline to the growth medium induced, with only one exception, higher in vitro arginase, OCT, and arginine deiminase activities. Not only the substrates for the respective enzymes induced higher in vitro activities, but also addition of eg external ornithine induced considerably higher in vitro arginase and arginine deiminase activities. The cells were grown for four days before respective enzymes were measured. During this growth period, external ornithine is rapidly converted to arginine/cyanophycin and induces higher in vitro arginase and arginine deiminase activities (Table 1). However, external citrulline might not be metabolized as quickly as ornithine and will therefore not induce any significant changes in the in vitro arginase, OCT, or arginine deiminase activities (Table 1). These results sharply contrasts with those reported for *Anabaena variabilis* (Hood and Carr 1971) and *Aphanocapsa* PCC 6308 (Weathers et al. 1978), in which almost no alterations in the in vitro enzyme activities where observed when the cells were grown supplemented with arginine. However, in *Oscillatoria chalybea* the presence of arginine, ornithine or citrulline in the growth medium induced higher in vitro arginase activities (Bednarz and Schmid 1991).

Nostoc PCC 73102 is a facultative photoheterotroph (Rippka et al. 1979), with the capacity of high in vivo nitrogenase activity in darkness(Lindblad1992). Nitrogen

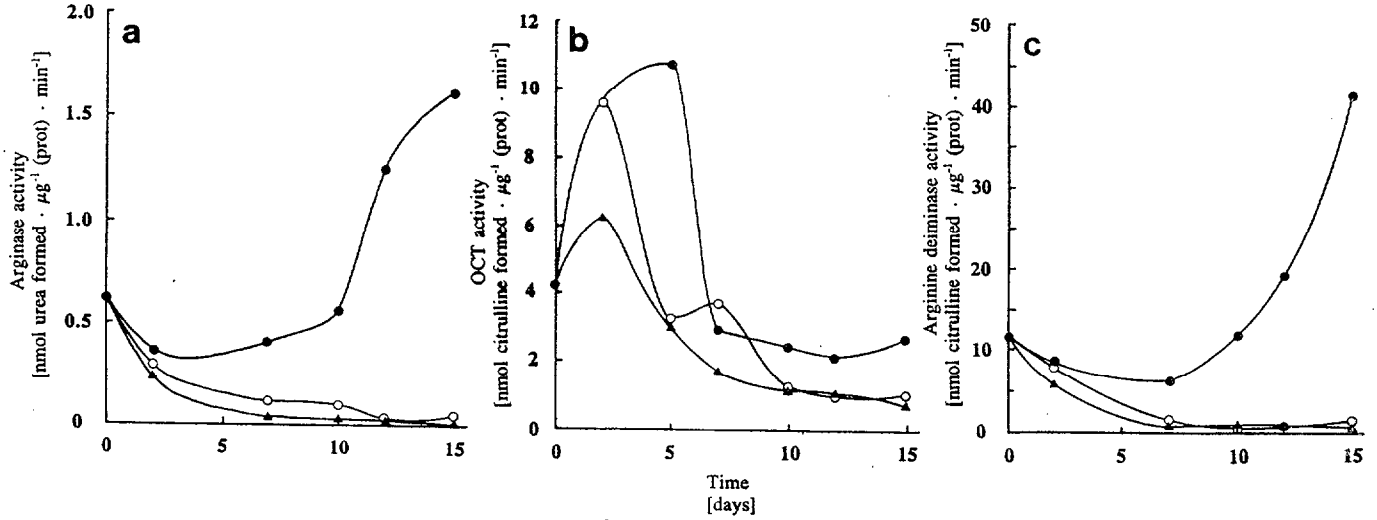

Fig. 3a–c. In vitro arginase *(a)*, ornithine carbamoyl transferase (OCT) *(b)*, and arginine deiminase *(c)* activities in *Nostoc* PCC 73102. The cells were grown in light (○), or darkness without (●) external carbon, or in darkness with (▲) the addition of external carbon (30 mM glucose and 30 mM fructose), before being analized for in vitro enzyme activities. Means [SD did not exceed ± 6% (n = 3)]

might be stored as cyanophycin either in the polar nodules of the heterocysts (Lindblad 1992) or in the cyanophycin granules present in the vegetative cells (not shown). Cells grown in light, or in darkness with the addition of external carbon, showed high in vivo nitrogenase activities (Fig. 2; Lindblad 1992) and decreased levels of both in vitro arginase and arginine deiminase activities (Fig. 3). However, growth in darkness resulted in low in vivo nitrogenase activity (Fig. 2) and increased in vitro arginase and arginine deiminase activities (Fig. 3). External carbon, added as a combination of glucose and fructose to cells growing in darkness, stimulated the in vivo nitrogenase activity and repressed the induction of the catabolic enzymes arginase and arginine deiminase. The external carbohydrates may serve as substrates stimulating the synthesis of both ATP through oxidative phosphorylation and NADPH by glycolysis (Bottomley and Stewart1977;SprentandSprent1990;Steinberg and Meeks 1991; Haselkorn and Buikema 1992), both required for nitrogenase activity in a cell-free system. In light, cyclic photophosphorylation (PSI) in the heterocysts may provide ATP to carry out biological nitrogen fixation (Tel-Or and Stewart 1977; Carr 1983; Sprent and Sprent 1990; Haselkorn and Buikema 1992).

An earlier study (Lindblad 1989) demonstrated that addition or either arginine, ornithine or citrulline to light grown cells *Nostoc* PCC 73102 resulted in decreased levels of in vivo nitrogenase activities. These amino compounds supplied the cells with a nitrogen source, thus reducing the demand(s) for energy requiring biological nitrogen fixation. However, in darkness, the ATP, produced mainly by oxidative phosphorylation (Bottomley and Stewart 1977;Tel-Or and Stewart1977;Houchins 1985;Haselkornand Buikema 1992), is not enough to support high levels of nitrogenase activity. The available nitrogen becomes insufficient to cover the metabolic demands of the cells. Consequently, the internal nitrogen storage compound, cyanophycin, might be mobilized, and, by induction of the catabolic enzymes arginase and arginine deiminase, arginine will be converted to a more available form of nitrogen. In *Anabaena cylindrica* cyanophycin has both a dynamic role in the nitrogen metabolism as well as a storage function (Mackerras et al. 1990a).Moreover, in cells grown in a dark/light cycle, the synthesis of cyanophycin continued in darkness, implying a function as a temporary storage that accumulates in the light for later amino compound synthesis in darkness (Mackerras et al. 1990a).

Addition of external arginine to the growth medium induced higher in vitro OCT activities. Moreover, as described earlier (Jansson et al. 1993), in addition to the previously described 80 kDa enzyme (Lindblad 1992), a second in vitro activeOCTwith a molecularmass of approximately 118 kDa appeared when the cells were grown with the addition of external arginine (Jansson et al. 1993).

In conclusion, arginase, OCT and arginine deiminase are present in cell-free extracts of the filamentous heterocystous cyanobacterium *Nostoc* PCC 73102. Their in vitro activities are siginificantly regulated by the amino compounds arginine, ornithine and citrulline.

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CURRENT MICROBIOLOGY Vol. 26 (1993), pp. 75–78

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Ornithine Cycle in *Nostoc* PCC 73102: Stimulation of In Vitro Ornithine Carbamoyl Transferase Activity by Addition of Arginine

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Abstract. Cells of *Nostoc* PCC 73102, a free-living cyanobacterium originally isolated from the cycad *Macrozamia*, were cultured under different conditions and examined for the presence of *in vitro* active ornithine carbamoyl transferase (OCT). Cells grown in darkness showed a significant increase of *in vitro* OCT activity compared with the activity when grown in light. Addition of external arginine in the growth medium increased *in vitro* OCT activity both in light and in darkness. Moreover, the highest *in vitro* OCT activity was observed in cells grown in darkness and with the addition of external arginine, a sevenfold increase compared with cells grown in light. Native-PAGE in combination with on gel OCT activity stain demonstrated that external arginine induced the presence of two *in vitro* active OCT. In addition to the previously described 80 kDa OCT [Physiol Plant 84:275–282, 1992], a second *in vitro* active enzyme with a molecular weight of approximately 118 kDa appeared. Western immunoblots, with native cell-free extracts and antibodies directed either against native or denatured OCT purified from *Pisum sativum*, confirmed that both enzymes were OCT. Moreover, with a denatured cell-free extract only one polypeptide, with a molecular weight of about 40 kDa, was recognized, indicating that the second *in vitro* active OCT might be a trimer with three identical subunits.

Cyanobacteria can establish symbioses with a number of higher plants [15]. In the *Macrozamia-Nostoc* symbiosis, glutamine and citrulline have been identified as the main N-solutes transported in the xylem from the coralloid root to the rest of the cycad. $^{15}N_2$ experiments demonstrated that both amino compounds were involved in the transfer of fixed nitrogen [13]. Citrulline is synthesized from NH_4^+ or glutamine, CO_2, and ornithine with the two enzymes carbamoyl phosphate synthetase (CPS) and ornithine carbamoyl transferase (OCT, also named ornithine transcarbamoylase; EC 2.1.3.3) [9, 16]. Earlier studies have demonstrated that CPS and OCT are present in cell-free extracts of several filamentous heterocystous cyanobacteria [1, 3, 5, 7, 11]. In light-grown, nitrogen-fixing *Nostoc* PCC 73102, a free-living filamentous cyanobacterium originally isolated from *Macrozamia*, both CPS [10] and OCT [11] have been found to be present in both the nitrogen-fixing heterocysts and in the photosynthetic vegetative cells. Moreover, OCT was shown to be an *in vitro* active enzyme with a molecular weight of approximately 80 kDa and, based on SDS-PAGE/Western immunoblots, suggested to consist of two identical subunits with molecular weights of approximately 38 kDa [11].

OCT can have both an anabolic and a catabolic function. Anabolic OCT, catalyzing the formation of citrulline, is usually a trimeric molecule where the identical subunits have a molecular mass of 35–39 kDa [8, 19]. Catabolic OCT, producing carbamoylphosphate and ornithine from citrulline and inorganic phosphate, is, with only few exceptions, larger than the anabolic OCT and composed of six, eight, nine, or more identical subunits [8, 19]. In bacteria, catabolic OCT can be induced by including arginine in the growth medium [6, 8]. However, no catabolic enzyme could be induced by arginine in the three microalgae *Chlorella autotrophica*, *Chlorella saccharophila*, and *Dunaliella tertiolecta* [6]. These three microalgae all have arginine deiminase activity, where arginine is converted directly to citrulline and ammonium [6].

In the present work we continue our characterization of the citrulline synthesis in the free-living cyanobacterium *Nostoc* PCC 73102. We demon-

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strate that it is possible to induce higher OCT activity as well as a second *in vitro* active OCT by including arginine in the growth medium.

Materials and Methods

Organism and culture condition. Cells of *Nostoc* PCC 73102 (ATCC 29133), a free-living cyanobacterium originally isolated from the cycad *Macrozamia*, were grown in $BG11_0$-medium [18], as described [11]. Experimental cells were grown in $BG11_0$-HEPES ($BG11_0$ containing 10 mM HEPES-NaOH, pH 7.5) without or with the addition of 5 mM arginine. Cells of *Nostoc* PCC 73102 were also grown in $BG11_0$-HEPES with different pH: (i) $BG11_0$-HEPES pH 6.0, (ii) $BG11_0$-HEPES pH 7.0, (iii) $BG11_0$-HEPES pH 8.0, and (iv) $BG11_0$-HEPES pH 9.0.

***In vitro* ornithine carbamoyl transferase (OCT) activity.** OCT (EC 2.1.3.3.) was measured according to a modified method of Boyde and Rahmatulla [2], based on the colorimetric detection of citrulline with diacetyl monoxime (DAMO, systematic name: 2,3-butanedione monoxime). Cells were harvested (3000 *g*, 10 min), broken by sonication (MSE, Ultrasonic Power unit, 12-63, UK) in buffer [200 mM Tris-HCl (pH 8.5) containing 2 mM dithiothreitol] and centrifuged (14,000 *g*, 30 min, 4°C) before the supernatants were used in the assays. The reaction mixture contained 200 mM HEPES-NaOH (pH 8.5), 10 mM L-ornithine, 10 mM carbamoylphosphate-dilithium salt (CP) in a final volume of 0.1 ml. The reaction was carried out at 37°C for 15 min. The mixture was deproteinized and the enzyme reaction stopped by addition of 100 µl of 10% trichloroacetic acid; the mixture was then centrifuged for 5 min and the supernatant collected. A sample without the addition of CP was used as background level (control). The acidified reaction mixture was combined with 400 µl acid-ferric solution (1 *N* H_3PO_4, 9 *N* H_2SO_4, and 5 mM ferric ammonium sulfate) and 200 µl DAMO solution [50 mM DAMO and 20 mM antipyrine (2,3-dimethyl-1-phenyl-3-pyrazolin) added just before use]. The samples were mixed vigorously in a Vortex mixer (KEBO-Lab REAX 2000, Stockholm, Sweden), boiled at 100°C for 30 min, allowed to cool to room temperature before the absorbance was measured at 464 nm with a Hitachi U-2000 spectrophotometer (Tokyo, Japan). The *in vitro* OCT activities were then calculated by use of a standard curve, linear between 0 and 0.25 µmol of citrulline. Total amounts of protein in the cell-free extracts were calculated according to [14] with bovine serum albumin to standardize the assay procedure.

Native-PAGE and *in vitro* staining for OCT activity. The cyanobacterial cells were harvested by centrifugation (2500 *g*, 10 min) and resuspended in OCT-lysis buffer [10 mM HEPES-NaOH, 0.5% (w/v) Triton X-100, 2 mM dithiothreitol (pH 7.4)]. The cell suspension was sonicated (see above) on ice and centrifuged (13,000 *g*, 4°C, 10 min). By using Pharmacia PhastSystem (Pharmacia Biotechnology Ltd., Uppsala, Sweden) native-PAGE (Polyacrylamide gel electrophoresis) was performed on 10–15% gradient gels [11]. Two identical gels were run simultaneously; one stained for total protein profiles with Coomassie blue, and the second one stained for OCT activity by use of the method of Farkas et al. [4], outlined briefly below. The gel is incubated in a reaction mixture containing the substrates for OCT [5 mM ornithine and 15 mM carbamoylphosphate in 270 mM triethanolamine (pH 7.7)] for 10 min at 37°C. Through the activity of OCT, citrulline and inorganic phosphate are produced. The phosphate reacts with a 1 : 1 mixture of 1% nitric acid and 40 mM ammonium molybdate, and phosphomolybdic acid is formed. In the last step phosphomolybdic acid is reduced with 10% ascorbic acid, and the site(s) of the *in vitro* active OCT will appear as a blue band/precipitate. The sizes of the *in vitro* active OCT were calculated with high-molecular-weight markers (Pharmacia) as standards.

Table 1. *In vitro* ornithine carbamoyl transferase (OCT) activity in *Nostoc* PCC 73102. The cyanobacterial cells were grown for 4 days in light or darkness, without or with the addition of external arginine (5 mM) in the growth medium before being assayed for the presence of *in vitro* active OCT

Growth condition	nmol citrulline formed · µg protein^{-1} · min^{-1}	
Light	64 ± 6[a]	(100)[b]
Light + arginine	136 ± 23	(212)
Darkness	140 ± 9	(219)
Darkness + arginine	450 ± 19	(703)

[a] Means ± SE (n = 3).
[b] % activity compared with cells grown in light without the addition of external arginine in the growth medium.

Documentation of OCT activity stain. The on gel visualized OCT activities were viewed on a TV-screen (DAGE-MTI HR1000, USA)/saved on a diskette, by use of a Videocamera (DAGE-MTI CCD72, USA) and a Quantel image processor (Quantel, UK). Recalled images were photographed (Kodak TMAX 100) with a Polaroid FreezeFrame Video Recorder.

SDS-PAGE. The native cell-free extracts were denaturated by adding solubilization buffer [Tris-HCl (pH 8.0), containing 1 mM EDTA, 5% β-mercaptoethanol, 2.5% SDS, and 0.5% Triton X-100)] in a 1 : 1 ratio and were then boiled for 5 min. SDS-PAGE was performed on 10–15% gradient gels by use of Pharmacia PhastSystem [12].

Western immunoblot. The separated cyanobacterial proteins were transferred onto a nitrocellulose membrane before incubation with antibodies directed against either the native enzyme (1 : 400 dilution) or the denatured OCT (1 : 250 dilution) purified from *Pisum sativum* (rabbit-anti-OCT-antiserum) [17]. The recognition of the antibodies with the antigen(s) was visualized with goat-anti-rabbit IgG antibodies conjugated with horseradish peroxidase and chloro-1-naphthol as substrate [11, 12]. The results were documented with a Videocamera, a Quantel image processor, and a Polaroid FreezeFrame Video Recorder as described above.

Results and Discussion

By using a modified colorimetric method it was possible to measure *in vitro* OCT activity in the cyanobacterium *Nostoc* PCC 73102. Cells grown in light showed a value of 64 ± 6 (means ± SE, $n = 3$) nmol citrulline formed · µg protein^{-1} · min^{-1} (Table 1). However, by excluding light for 4 days, the specific

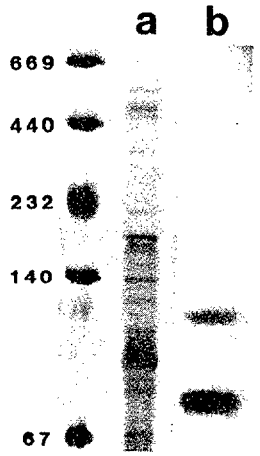

Fig. 1. Nitrogen-fixing *Nostoc* PCC 73102 contains two *in vitro* active OCTs when grown in darkness and with the addition of external arginine in the growth medium. Native-PAGE stained for total protein with Coomassie blue (a), and activity stain of *in vitro* active OCT (b). The two OCTs have molecular weights of approximately 80 and 118 kDa, respectively. High-molecular-weight markers (in kDa) are shown to the left.

in vitro OCT activity doubled. A similar increase was observed when arginine was added to the light-grown cells. Moreover, a combination of darkness and addition of external arginine induced a sevenfold stimulation of the *in vitro* OCT activity (Table 1). Interestingly, when native-PAGE and on gel OCT activity stain were used, two *in vitro* OCTs were present in cells grown with the addition of external arginine in the growth medium. In addition to the previously described 80 kDa enzyme [11], a second, *in vitro* active OCT of approximately 118 kDa appeared (Fig. 1). The 118-kDa enzyme was detectable after 26 h of induction (darkness + arginine) but reached maximal *in vitro* on gel activity after 70 h. Since the induction of the second *in vitro* active OCT was more pronounced in cells grown in darkness, these were chosen for further experiments.

Aspartate carbamoyl transferase (ACT, EC 2.1.3.2) is another enzyme generating inorganic phosphorus from carbamoylphosphate that theoretically could give a positive response with the method used. By replacing ornithine with aspartic acid in the reaction mixture, we examined the presence of *in vitro* active ACT in the cell-free extracts. No activity could be observed, demonstrating that the method used [4] is specific for OCT and that the second enzyme is OCT.

In the bacterium *Aeromonas formicans* OCT is a nonamer under acidic conditions (pH 6.0), but a trimer under more basic pH (8.0) [19]. By growing

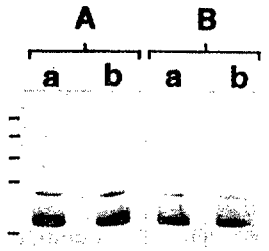

Fig. 2. Western immunoblotting of native cell-free extracts of *Nostoc* PCC 73102 grown in darkness and with the addition of external arginine in the growth medium. Before being visualized, with 4-chloro-1-naphthol as substrate, the cell-free extracts were electroblotted onto nitrocellulose membranes and incubated either with polyclonad rabbit-anti-native OCT antiserum (A) or rabbit-anti-denatured OCT antiserum (B), followed by goat-anti-rabbit IgG conjugated to horseradish peroxidase. (a) and (b) represent two individual cyanobacterial cultures. The positions of high-molecular-weight markers (see Fig. 1) are indicated to the left.

Nostoc PCC 73102 in darkness and in media with different pHs (6, 7, 8, or 9), the direct influence of pH on the presence of *in vitro* active OCT was evaluated. Under all pHs tested, only one *in vitro* active OCT, with a molecular weight of about 80 kDa, could be observed. Thus, the presence of the 118 kDa *in vitro* active enzyme is not an effect of low/high pH, but a response to the amino acid added. In several bacteria a catabolic OCT has been reported to be induced by external addition of arginine [8, 19]. Under the same conditions a second, larger OCT was induced in *Nostoc* PCC 73102. However, compared with other bacterial OCTs [8, 19], the cyanobacterial enzymes are, in general, smaller proteins (Fig. 1) [1, 11].

Native-PAGE/Western immunoblots and antibodies directed against either native or denatured OCT purified from *Pisum sativum* demonstrated recognitions against both the 80- and the 118-kDa enzymes (Fig. 2). This clearly demonstrates that both *in vitro* active enzymes are OCT, and that they share homology on subunit composition. Moreover, with a denatured cell-free extract and SDS-PAGE/Western immunoblots, only one single band, with a molecular weight of approximately 40 kDa, appeared (not shown). The 80-kDa enzyme was earlier suggested to be a dimer with two identical subunits [11], and the present data suggest that the 118-kDa enzyme might be a trimer with three identical subunits.

Nostoc PCC 73102 grown in darkness exhibits no significant nitrogenase activity [11]. Instead, nitrogen might be made available from the storage compound cyanophycin, a multipeptide consisting of arginine and aspartic acid in a 1 : 1 ratio [3]. The

released arginine may be converted either to citrulline directly by arginine deiminase, or to ornithine followed by citrulline by arginase and OCT, respectively. Addition of arginine, externally or by the catabolism of cyanophycin, will induce higher *in vitro* OCT activity (Table 1). Moreover, external arginine also induces a second, larger *in vitro* active OCT (Figs. 1, 2). The physiological function, anabolic/catabolic role, of this second *in vitro* active OCT requires further experiments.

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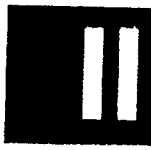

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Osmotic-adjustement in the cyanobacterium *Spirulina platensis:* Presence of an α-glucosidase

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Cell-free extracts of *Spirulina platensis* grown under a hypersaline condition showed substantial levels of in vitro α-D-glucosidase activities. The activity of this enzyme increased upon transfer to hyposaline medium. The increase in *in vitro* activity of the α-D-glucosidase in the down-NaCl-shock cells was prevented by addition of an inhibitor of either RNA biosynthesis (streptomycin or methyl-purine), or protein biosynthesis (chloramphenicol). A possible involvement of the cyanobacterial α-D-glucosidase in the osmotic adjustement of *S. platensis* is discussed.

Additional key words – Cyanobacteria, glycosyl-glycerol, saline stress.

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INTRODUCTION

The utilization of cyanobacteria as a source of biomass and for the extraction of economically important metabolites has received great attention (Dubinsky *et al.*, 1978; Shefel and Soeder, 1980; Aaronson and Dubinsky, 1982; Vonshak, 1990). These organisms occur in environments which show dramatic changes in various environmental parameters such as salinity. Cyanobacteria exposed to salt accumulate osmotically active compounds to generate a positive hydrostatic pressure (Stal and Reed, 1987). The accumulation of various osmolytes makes it possible for the organisms to adjust to different osmotic pressure, the salinity of the medium, growth conditions, temperature and the light/dark conditions (Müller and Wegmann, 1978 *a* and *b;* Setter and Greenway, 1983; Warr *et al.*, 1985 *a* and *b*). Among the most studied cyanobacteria are *Spirulina* spp., a filamentous, non-heterocystous organism which has the unique capacity to grow at high temperature in highly alkaline waters (Ciferri, 1983; Richmond, 1988; Vonshak, 1990). Cultivation of *Spirulina* species in sea-water has become an alternative to improve industrial biomass production. Arid areas where the climatic conditions are favorable for outdoor cultivations and with available sea-water are ideal for massive culture of this halotolerant organisms (Materassi *et al.*, 1984; Tredici *et al.*, 1986). To determine the suitability of sea-water for mass culture of different *Spirulina* species/strains, it is necessary to understand the mechanism(s) of adaptation to high concentrations of NaCl, and its physiological and biochemical effects, on the exposed cells (Materassi *et al.*, 1984; Warr *et al.*, 1985 *a;* Vonshak *et al.*, 1988).

Earlier studies with *S. platensis* have demonstrated an intracellular osmotic adjustement of low-

molecular-weight carbohydrates, synthesis and accumulation of glycosyl-glycerol [*O*-α-D-glucopyranosyl-(1 → 2)-glucerol] and trehalose, in response to high concentrations of NaCl (Warr *et al.*, 1985 *a* and *b*, 1987). Glucosyl-glycerol is a nine carbon heteroside (Kollman *et al.*, 1979), a structural analog to floridoside (galactosyl-glycerol) found in Rhodophyta (Reed *et al.*, 1984). Glucosyl-glycerol, the major osmoticum, is accumulated in proportion to the external salinity (Warr *et al.*, 1985 *a*) When cells of *S. platensis* were subjected to a hyperosmotic shock (transferred from low to high salt concentration) the accumulation of glucosyl-glycerol was accompanied by a decrease in glycogen content. Similarly, a hypo-osmotic shock resulted in a decrease in glucosyl-glycerol content and an increase in the glycogen level, indicating that glucosyl-glycerol/glycogen interconversions may be partially responsible for the changes in glucosyl-glycerol content of *S. platensis* (Warr *et al.*, 1985 *a*).

In red algae it has been demonstrated that the concentration of the osmoticum floridoside (galactosyl-(1 → 2)-glycerol) increased upon exposure to an elevated external osmotic pressure (Kauss, 1968; Kirst and Bisson, 1979; Reed *et al.*, 1980; Reed, 1985). Floridoside is degraded by an α-galactoridase. The activity of this enzyme, regulated at the level of its synthesis, increased when the algae were transferred from hypersaline to hyposaline medium and decreased when it was transferred back to hypersaline medium (Yu and Pedersén, 1990 *a* and *b*).

Isofloridoside (galactosyl-(1 → 1)-glycerol), an isomeric form of floridoside, has been described as the osmolite in the chrysophyte *Poterioochromonas malhamensis*, an unicellular fresh-water flagellate (Dey, 1980). An increase in external concentration of NaCl caused the algal cells to shrink within 1-2 min. However, the cell volume recovered within 2 h. This recovery was accompanied by an increase in the internal level of isofloridoside (Kauss, 1973). Reducing the concentration of NaCl resulted in a rapid decrease in the internal level of isofloridoside (Kauss, 1979). An α-galactosidase isolated from *P. malhamensis* is involved in regulating the level of isofloridoside (Dey and Kauss, 1981). Activity of this α-galactosidase has been shown to be regulated at the transcription level as the addition of a protein synthesis inhibitor abolished the induction of this enzyme under high concentrations of NaCl (Dey, 1980).

We have examined the presence of an α-glucosidase in cell-free extracts of the filamentous halotolerant cyanobacterium *S. platensis*, and determined its responses to hypo-osmotic shock.

MATERIALS AND METHODS

Organism and growth condition. Axenic *Spirulina platensis* Geitler (Sammlung von Algenkulturen, Göttigen Universität, Germany) cells were maintained in 50 ml Erlenmeyer flasks containing 25 ml of Zarrouk medium (Zarrouk, 1966). Experimental cells were culturel in Zarrouk medium supplemented with 1 or 4 % NaCl (Zarrouk medium contains 0.1 % NaCl), on a shaker, at 25°C, and under continuous light [THORN Polylux 4000, and OSRAM Warmton Warm White (400-700 nm), providing 220 μmol m^{-2} s^{-1} at the surface of the flasks]. After 4 days of growth (*i.e.* cells in exponential growth phase), the cultures were harvested and resuspended in fresh Zarrouk medium (*i.e.* 0.1 % NaCl). Samples were taken after 5, 10, 15, 20 and 25 h for the measurements of *in vitro* α-glucosidase activity.

Preparation of cell-free extracts. Cells of *S. platensis* were harvested (glassfilter; Whatman GF/C), and ground in 3 ml chilled (4°C) phosphate buffer [50 mM NaH_2PO_4-NaOH (pH 7.4) containing 14 mM β-mercaptoethanol], at room temperature, using a chilled (4°C) mortar-pestle. After centrifugation (2000 × *g*, 4°C, 10 min) the supernatants were immediately used for *in vitro* α-glucosidase activity measurements.

Analytical determinations. The *in vitro* activity of α-glucosidase (EC 3.2.1.20) was assayed by incubating 150 μl of cell-free extract with 100 μl of 13.5 mM *p*-nitrophenyl-α-D-glucopyranoside, 100 μl of phosphate buffer (see above) and distilled water to a final volume of 540 μl. After incubating at 30°C for 30 min, the reaction was terminated by the addition of 360 μl of 1 M Na_2CO_3. Liberated nitrophenol was measured at 405 nm using a Hitachi U-2000 spectrophotometer. Specific activity of α-glucosidase was expressed as pkat mg^{-1} protein. Total amount of proteins in the cell-free extracts were calculated using the method described by Peterson (1983), and bovine serum albumin as a standard.

Inhibitor studies. Cells of *S. platensis* grown in Zarrouk medium with the addition of 4 % NaCl were harvested and transferred to 25 ml fresh Zarrouk medium (0.1 % NaCl) without or with the addition of either a transcription inhibitor [streptomycin (75 μg ml^{-1}) or methylpurine (250 μg ml^{-1})], or a protein synthesis inhibitor [chloramphenicol (50 μg ml^{-1})]. Samples were then taken for measurements of *in vitro* α-glucosidase activities after 5 h. Chemicals used were from Sigma (USA).

RESULTS

General properties

High *in vitro* activity of α-glucosidase was obtained when the cell-free extract from *S. platensis*

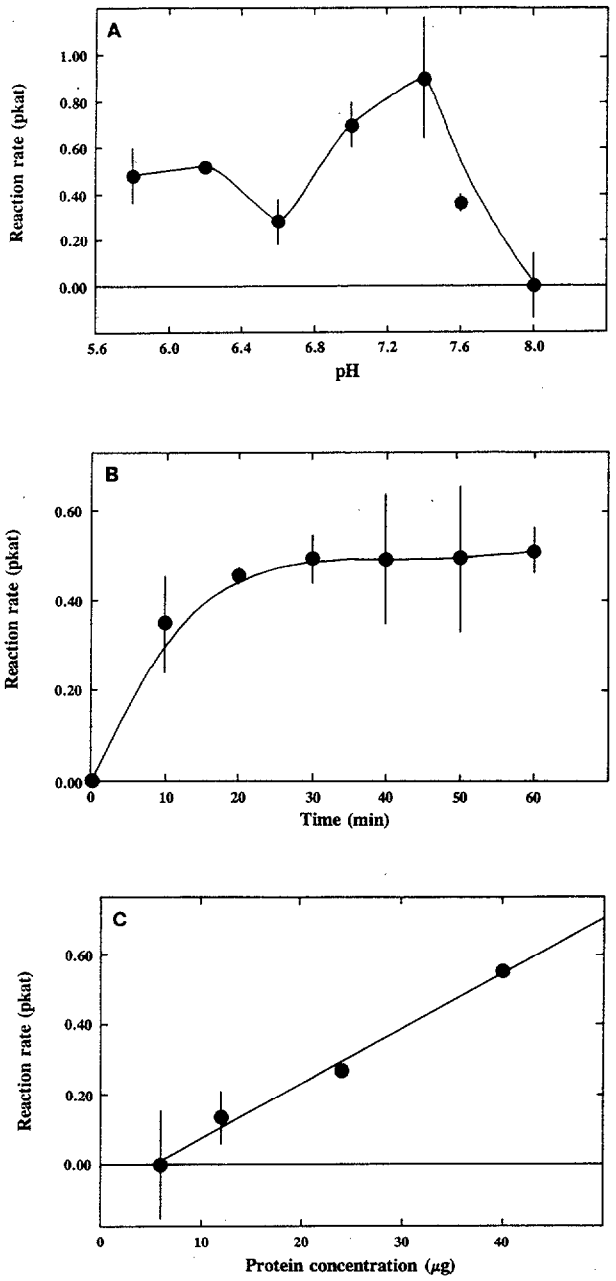

Figure 1. *Effect of pH* **(A)**, *incubation time* **(B)** *and total protein content* **(C)** *on the* in vitro *activity of α-glucosidase in cell-free extracts from* Spirulina platensis *grown in the light.* The results presented in **A** and **B** correspond to two separate cell-free extracts used in each of the pH or time incubations, respectively. Values given are means ± S.D. (n = 4).

was prepared and assayed in a phosphate buffer (50 mM NaH_2PO_4-NaOH). Early experiments using 50 mM Bicine-NaOH (pH 8.2) did not show any appreciable *in vitro* activities (data not shown). The optimal pH for activity of α-D-glucosidase was found to be 7.4 (*fig.* 1 A), and the cell-free extracts could be stored at +4°C for 24 h without losing activity. Figure 1 B shows the activity of α-glucosidase as a function of reaction time at 30°C and a pH of 7.4. Using a phosphate buffer with a pH of 7.4 and incubation time of 30 min, we checked the relation between total amount of protein in the cell-free extract versus the enzyme activity. Ten μg, or less, of total protein content in the *in vitro* assay did not show any detectable level of α-glucosidase activity. However, a linear response was observed when using total protein concentrations between 10 and 40 μg (*fig.* 1 C).

Substrate specificity of α-glucosidase

The enzyme α-glucosidase showed activity only when using *p*-nitrophenyl-α-D-glucopyranoside as the substrate. Alternative substrates, methyl-α-D-glucoside, trehalose, and maltose, did not support any α-glucosidase activity (data not shown).

Effect of down-shock on the *in vitro* enzyme activity

Cell-free extracts of *S. platensis* grown under a hypersaline condition [Zarrouk medium (0.1 % NaCl) supplemented with either 1 or 4 % NaCl] showed detectable levels of *in vitro* α-glucosidase activities. When cells cultured with the addition of 4 % NaCl were transferred to a hyposaline condition [Zarrouk medium (0.1 % NaCl)] the *in vitro* α-glucosidase activity started to increase and reached a maximal level approximately 10 h after the transfer. Thereafter,

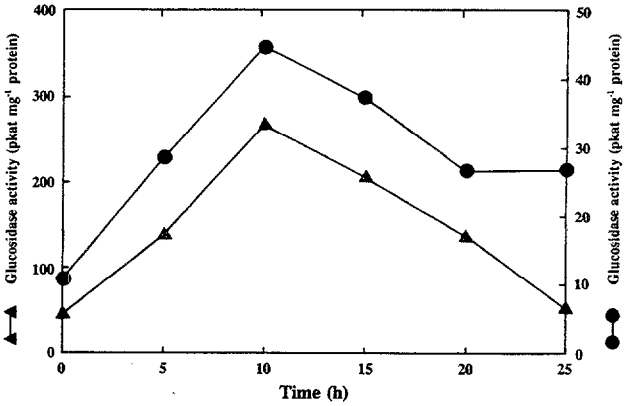

Figure 2. *Effects of down-shock on the* in vitro *activity of α-glucosidase in cell-free extracts from* Spirulina platensis *grown in the light.* At time zero, a culture of *S. platensis* grown at the salinity of 4 % NaCl (▲), or 1 % NaCl (●), was transferred to a medium containing 0.1 % NaCl (Zarrouk medium). Samples were then taken for measurements of *in vitro* α-glucosidase activities during 25 h.

the activity decreased and restored the initial level after 25 h (*fig.* 2). A similar response was observed in cultures down-shocked from 1 % to 0.1 % NaCl. The α-glucosidase activity increased over 10 h of hypo-osmotic shock before starting to decrease and reach a new level after 20 h (*fig.* 2). Considerable higher *in vitro* α-glucosidase activities were found in cells transferred from 4 % NaCl to Zarrouk medium compared with cells transferred from 1 % NaCl to Zarrouk (*fig.* 2).

Effect of inhibitors of RNA and protein biosynthesis on the activity of α-glucosidase

The increase in *in vitro* activity of α-glucosidase in the down-NaCl-shock was prevented by addition of either an inhibitor of RNA biosynthesis (streptomycin or methyl-purine), or an inhibitor of protein biosynthesis (chloramphenicol) (*tab.* 1).

Table 1. *Effect of addition of a transcription inhibitor (streptomycin or methyl-purine), or a protein synthesis inhibitor (chloramphenicol) on the* in vitro *activity of α-glucosidase from* Spirulina platensis *grown in the light.* At time zero, a culture of *S. platensis* grown at the salinity of 4 % NaCl was transferred to medium containing 0.1 % NaCl (Zarrouk medium) without or with the addition of an inhibitor. Samples were then taken for measurements of *in vitro* α-glucosidase activities after 5 h of incubation. Values given, in pkat mg^{-1} protein, are means ± S.D. (n = 3).

Experimental conditions	α-Glucosidase activity *(pkat mg^{-1} protein)*	
Control (no addition)	85.3 ± 0.4	(1204)[a]
+ Streptomycin *(75 μg ml^{-1})*	11.2 ± 0.5	(159)
+ Methyl-purine *(250 μg ml^{-1})*	8.8 ± 0.5	(125)
+ Chloramphenicol *(50 μg ml^{-1})*	0.7 ± 0.06	(10)

[a]Relative activity compared to that observed at time zero. 100 %, 7.1 ± 0.3 pkat mg^{-1} protein (n = 3) corresponds to cultures grown at salinity of 4 % NaCl.

DISCUSSION

This is, to our knowledge, the first report describing the existence of an α-glucosidase in the filamentous halotolerant cyanobacterium *S. platensis*. The enzyme α-glucosidase showed no activity with a variety of glucosidase substrates with the exception of *p*-nitrophenyl-α-D-glucopyranoside. The optimal pH profile showed a peak of 7.4. This contrasts to that of higher plants which lie in the acidic range, pH 4-5 (Dey and Campillo, 1984). However, in agreement with α-glucosidase from other sources (Dey and Campillo, 1984), no cofactors were necessary for the catalytic activity of α-glucosidase from *S. platensis*.

It is known that the intracellular pool of the osmoticum glucosyl-glycerol in *S. platensis* increases at high external osmotic pressure; in response to a hypo-osmotic shock this glucosyl-glycerol content is converted into glycogen (Warr *et al.*, 1985 *a*). Interestingly, higher *in vitro* activities of α-glucosidase were detected in cultures transferred from 4 % NaCl compared to those transferred from 1 %, indicating a correlation between the pool size of glucosyl-glycerol and α-glucosidase activity. Similar results have been found with the red macroalgae *Gracilaria teniuistipitata* and *G. sordida*, when transferred from a medium of 5 % salinity to hyposaline medium of 1 % salinity, the activity of the enzyme α-galactosidase increased steadily over 24 h and thereafter remained constant (Yu and Pedersén, 1990 *a*). In contrast, the activity of α-glucosidase in the cells of *S. platensis* began to decrease rapidly after 10 h of increase, and a new level, close to the initial level of activity, was reached. Our data, and those obtained from the red algae (Yu and Petersén, 1990 *a*), differ from those reported for *Poterioocromonas malhamensis* (Kreuzer and Kauss, 1980). In this microalgae, high activities of α-galactosidase was always present in cells exposed to high salinities.

In *S. platensis*, the increase in α-glucosidase induced by down-shock was prevented by incubating the cells with an inhibitor of either protein or RNA biosynthesis; chloramphenicol, streptomycin or methyl-purine (*tab.* 1). The results suggest that α-glucosidase is a *de novo* synthesed enzyme rather than a pre-existing form(s) which can be either active or inactive. In agreement with this, the osmoregulatory enzyme α-galactosidase in *P. malhamensis* also seems to be regulated, based on inhibitor studies, on the level of its synthesis (Kreuzer and Kauss, 1980). Moreover, the activity of the osmoregulatory enzyme α-galactosidase from the red algae *G. tenuistipitata* and *G. sordida* decreased by addition of either inhibitors of RNA or protein synthesis. However, the responses to theses inhibitors were not immediate (Yu and Pedersén, 1990 *a*).

In conclusion, the present study clearly demonstrates the presence of a functionnal α-glucosidase in the filamentous halotolerant cyanobacterium *S. platensis*. Moreover, a substantial stimulation in *in vitro* activity, regulated on the level of transcription, is observed upon transfer from a hypersaline to a hyposaline condition.

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IV

Ms for *Planta*

Occurrence and immunolocalization of an α-glucan phosphorylase in the cyanobacterium *Spirulina platensis*

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Abstract. Cell-free extracts of the filamentous, non-heterocystous cyanobacterium *Spirulina platensis* were examined for the presence of an α-1,4-glucan phosphorylase (EC 2.4.1.1) by native-PAGE/*in situ* activity stain, SDS-PAGE/ and native-PAGE/Western immunoblots. The cellular and subcellular localization of the cyanobacterial phosphorylase were investigated using immunogold labeling and transmission electron microscopy. Native-PAGE in combination with on-gel phosphorylase activity stain revealed the presence of one *in vitro* active phosphorylase with a molecular mass of approximately 219 kD. Moreover, native-PAGE/Western immunoblot demonstrated that a protein of about 220 kD was immunologically related to a starch phosphorylase purified from the red seaweed *Gracilaria chilensis*. One polypeptide, with a molecular mass of about 47 kD, was recognized under denaturing conditions. Interestingly, immunolocalization showed an intensive label associated with the carboxysomes, about 19 times more phosphorylase-gold particles per carboxysomal cell area compared to in the cytoplasm. However, recalculations of the number of gold particles per cell demonstrated that the carboxysomal fraction of phosphorylase contains only about one third of the total amount of gold-labeled cyanobacterial phosphorylase observed in a *Spirulina platensis* cell.

Key words: Carboxysome - Immunogold localization - *Spirulina* - Starch phosphorylase

Introduction

Cyanobacterial carbon reserves (accumulating in so called glycogen granules) are highly branched polyglucans with predominantly α-1:4 linkages, and with α-1:6 linkages at the branches (Klein and Cronquist 1967; Shively 1988). Although this polymer is termed glycogen, its configuration is identical to the amylopectin fraction of starch found in higher plants (Meeuse 1962), and with the same linkage configurations as floridean starch in Rhodophyta (Klyn 1943; Blinks 1951). It is well established that glycogen is the major carbon and energy reserve in cyanobacteria. It is synthesized during photoautotrophic growth (Smith 1982; Shively 1988) and accumulates in the cells when growth is restricted due to nutrient deficiency, *e.g.* nitrogen or phosphorous starvation (Allen and Smith 1969; Stevens et al. 1981; De Philippis et al. 1992), when grown under suboptimal temperature and irradiation (Ciferri 1983; Van Eykenlenburg 1980), by a sudden increase in energy input, *e.g* a shift from low to high irridiance (Ernst and Böger 1985; Post 1987; De Philippis et al. 1992), or in response to a decrease of external salinity (Warr et al. 1985). Once conditions for balanced growth are re-established, accumulated glycogen is rapidly metabolized to yield energy and carbon for the cell metabolism (Warr et al. 1985; De Philippis et al. 1992). Therefore it has been suggested that glycogen acts as a dynamic reserve with a dual function of both storage product and a buffer substance, separating the process of carbon supply from its utilization in other biosynthetic process (Carr 1988; Shively 1988). Its role in regulation of buoyancy

in strains containing gas vesicles, *e.g. Spirulina platensis*, should also be noted (Kromkamp 1986). The polymer is deposited as granules, termed glycogen granules (Stanier 1988), between the thylakoid membranes in the cytoplasm of the cell (Fay 1983; Shively 1988).

α-Glucan phosphorylase (EC 2.4.1.1) catalyzes the interconversion between α-1,4-glucan and glucose-1-phosphate (Fredrick 1971; Shively 1988). In general, phosphorylases are present in animals (Graves and Wang 1972; Fletterick 1980; Kruger and ap Rees 1983; Nakamura and Imamaura 1983; Pan et al. 1988), higher plants (Steup and Melkonian 1981; Steup 1988), a few species of both macro- and microalgae, including cyanobacteria (Fredrick 1962; Fukui 1983; Steup 1988; Yu and Pedersén 1991a, 1991b). In higher plants, usually two or more isoenzymes are present (Steup and Melkonian 1981; Chang et al. 1987; Preiss 1988; Steup 1988). These differences in the phosphorylases forms may be related to the heterogeneity of the starch molecules and to the structure of the starch granules (Beck and Zeigler 1989). Moreover, substantial evidences exist for inter-species and intra-species differences, and even differences within the same tissue of one individual organism with regard to regulatory properties and subcellular localization(s) of the phosphorylases (Fredrick 1962).

When using cell-free extract of the red alga *Gracilaria chilensis* and native-PAGE/*in vitro* stain, only one band of phosphorylase activity was observed (Yu and Pedersén 1991a, 1991b). The same result was obtained from ten other species of red seaweeds (Yu and Pedersén 1991b). The molecular masses of

these phosphorylases were estimated to be between 200 and 243 kD (Yu and Pedersén 1991a, 1991b). Moreover, using immunogold labeling/TEM this α-1,4-glucan phosphorylase has recently been subcellulary localized around the floridean starch granules in the cytosol of both *G. chilensis* and *G. tenuistipitata* and in the pyrenoid of the green alga *Enteromorpha intestinalis* (Yu et al. 1993).

In the present work we examine the presence of an *in vitro* functional α-glucan phosphorylase, and its cellular/subcellular localization, in the filamentous non-heterocystous cyanobacterium *Spirulina platensis*.

Material and methods

Organism and growth conditions. Spirulina platensis Geitler, a filamentous non-heterocystous cyanobacterium, was obtained from Sammlung von Algenkulturen, Göttigen Universität, Germany. Axenic cells were grown in Zarrouk medium (Zarrouk 1966) as described earlier (Martel et al. 1992).

Native-PolyAcrylamide Gel Electrophoresis (native-PAGE). Cells of *S. platensis* were harvested (Glassfilter, Whatman GF/C, Maidstone, UK), removed from the filter and resuspended in the extraction buffer [50 mM Bicine-NaOH containing 14 mM ß-mercaptoethanol (pH 8.25)]. The cell suspension was sonicated (20 MHz, 1.8 A; MSE, Ultrasonic Power Unit, 12-63, London, UK) for 3 x 1 min on ice and centrifuged (13,000 xg, 4°C, 30 min). Native-PAGE was performed on 10-15% gradient gels using a PhastSystem (Pharmacia Biotechnology Ltd., Uppsala, Sweden; see Lindblad 1992). Two gels were run; one stained for total protein using Coomassie brilliant blue, and the other stained for *in vitro* phosphorylase activity as detailed below.

Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE). Cells of *S. platensis* were harvested (see above), denatured by adding solubilisation buffer [Tris-HCl (pH 8.0), containing 1 mM EDTA, 5% ß-mercaptoethanol, 2.5% SDS and 5% Triton X-100)] in a 1:1 ratio and boiled for 5 min. SDS-PAGE was performed on 10-15% gradient gels by using a PhastSystem (Lindblad and Sellstedt 1991; Lindblad 1992).

In vitro staining for phosphorylase activity. Following native-PAGE, the gel was rinsed with chilled (4°C) 0.1 M

citrate-NaOH (pH 6.0) for 8 min, and then incubated at 30°C overnight in a reaction mixture containing 0.1% (w/v) soluble starch (Merck, Germany) and 20 mM glucose-1-phosphate (Sigma, USA) in 0.1 M citrate-NaOH (pH 6.0). Active of phosphorylase is revealed as white starch bands, which stain blue with a I_2-solution (10 mM I_2 in 14 mM KI, stored at 4°C). The size of *in vitro* active phosphorylase was calculated with high molecular mass markers (Pharmacia) as standards.

Western immunoblot. The separated cyanobacterial proteins/polypeptides were transferred onto a nitrocellulose membrane, before being incubated overnight with primary antibodies directed against phosphorylase purified from *Gracilaria chilensis* (rabbit-anti-phosphorylase antiserum [1:400 dilution, incubation time 3 h; Yu and Pedersén 1991a]. The recognition of the antibodies with the antigen was visualized using goat-anti-rabbit IgG conjugated with horseradish peroxidase and 4-chloro-1-naphtol as substrate (see Lindblad and Sellstedt 1991; Lindblad 1992).

Documentation of phosphorylase activity stain and Western immunoblot. The on-gel visualized and phosphorylases were viewed on a TV-screen (DAGE-MTI HR 1000, USA)/saved on diskette, by using a Videocamera (DAGE-MTI CCD72, USA) and Cristal image processor (Quantel, Newbury, UK). Recalled images were photographed (Kodak T_{MAX}-100) with a Polaroid FreezeFrame Video Recorder.

Immunogold labeling. Fixation, embedding, sectioning and immunogold labeling were all performed as described earlier

(Lindblad and Sellstedt 1991), using polyclonal rabbit-anti-phosphorylase antiserum [1:400 dilution, incubation time 3 h; Yu and Pedersén 1991a] and a secondary IgG [goat-anti-rabbit IgG, 1:20 dilution, incubation time 1 h; BioCell, Cardiff, UK] conjugated to 5nm colloidal gold particles.

Transmission electron microscopy (TEM). TEM was performed using a Philips CM10 electron microscope operating at 60 kV.

Relative quantification by image analysis. Quantification of phosphorylase-gold labeling was performed using the particle analysis of a Crystal image processor as detailed previously (Lindblad and Sellstedt 1991).

Volume estimations. Total cell and carboxysomal volumes were calculated using a Crystal image processor (see above). The *Spirulina* cells were assumed to be cylinders, and the carboxysomes to be cubic. Numbers, individual and total carboxysomal volumes were determined for all carboxysomes within ten individual *Spirulina platensis* cells.

Results

Detection of in vitro functional phosphorylase using native polyacrylamide gel electrophoresis (native-PAGE) and in vitro activity stain. Phosphorylase activity was detected in the cell-free extracts of *Spirulina platensis* using native-PAGE in combination with *in vitro* activity stain (Fig. 1). A single band, with a molecular mass of approximately 219 ± 7 kD (mean ± SD, n=3) appeared, demonstrating the presence of an *in vitro* active and functional phosphorylase enzyme (Fig. 1B).

Specificity of phosphorylase antibodies. The specificity of polyclonal rabbit-anti-phosphorylase antiserum was examined by native-PAGE/ and SDS-PAGE/Western immunoblotting. Electrophoresed and blotted cell-free extracts of *S. platensis* were incubated with rabbit-anti-phosphorylase antiserum and visualized using a secondary antibody conjugated to horseradish peroxidase. Single bands with molecular masses of approximately 220 (Fig. 1C) and 47 (Fig. 2) were recognized for native-PAGE/ and SDS-PAGE/Western immunoblots, respectively.

Cellular/subcellular localization of phosphorylase protein. Treating thin sections with polyclonal rabbit-anti-phosphorylase antisera and secondary IgG conjugated with 5 nm colloidal particles, resulted in labeling of all *S. platensis* cells (Fig. 3A). The labeling intensity appeared to be higher in the carboxysomes compared to the rest of the cells (Fig. 3B).

Relative quantification of the phosphorylase-gold labeling. Phosphorylase-gold labeling intensities were quantified using a digital image analyzer. The labeling associated with the carboxysomes was calculated to be 690 ± 105 (mean ± SD) gold particles μm^{-2} compared to 36 ± 14 (means ± SD) in the rest of the cell (Tab. 1). The relative volumes of individual cells as well as the numbers and volumes of the individual carboxysomes were estimated from the areas measured with image analyzer. From these data the number of gold particles in an individual cell was calculated to be higher in the cytoplasm compared to the carboxysomes (Table 1). Assuming a linear correlation between the number of gold particles and the amount of antigen, this indicates that the majority of the α-glucan phosphorylase is found in the cytoplasm of *Spirulina platensis*, even though the density per cell area is higher in the carboxysomes.

Discussion

The present study clearly demonstrates the presence of an *in vitro* functional α-glucan phosphorylase (EC 2.4.1.1) in the halotolerant cyanobacterium *Spirulina platensis*. Antibodies raised against a starch phosphorylase purified from the red seaweed *Gracilaria chilensis* (Yu and Pedersén 1991a; 1991b), specifically recognized a cyanobacterial protein/polypeptide with a molecular mass of approximately 220 and 47 kD, data obtained using native-PAGE/ and SDS-PAGE/Western immunoblots, respectively. The molecular mass of the cyanobacterial polypeptide appears to be approximately half of that reported for starch phosphorylase in red seaweeds (Yu and Pedersén 1991a; 1991b) and of glycogen phosphorylase in rabbit muscle (Titani et al. 1977). In higher plant, more than two isoenzymes are usually present, while in the cell-free extracts of *S. platensis*, like in red seaweeds (Yu and Pedersén 1991a; 1991b), only one band of phosphorylase activity was observed corresponding to a molecular mass of approximately 219 kD.

The carboxysomes are polyedral bodies mainly composed of proteins (Lang and Whitton 1973; Stewart and Codd 1975). SDS-PAGE analysis revealed the presence of seven to fifteen polypeptides in the cyanobacterial and bacterial carboxysomes (Biederman and Westphal 1979; Lanaras and Codd 1981; Prince et al. 1992). The most abundant are the large and small subunit of Ribulose-bisphosphate carboxylase/oxygenated (Rubisco), the key enzyme of CO_2 fixation via the reductive pentose phosphate pathway (Codd and Marsden 1984; Shively 1988). The identity of

the other carboxysomal polypeptides remains unknown. Recently, a new method for the isolation of carboxysomes was developed, which allowed the identification of a specific and inducible carboxysomal carbonic anhydrase in the unicellular cyanobacterium *Synechococcus* PCC 7942 (Price et al. 1992). The cyanobacterial phosphorylase might correlate with the 43 and/or 47 kD polypeptides, observed on SDS-gels using carboxysomes purified from the cyanobacterium *Chlorogloepsis fritschii* (Lanaras and Codd 1981) and the nitrate-oxidizing bacterium *Nitrobacter agilis* (Bierderman and Westphal 1979), respectively.

By using immunogold labeling technique, we found that the cyanobacterial phosphorylase is located in both the thylakoid area, the site of glycogen accumulation, and in the carboxysomes of *S. platensis* cells. Relative quantifications of the phosphorylase-gold particles revealed about 19 times more phosphorylase per cell area in the carboxysomes compared to in the cytoplasm, the thylakoid area. However, recalculations of relative number of particles per volume revealed that about one third of the phosphorylase was confined to the carboxysomes, the majority being cytoplasmatic. The possibility exists that the non-active form of phosphorylase is located in the carboxysomes, while an active form is found in the cytoplasm; the immunogold labeling technique only detects recognizable antigens, which could be either active or inactive. Moreover, this interpretation assumes a linear correlation between the number of gold-particles and the amount of antigen.

Further studies are needed in order to understand the physiological role of the cyanobacterial starch phosphorylase, and the correlation of its presence in both the carboxysomes and the cytoplasm of the filamentous halotolerant cyanobacterium *Spirulina platensis*.

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T a b l e [1]

Table 1. Relative quantification of phosphorylase-gold labeling in *Spirulina platensis* cells using polyclonal rabbit-anti-phosphorylase antiserum. Values given are number of gold particles μm^{-2} *Spirulina* cell, relative carboxysomal volume, and relative number of gold particles · $cell^{-1}$. Means ± SD (n= 10)

Parameter	Cytoplasm	Carboxysome
Phosphorylase-gold labeling (particles · μm^{-2})	36 (± 14)	690 (± 105)
Gold particles counted	1933	2646
Relative frequencies, gold particles · $area^{-1}$	1 (0.05)	19.2 (1)
Relative volume	1[a] (32.1)	0.03 (1)
Relative frequencies, gold particles · $cell^{-1}$	1.8 (1)	1 (0.5)

[a] 1.0 corresponds to 11.2 μm^3

F i g u r e s l e g e n d s [1 - 3]

Figure 1. Native-PAGE of total proteins (A), *in vitro* phosphorylase activity stain (B), and Western immunoblot (C) of cell-free extracts of *Spirulina platensis*. In (C), the cell-free extracts were electroblotted onto a nitrocellulose membrane and incubated with polyclonal rabbit-anti-phosphorylase antiserum, and goat-anti-rabbit IgG conjugated to horseradish peroxidase before visualized with 4-chloro-1-naphtol. The positions, from the top to the bottom, of high molecular mass markers; thyroglobulin (669 kD), ferretin (440 kD), catalase (232 kD), lactate dehydrogenase (140 kD), and albumin (67 kD), are shown to the left.

Figure 2. SDS-PAGE/Western immunoblot of a denaturing cell-free extracts of *Spirulina platensis*. Details as in Figure 1. The positions, from the top to the bottom, of low molecular mass markers; phosphorylase b (94 kD), albumin (67 kD), ovoalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20.1 kD), and α-lactalbumin (14.4 kD), are indicated to the left.

Figure 3. Immunogold localization of phosphorylase in *Spirulina platensis* grown in Zarrouk medium, using polyclonal rabbit-anti-phosphorylase antiserum and goat-anti-rabbit antiserum conjugated with 5 nm colloidal gold particles before viewed in a Philips CM transmission electron microscope (A). Note the high label associated with the carboxysomes (c) (B). Bars = 1.0 μm.

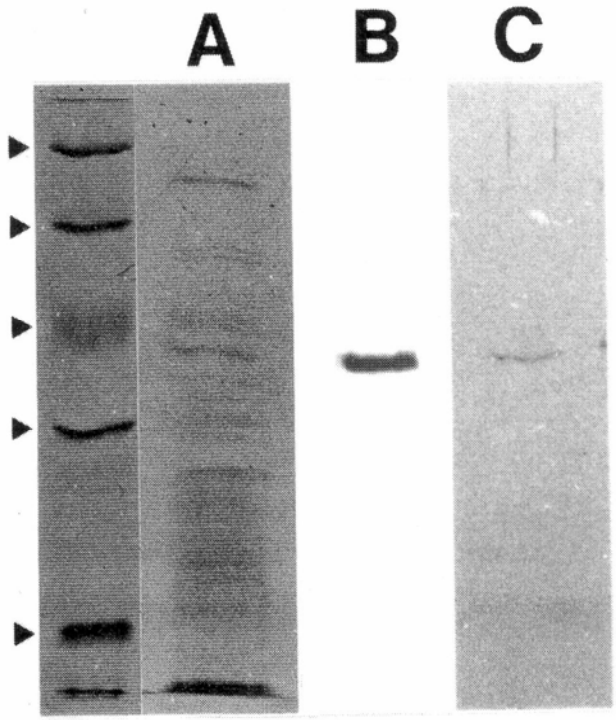

Fig 1

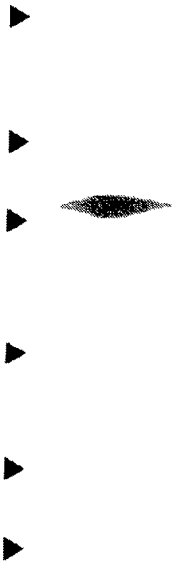

Fig **2**

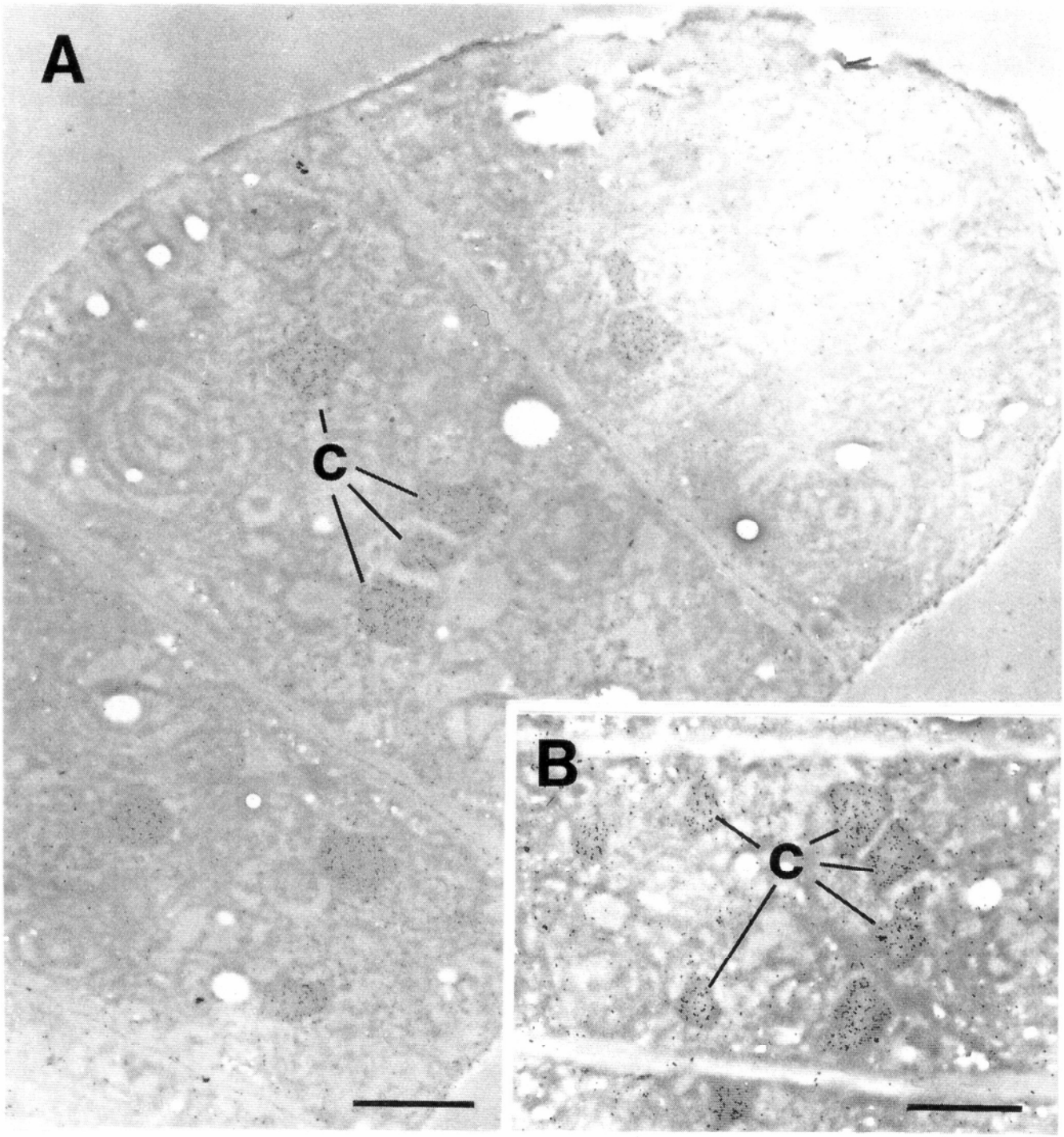

Fig **3**

V

α-Glucan phosphorylase in the unicellular cyanobacteria *Synechocystis* PCC 6803, *Synechococcus* PCC 6301 and *Synechococcus* PCC 7942

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Summary. Cell-free extracts of the unicellular cyanobacteria *Synechocystis* PCC 6803, *Synechococcus* PCC 6301 and *S.* PCC 7942 were examined for the presence of α-1,4-glucan phosphorylase (EC 2.4.1.1). Native-PAGE/*in vitro* activity stain revealed the occurrence of two forms *in Synechocystis* PCC 6803 and *Synechococcus* PCC 6301, and three forms in *Synechococcus* PCC 7942. SDS-PAGE/Western immunoblot demonstrated that a polypeptide of 71 kD, 58 kD and 57 kD for *Synechocystis* PCC 6803, *Synechococcus* PCC 7942 and *S.* PCC 6301, respectively, was immunologically related to a starch phosphorylase purified from the red seaweed *Gracilaria chilensis*. Localization of the α-glucan phosphorylase in thin sections of the unicellular cyanobacteria was performed using immunogold labeling and transmission electron microscopy. The enzyme was localized in both the cytoplasm and in the carboxysomes of both *Synechoccocus* strains. Interestingly, as earlier shown for the filamentous cyanobacterium *Spirulina platensis*, the labeling was more intensive in the carboxysomes compared with the rest of the cell. However, in *Synechocystis* 6803 the enzyme was localized within the thylakoid area and not associated with the carboxysomes.

Keywords: α-Glucan phosphorylase; Carboxysome; Cyanobacteria; Immunogold localization; *Synechococcus*; *Synechocystis*

Introduction

α-Glucan phosphorylase (EC 2.4.1.1) catalyzes the interconversion between α-1,4-glucan and glucose-1-phosphate (Fredrick 1971, Shively 1988). The presence of phosphorylase has been demonstrated in animals (Graves and Wang 1972, Fletterick 1980, Kruger and ap Rees 1983, Nakamura and Imamaura 1983, Pan et al. 1988), higher plants (Steup and Melkonian 1981, Steup 1988) and in a few species of both macro- and microalgae, including cyanobacteria (Fredrick 1962, Fukui 1983, Steup 1988, Yu and Pedersén 1991 a, b, Martel et al. 1993). In green plants, at least two isoenzymes of starch phosphorylase are usually present, one in the chloroplast, where starch is found, another in the cytosol (Gerbrandy and Verleur 1971, Richardson and Matheson 1977, Steup and Melkonian 1981, Shimomura et al. 1982). However, in the cell-free extracts of the filamentous cyanobacterium *Spirulina platensis* (Martel et al. 1993), like in red seaweeds (Yu and Pedersén 1991 a, b), only one active phosphorylase was observed when using native-PAGE/*in vitro* activity stain (Martel et al. 1993).

The different phosphorylase forms might be related to the heterogeneity of the starch molecules and/or to the structure of the starch granules (Beck and Zeigler 1989). Moreover, substantial evidences exist for inter-species and intra-species differences, and even differences within the same tissue of one individual organism with regard to regulatory properties and subcellular localization(s) of the phosphorylases (Fredrick 1962).

Antibodies directed against a starch α-glucan phosphorylase purified from the red seaweed *Gracilaria chilensis* (Yu and Pedersén 1991 a, b), specifically recognized a protein/polypetide in cell-free extracts of *S. platensis* with a molecular mass of approximately 220 and 47 kD in native-PAGE and SDS-PAGE Western immmunoblots, respectiviely (Martel et al. 1993). The molecular mass of the cyanobacterial polypeptide appeared to be approximately half of those reported for starch phosphorylase in red seaweeds (Yu and Pedersén 1991 a, b), higher plants () and of glycogen phosphorylase in animals (Titani et al. 1977).

Using immunogold labeling/TEM, α-glucan phosphorylase was subcellularly localized around the floridean starch granules in the cytosol of red seaweeds, and in the pyrenoid of *Enteromorpha intestinalis* (Yu et al. 1993). However, phosphorylase in filamentous cyanobacterium *S. platensis* was subcellulary localized to both the cytoplasm and the carboxysomes, and not exclusively around the glycogen granules (Martel et al. 1993). The labeling associated with the carboxysomes was about 19 times higher per cell area compared to the cytoplasm. However, recalculations of relative number of particles per volume revealed that approximately one third of the phosphorylase was confined to the carboxysomes, the rest being in the cytoplasm (Martel et al. 1993).

The present communication reports on the occurrence of α-glucan phosphorylase and its isoforms in the unicellular cyanobacteria *Synechocystis* PCC 6803, *Synechococcus* PCC 6301 and *Synechococcus* PCC 7942.

Material and methods

Organisms and growth conditions

The unicellular cyanobacteria *Synechocystis* PCC 6803, *Synechococcus* PCC 6301 (*Anacystis nidulans*; 1402-1 Göttingen Algenkulturen) and *Synechococcus* PCC 7942 (*Synechococcus* R2) were grown in liquid BG11 medium (mineral medium containing combined nitrogen; Rippka et al. 1979), on a shaker, at 25° C, and continious light [THORN Polylux 4000, and OSRAM Warmton Warm White (400-700 nm)] providing 80 μmol $\cdot$ m^{-2} $\cdot$ s^{-1}.

Native-PolyAcrylamide Gel Electrophoresis (native-PAGE)

Cells were harvested by centrifugation (13,000 xg, 10 min) and resuspended in the extraction buffer [10mM HEPES-NaOH, 0.5 % (w/v) Triton X-100, 2 mM dithiothreitol (pH 7.4)]. The cell suspension was sonicated (20 MHz, 1.8 A; MSE, Ultrasonic Power Unit, 12-63, London, UK) for 3 x 1 min on ice and centrifuged (13,000 xg, 4°C, 30 min). Native-PAGE was performed on 10-15% gradient gels using a PhastSystem (Pharmacia Biotechnology Ltd., Uppsala, Sweden; Lindblad 1992). Following native-PAGE, phosphorylase activity was stained based its ability in forming starch in the presence of glucose-1-phosphate and the primer soluble starch. Controls were performed by omitting either glucose-1-phosphate or the primer soluble starch.

Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) and Western immunoblot

The native cell-free extracts were denaturated by adding solubilisation buffer [Tris-HCl (pH 8.0), containing 1 mM EDTA, 5% ß-mercaptoethanol, 2.5% SDS and 5% Triton X-100)] in a 1:1 ratio and then boiled for 5 min. SDS-PAGE was performed

on 10-15% gradient gels by using a PhastSystem (Lindblad and Sellstedt 1991).

The separated polypeptides were then used for Western immunoblot analysis as described previously (Martel et al. 1993).

Documentation of phosphorylase activity stain and Western immunoblot

The on-gel visualized phosphorylases were viewed on a TV-screen (DAGE-MTI HR 1000, USA) by using a Videocamera (DAGE-MTI CCD72, USA) and a Crystal image processor (Quantel, Newbury, UK). Images were photographed (Kodak T_{MAX}-100) with a Polaroid FreezeFrame Video Recorder.

Ultrastructural characterization

Cells were prepared for ultrastructural characterization and subsequently visualized by transmission electron microscopy as detailed earlier (Lindblad et al. 1985).

Phosphorylase-immunogold labelling

Fixation, embedding, sectioning and immunogold labeling were all performed as described earlier (Lindblad & Sellstedt 1991, Martel et al. 1993), using polyclonal rabbit-anti-phosphorylase antiserum [1:400 dilution, incubation time 3 h; Yu & Pedersén 1991a] and secondary IgG [goat-anti-rabbit IgG, 1:20 dilution, incubation time 1 h; BioCell, Cardiff, UK] conjugated to 5nm colloidal gold particles.

Transmission electron microscopy (TEM)

TEM was performed using a Philips CM10 electron microscope operating at 60 kV.

Results and Discussion

α-Glucan phosphorylase activity was detected in cell-free extracts of the unicellular cyanobacteria *Synechocystis* PCC 6803, *Synechococcus* PCC 6301 and *S.* PCC 7942. Native-PAGE/*in vitro* activity stain (Fig. 1) demonstrated two phosphorylase isoforms in both *Synechocystis* 6803 (96 kD and 123 kD) and *Synechococcus* 7942 (234 kD and 248 kD). However, in cell-free extracts of *Synechococcus* 6301 three forms with molecular masses of approximately 92 kD, 224 kD and 251 kD were observed. Previous studies showed one band of phosphorylase activity in cell-free extracts of the filamentous non-heterocystous cyanobacterium *Spirulina platensis* (Martel et al. 1993) as well as in red seaweeds (Yu and Pedersén 1991 a, b). In higher plants two or more forms are found, *e.g.* in potato nine forms have been detected. This multiplicity of the starch enzymes may be a biochemical response to the heterogeneity of the starch molecule and/or its structure (Beck and Zeigler 1983). Starch composition varies not only owing to the transition between particulate (granular) and soluble forms, but also as a result of alteration in amylase-to-amylopectin ratios (Beck and Zeigler 1983). Hence, different structural types of starch might require different enzyme forms with altered specificity. In addition to substrate specificity and regulatory properties, Beck and Zeigler (1983) suggested that compartmentalization may represent another factor encountered in the investigation of the multiplicity of starch-metabolizing enzymes.

The specificity of the polyclonal rabbit-anti-phosphoryalse antiserum from *Gracilaria chilensis* was examined by SDS-

PAGE/Western immunoblotting. Only one subunit of phosphorylase was recognized. The molecular masses were 71, 58 and 57 kD for *Synechocystis* 6803, *Synechococcus* 7942 and *S.* 6301, respectively (Fig. 2). The molecular masses of unicellular cyanobacterial phosphoryalse polypeptide appeared to be smaller than those detected earlier for green plants (Fukui 1983), red seaweeds (Yu and Pedersén 1991a, 1991b) and glycogen phosphorylase in rabbit muscle (Titani et al. 1977). However, the present polypeptide is larger than the 47 kD species observed in the filamentous cyanobacterium *Spirulina platensis*.

The general ultrastructure of the three unicellular strains are shown in Fig. 3. Cells present a characteristic peripherical arrangement of thylakoids enclosing a central region, the centroplasm (see Stanier 1988). Inclusion granules such as carboxysomes, cyanophycin granules and glycogen granules can be distinguished.

Treating thin sections with polyclonal rabbit-anti-phosphorylase antisera followed by goat-anti-rabbit IgG conjugated with 5 nm colloidal particles, resulted in labeling of all cells. In agreement with earlier studies on *S. platensis* (Martel et al. 1993), the labeling intensity apperared to be higher in the carboxysomes of *Synechococcus* 6301 and *S.* 7942 (Fig. 4). However, in *Synechocystis* 6803 the phosphorylase was localized only in the thylakoid area, the site for glycogen granules, and not in the carboxysomes. Hence, we observed differences with regard to the occurrence and subcellular localization of phosphorylase among different cyanobacteria (Figs. 1, 2, 4; Martel et al. 1993). We suggest

that this might be related to the heterogenity of the molecular and the structure of the glycogen granules within these prokaryotic organisms.

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F i g u r e s l e g e n d s [1-4]

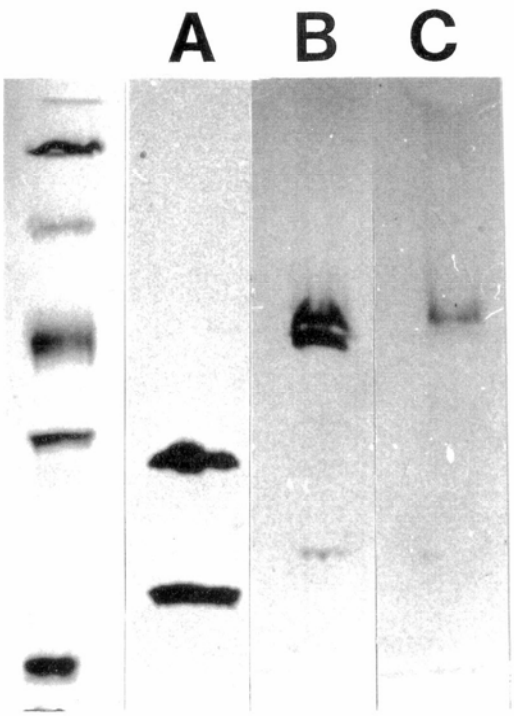
Figure 1. Native-PAGE/*in vitro* phosphorylase activity stain of cell-free extracts of *Synechocystis* PCC 6803 (**A**), *Synechococcus* PCC 6301 (**B**), *Synechococcus* PCC 7942 (**C**). The positions, from the top to the bottom, of high molecular mass markers; thyroglobulin (669 kD), ferretin (440 kD), catalase (232 kD), lactate dehydrogenase (140 kD), and albumin (67 kD), are shown to the left.

Figure 2. SDS-PAGE/Western immunoblot of denaturated cell-free extracts of *Synechocystis* PCC 6803 (**A**), *Synechococcus* PCC 6301 (**B**), *Synechococcus* PCC 7942 (**C**). The positions, from the top to the bottom, of low molecular mass markers; phosphorylase b (94 kD), albumin (67 kD), ovoalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20.1 kD), and α-lactalbumin (14.4 kD), are indicated to the left.

Figure 3. Transmission electron micrographs of *Synechocystis* PCC 6803 (**A**), *Synechococcus* PCC 6301 (**B**), *Synechococcus* PCC 7942 (**C**) grown in BG11 medium. The centroplasm (**cp**), where the carboxysomes (**cb**) are localized, is sorrounded by thylakoid membranes (**th**). Bars = 0.5 μm.

Figure 4. Immunogold localization of phosphorylase in *Synechocystis* PCC 6803 (**A**), *Synechococcus* PCC 6301 (**B**), *Synechococcus* PCC 7942 (**C**). The thin sections were incubated with polyclonal rabbit-anti-phosphorylase antiserum followed by goat-anti-rabbit antiserum conjugated with 5 nm colloidal

gold particles before being viewed in a Philips CM transmission electron microscope. Bars = 1.0 μm.



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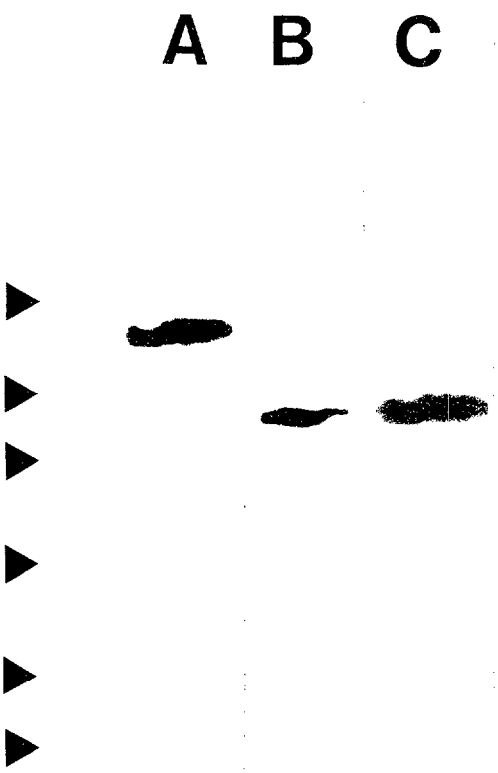
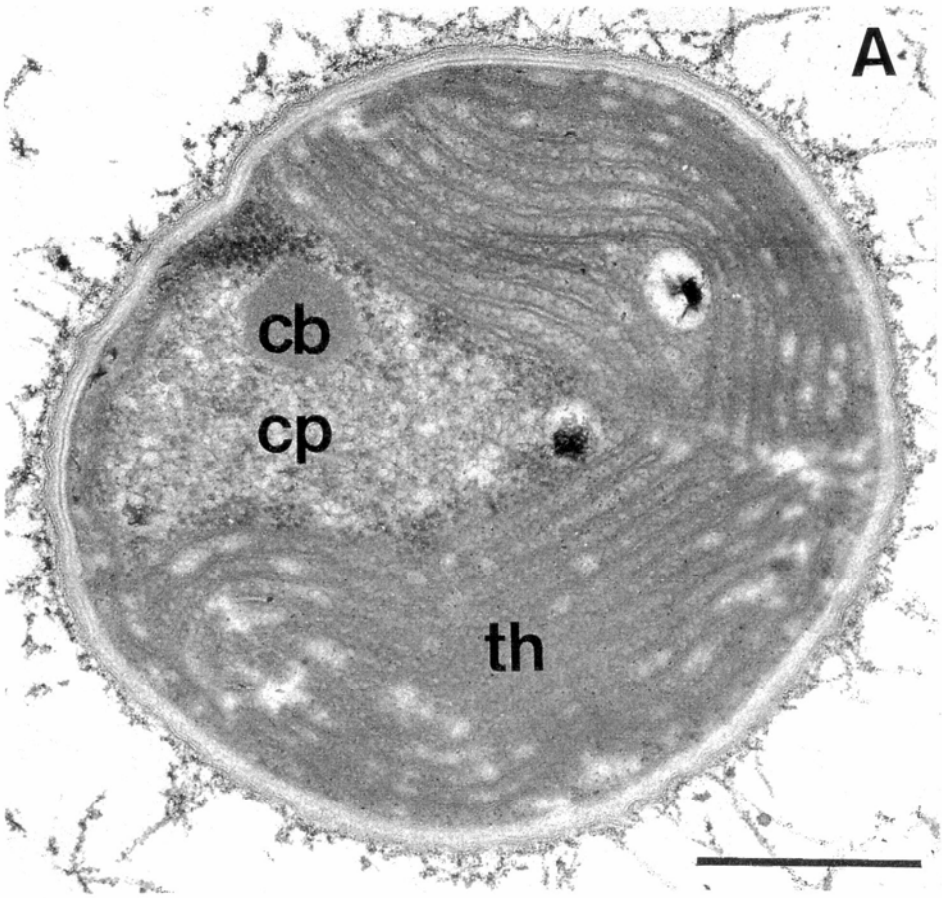
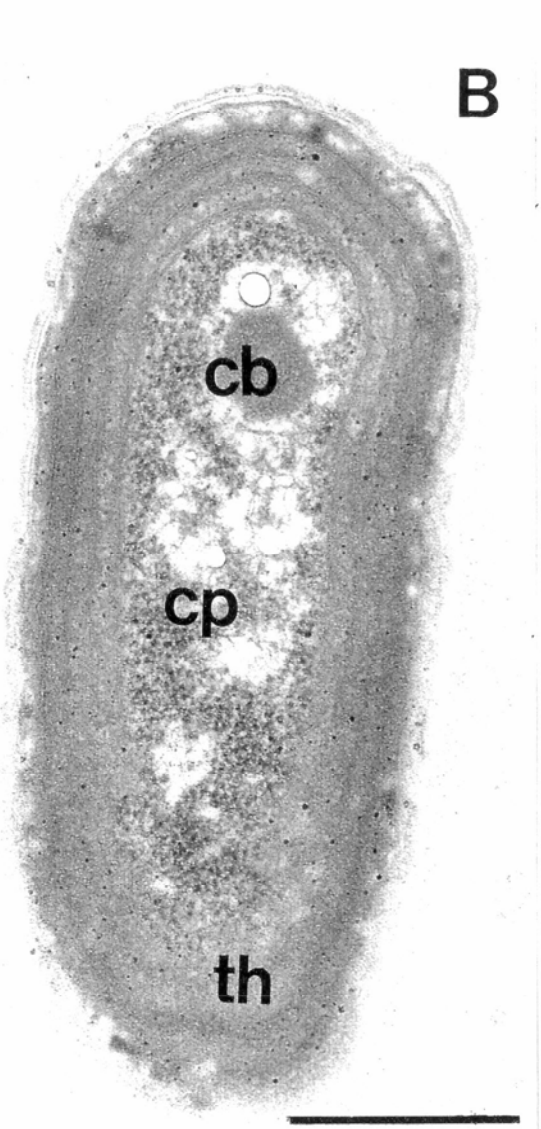


Fig 2

Fig 3





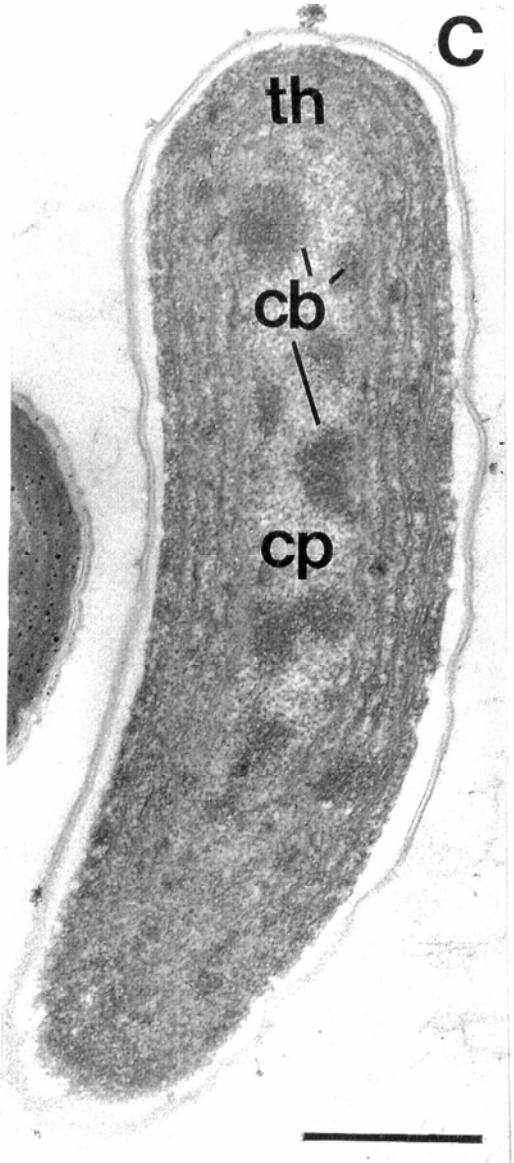


Fig 4

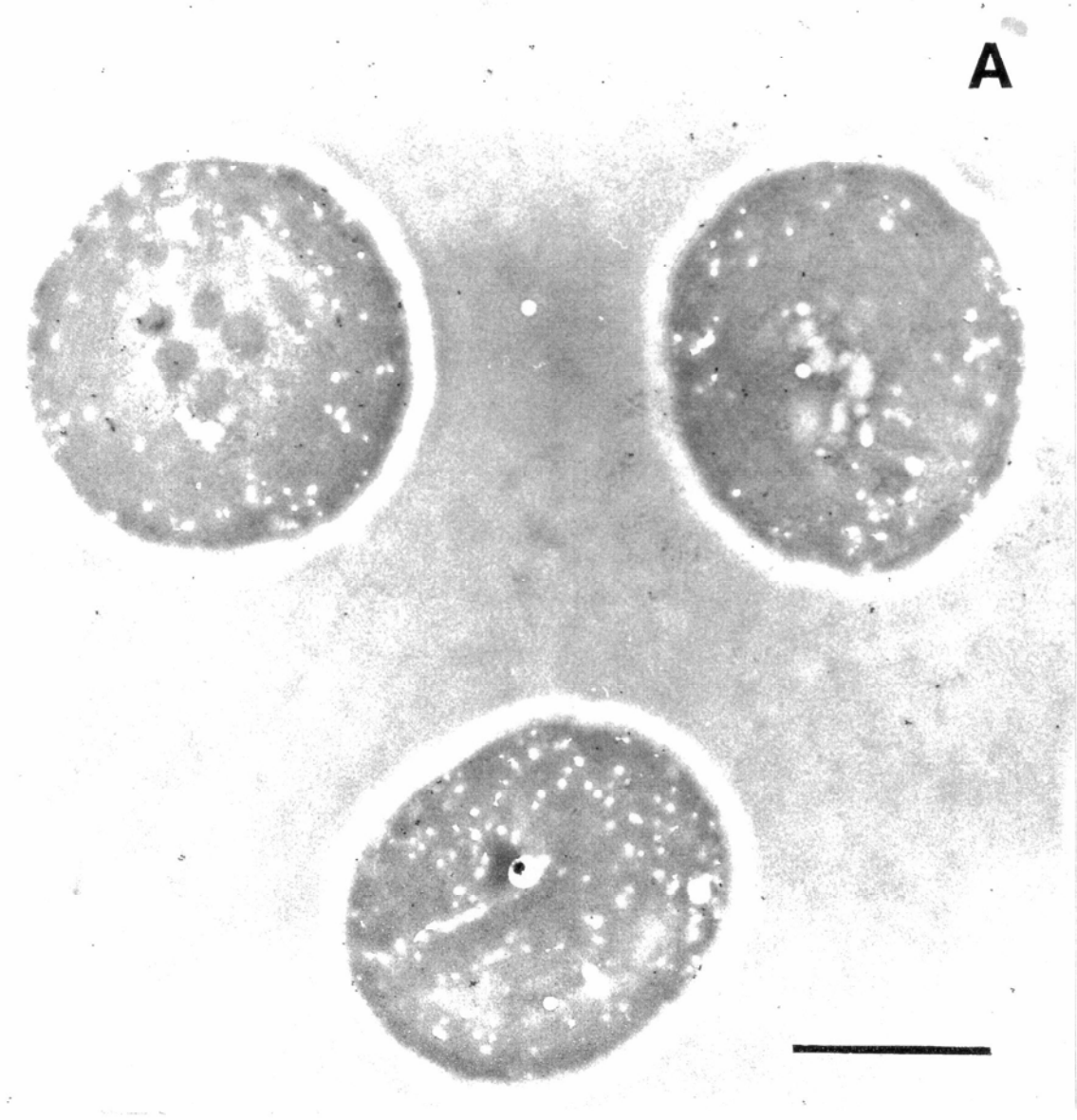


Fig 4