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CENTRO DE INVESTIGACION SOBRE FIJACION DE NITROGENO

GENETICA Y FISIOLOGIA DE LA GLUTAMINO SINTETASA DE *Neurospora crassa* EN AUXOTROFOS DE GLUTAMINA Y EN SUS REVERTANTES.

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PRESENTA

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RESUMEN

En *Neurospora crassa* se han descrito dos polipéptidos (α y β) responsables de la síntesis de glutamina. La expresión de estos polipéptidos esta regulada por la fuente de nitrógeno presente en el medio de cultivo. En cultivos limitados de amonio se encuentra una glutamino sintetasa (GS) tetramérica, formada principalmente por polipéptidos α, en tanto que en exceso de nitrógeno la enzima se encuentra estructurada como un octámero compuesto predominantemente por monómeros β.

Se han aislado una serie de mutantes que requieren glutamina para crecer. Las mutaciones responsables de la auxotrofía por glutamina se encuentran altamente ligadas entre si y probablemente se localizan en el gene estructural para el polipéptido β. Estas mutaciones se pueden dividir en dos tipos: aquellas que causan una disminución en el peso molecular del monómero β, generando un nuevo polipéptido de 30,000 daltones (γ), y aquellas que sin alterar sensiblemente el peso molecular de β, modifican su punto isoeléctrico (β'). Las revertantes aisladas a partir de cualquiera de estos dos tipos de mutantes, solo conservan el polipéptido α de GS perdiendo el monómero alterado γ o β'.

En base a estos datos se ha propuesto un modelo que explica la disminución de la actividad de GS en los auxótrofos de glutamina como resultado de la interacción de los monómeros α normales con los β alterados, formando enzimas hibridas inactivas.

INTRODUCCION

Conceptos iniciales.

La asimilación de nitrógeno es un proceso mediante el cual el nitrógeno del medio ambiente es incorporado al interior de una célula y combinando, de tal manera, que pueda ser usado en la biosíntesis de las moléculas básicas, como aminoácidos, nucleótidos y aminoazucares que a su vez sirven de eslabones en la fabricación de las macromoléculas propias de cada organismo.

En la naturaleza el nitrógeno se encuentra de tres maneras: a) Como nitrógeno molecular en estado gaseoso, en estas condiciones solo es aprovechable por aquellos microorganismos fijadores de nitrógeno que lo reducen a amonio; b) En forma de sales inorgánicas más o menos oxidadas como nitratos y nitritos o altamente reducidas como sales de amonio, este tipo de compuestos son utilizables como fuentes de nitrógeno por un espectro muy amplio de organismos; c) Como nitrógeno orgánico (aminas, amidas, etc.) que son compuestos derivados del metabolismo de seres vivos que para ser asimilados, requieren de sistemas de degradación más o menos específicos. Estos compuestos son biológicamente interconvertibles dando lugar a lo que se denomina el ciclo del nitrógeno (Fig. 1).

Asimilación de nitrógeno en procariotes.

Los microorganismos procariotes pueden usar una gran variedad de compuestos como fuente de nitrógeno. Esta variedad va desde el nitrógeno

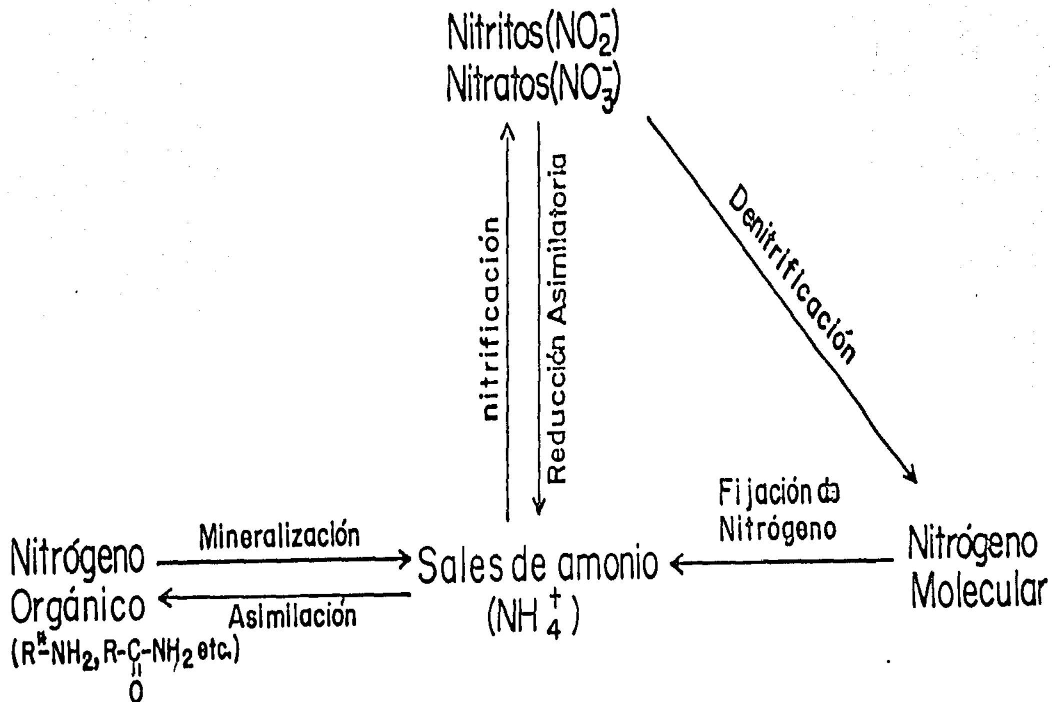

Fig. 1. Ciclo Biológico del Nitrógeno.

R^* esqueletos de carbono.

molecular hasta el nitrógeno presente en aminoácidos, nucleótidos etc. Para que estos compuestos sean asimilados es indispensable su conversión a amonio, de donde se desprende que el amonio es la fuente preferida de nitrógeno por las bacterias. Aún y cuando las capacidades y las maneras de como estos compuestos son usados, varían dependiendo del organismo, en todos ellos los aminoácidos glutamato y glutamina funcionan como los donadores del nitrógeno celular, estos aminoácidos son interconvertibles por una serie de enzimas (Fig. 2) ampliamente distribuídas a lo largo de la escala filogenética.

En *Klebsiella* y *Rhizobium* el amonio es asimilado por la deshidrogenasa glutámica, (L-glutamato:$NADP^+$oxidoreductasa, E.C.1.4.1.4.) o por la glutamino sintetasa (L-glutamato:amonio ligasa, E.C.6.3.1.2.).(84,37). La utilización alternativa de una u otra de estas enzimas, depende de la concentración de amonio presente en el medio. Cuando esta concentración es menor a 0.1 mM ó el amonio proviene enzimáticamente de otro compuesto, la glutamino sintetasa (GS) es la unica enzima con la afinidad necesaria para asimilarlo, esta glutamina es a su vez utilizada como substrato por la glutamato sintasa (L-glutamato:$NADP^+$oxidoreductasa o GOGAT E.C.1.4.13.) para la síntesis de acido glutámico (60), por lo que mutantes que carecen de actividad de GOGAT son incapaces de crecer en presencia de bajas concentraciones de amonio (7).

Por otro lado, en condiciones de exceso de amonio este es asimilado inicialmente por la GDH biosintética (84).

En *E. coli* y *Salmonella* a pesar de que las tres enzimas (GS, GDH y GOGAT) han sido descritas, la regulación de su expresión no se encuentra coordinada como en el caso de *Klebsiella* (84,6).

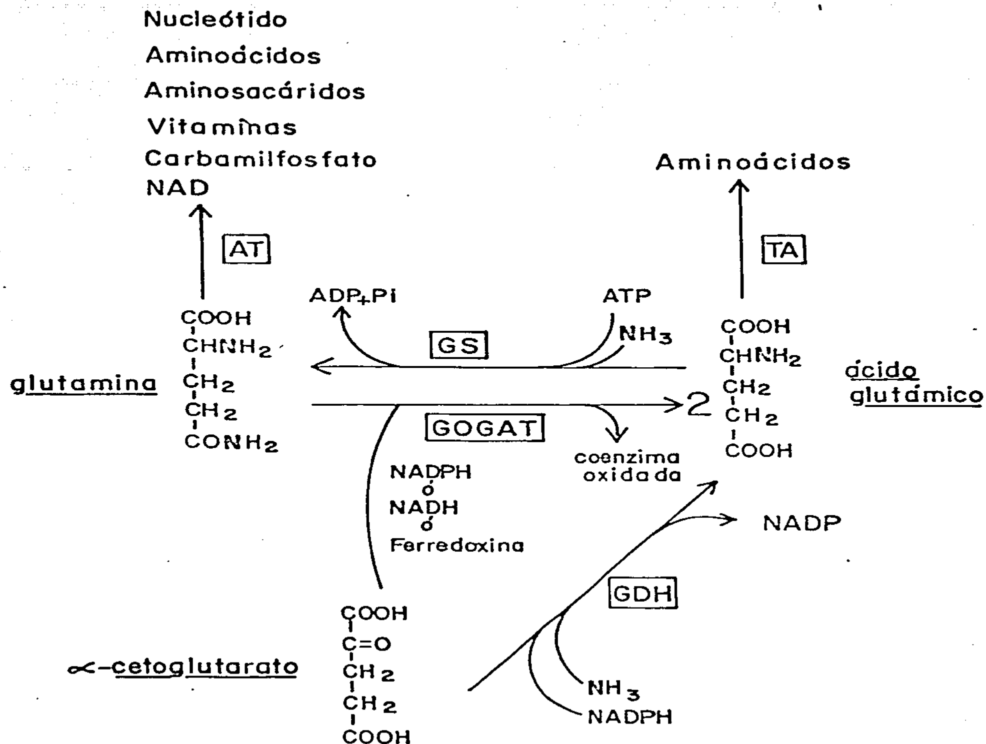

Fig. 2. Asimilación y distribución de nitrógeno.
GDH. deshidrogenasa glutámica, GOGAT- glutamato sintasa
GS. Glutamino sintetasa, AT - amido transferasa, TA- transaminasa

Por último, existen algunos microorganismos, como por ejemplo *Anabaena* y *Bacillus subtidis*, en los cuales la única vía de asimilación de amonio es a través de las enzimas GS y GOGAT (13,16,87).

En enterobacterias se han descrito dos niveles de regulación de GS: a nivel de su actividad y a nivel de su síntesis. La regulación de la actividad enzimática de la GS se lleva a cabo por diversos mecanismos: a) Por la interconversión entre una forma relajada (inactiva) y una forma rigida (activa), mediada por la concentración de los cationes divalentes MG^{2+} y Mn^{2+} (96). b) Por retroinhibición acumulativa por los productos finales del metabolismo de glutamina (26). c) Por un sistema de inactivación por adenilación. La adenilación y desadenilación de GS son catalizadas por la enzima adeniltransferasa (ATasa). Esta enzima cataliza la reacción de adenilación estimulada por la proteína P_{II} no modificada, y la reacción inversa (desadenilación) en presencia de la proteína P_{II} uridilada. La uridilación de la proteína P_{II} se lleva a cabo por la uridiltransferasa (UTasa). Esta enzima es activada por α-cetoglutarato e inhibida por glutamina, por lo que el estado de adenilación de GS es una función de la relación de las concentraciones intracelulares de α-cetoglutarato y glutamina (26).

El sistema que regula la síntesis de la GS en algunas enterobacterias tales como E. *coli*, *K. aerogenes* y *S. typhimurium* es bastante complejo. El sistema *gln* de estas bacterias esta compuesto por los *glnA*, *GlnB*, *glnD*, *glnE*, *glnF*, *glnG* y *glnL*. El gene *glnA* que es el gene estructural para GS, mutaciones en este gene resultan en el fenotipo Gln^- (15,38,56). Este gene está localizado en el minuto 86 del cromosoma de *E. coli* y en las

posiciones equivalentes de los cromosomas de K. *aerogenes* y S. *typhimurium*, en todos los casos está ligado por transducción al operón *rha*.

El gene *glnF*, fué originalmente descrito en S. *typhimurium*, al aislar mutaciones no ligadas a *glnA* que conferian auxotrofia por glutamina. Este gene se encuentra localizado en el minuto 68 del mapa de E. *coli* y está ligado por transducción al gene *argG* (24). Todas las mutantes *GlnF* analizadas hasta la fecha, son incapaces de producir concentraciones altas de glutamino sintetasa y la que producen es insuficiente para crecer en medios sin glutamina. Simultaneamente a la falta de inducción de GS en respuesta a una limitación de nitrógeno, estas cepas tampoco pueden inducir algunas enzimas catabólicas, como histidasa o prolino-oxidasa, (fenotipo Ntr^-). El gene *glnG* fué identificado porque mutaciones en este gene podían suprimir el fenotipo Gln^- causado por mutaciones en el gene *glnF* (39,71). Este tipo de mutaciones resultan en una síntesis baja y constitutiva de GS. Con base en estos datos se propone que los productos de *glnG* y *GlnF* se requieren para activar la expresión del gene *glnA*. Por otro lado, las cepas con mutaciones que causan una ausencia total del producto de *glnG* (por inserción de un transposon) producen un poco más de GS que aquellas con una mutación puntual en *glnG* (57). Esto sugiere que el producto de *glnG* también participa en la represión del gene *glnA*.

El gene *glnB* codifica para la proteina P_{II} (22). Esta proteína participa en el sistema de adenilación-desadenilación de GS. Mutaciones en este gene que impiden la uridilación de P_{II}, además de provocar una adenilación elevada de GS, resultan en una disminución en la concentración de esta enzima.

Por otro lado, mutaciones que causan la pérdida total de P_{II} dan lugar a un fenotipo GlnC (expresión constitutiva de GS), estos datos sugieren que la proteína P_{II} no uridilada participa en la represión del gene *glnA*.

El gene *glnD*, es el gene estructural para la uridiltransferasa (UTasa) (5), mutaciones en este gene elevan el estado de adenilación de la GS y disminuyen su concentración. Ambos efectos parecen estar mediados por un incremento en la concentración de P_{II} a falta de UTasa. La auxotrofia por glutamina causada por las mutaciones en los genes *glnB* o *glnD* son suprimidas por segundas mutaciones que se encuentran altamente ligadas al gene *glnA* y que han sido identificadas en el gene *glnL*. El producto del gene *glnL* junto con el producto del gene *glnG* reprimen la expresión de GS. Pero para que *glnL* pueda participar en esta represión requiere de la presencia de la proteína P_{II} no uridilada (9). Finalmente se ha propuesto que el producto del gene *glnG* participa en la activación de operones catabólicos nitrogenados dado que mutaciones en este gene presentan el fenotipo Ntr^-.

En la familia *Rhizobiacea* se ha descrito la presencia de dos enzimas con actividad de GS (GS_I y GS_{II}) (23). La GS_I es semejante a la que se encuentra en enterobacterias ya que esta sujeta a regulación por adenilación en respuesta a un cambio en la concentración de amonio. La GS_{II} no esta sujeta al sistema de regulación por adenilación pero su síntesis es reprimida en respuesta a un incremento en la concentración de amonio (11). Por otro lado, se tiene evidencia en favor de una relación entre la regulación de la GS y la expresión de la nitrogenasa (50,51) ya que auxótrofos de glutamina de *Rhizobium* "cowpea" 32H1 no sintetizan la nitrogenasa y son incapaces de

nodular (51).

La presencia de dos GS ha sido descrita recientemente en otras bacterias tales como *Bacillus brevis* y *Bacillus caldolyticus* (25,94). De B. *brevis* se han purificado dos enzimas con actividad de GS que difieren en sus propiedades cinéticas. Las enzimas fueron aisladas a partir de 2 difererentes condiciones de cultivo: una en la cual el organismo es capaz de esporular y otra en la que no. Con base en esto, los autores sugieren una relación entre una de las formas de GS y la capacidad de esporulación en este microorganismo.

En el termofilo extremo B. *caldolyticus* también se ha descrito la presencia de dos glutamino sintetasas que difieren en su composición de aminoácidos, en sus propiedades cinéticas y en su espectro de respuesta a retroinhibidores. Es interesante hacer notar que esta regulación por retroinhibición se lleva a cabo de una manera complementaria ya que cada enzima responde solo a una parte de los productos finales del metabolismo de glutamina. La GSE_{I} es mas sensible a inhibición por glicina, alanina y serina, en tanto que la GSE_{II} es inhibida preferentemente por gln, AMP y 5-fosforilribosa-1-pirofosfato. Los autores proponen que la existencia de estas dos GS es una respuesta a la necesidad de mantener la termoestabilidad y la sensibilidad a todo el espectro de retroinhibidores que regulan la actividad de GS (95).

Asimilación de Nitrógeno en Plantas.

La mayoría de las plantas utilizan el nitrato como fuente preferida de nitrógeno, ya que es la forma más abundante en el suelo, inclusive las sales

de amonio, provenientes de fertilizantes, son oxidadas rápidamente a nitratos por los organismos del suelo. Aún y cuando un gran número de plantas son capaces de usar indistintamente amonio o nitratos como fuente de nitrógeno, el amonio no puede ser acumulado, ya que aun pequeñas concentraciones resultan tóxicas para la planta (28). En presencia de fertilizantes amoniacalaes el rendimiento de las plantas depende directamente de la capacidad que tengan para detoxificar este amonio y esta habilidad depende de la disponibilidad de cetoácidos (3,28). A diferencia de lo que sucede con el amonio, el nitrato puede ser acumulado hasta concentraciones relativamente altas sin daño para la planta.

Tanto los nitratos como el nitrógeno molecular (N_2) tienen que ser inicialmente reducidos a amonio, antes de ser incorporados en compuestos orgánicos. El amonio es primordialmente asimilado combinándolo con cetoácidos en forma de nitrógeno amino y amido.

Tradicionalmente se había considerado a la deshidrogenasa glutámica, como la primera enzima de la vía principal para la asimilación de amonio, ya que esta enzima proporcionaba el único mecanismo de alta actividad y amplia distribución para la síntesis de un nitrógeno α-amino a partir de amonio y α-cetoglutarato. La glutamino sintetasa era la única otra enzima que podría asimilar el amonio y que tenía una distribución y actividad equivalente a la de GDH, pero no se conocía ninguna manera de transferir este nitrógeno amido formado durante la asimilación a la posición α-amino para sintetizar aminoácidos.

En 1970 Tempest describe a la enzima GOGAT (60) que en conjunto con la GS permite a las bacterias la asimilación de amonio cuando este compuesto se encuentra en concentraciones limitantes. Posteriormente en plantas

fueron descritas dos GOGATS diferentes: la primera es NADPH dependiente y se encuentra en tejidos no verdes (17); la otra esta presente en hojas y es dependiente de ferrodoxina (42). En la actualidad se ha establecido que existe una amplia distribución de la GOGAT tanto en bacterias como en hongos y plantas. Aún y cuando se han descrito en varias plantas otras enzimas potencialmente capacitadas para incorporar amonio en compuestos orgánicos, hay muy poca evidencia a favor de que jueguen un papel significativo en la asimilación de amonio (62).

En la actualidad se han realizado experimentos tendientes a dilucidar cual es la vía principal de la asimilación de amonio en plantas. En hojas de chícharo alimentadas con nitrato ^{15}N , o con glutamina ^{15}N (amido) ó glutamato ^{15}N (amino), el isótopo se distribuye en proporciones similares, en la poza de aminoácidos libres y en proteínas, independientemente del substrato suministrado (46,62). Esta observación indica que la principal vía de asimilación en plantas es la de GS-GOGAT. Experimentos realizados administrando $^{15}NO_3^-$ viá la corriente transpiratoria de hojas cortadas de *Datura*, demostraron que la glutamina es el principal receptor del nitrógeno fotosintéticamente reducido (47,48). Estudios cinéticos de la aparición de compuestos marcados en raices de plántulas de arroz, muestran a la glutamina como el compuesto que se marca inicialmente, seguido en orden por el glutamato, aspartato y alanina en este orden (97). Finalmente, la cinética de asimilación de nitrógeno a tiempos cortos, se ha realizado usando el isotopo radioactivo ^{13}N en presencia de inhibidores específicos de las enzimas GS y GOGAT. Los resultados obtenidos han generado evidencia que el sistema de asimilación de amonio en nódulos provenientes de la asociación de *Rhizobium*

con raices de leguminosas (62). Es interesante hacer notar que exclusivamente las enzimas de la planta son las que participan en la asimilación del àmonio que proviene de la fijación de nitrógeno, ya que las enzimas del bacteroide se encuentran completamente reprimidas.

La presencia de la actividad de GS se ha descrito en semillas (20), plãntulas (85), hojas (86), raices (34,86) y en nódulos de raíz (59). Existen marcadas diferencias en la actividad presente en cada tejido (43, 86), siendo la actividad específica de GS mas elevada en hoja que en raíz. Este mismo patrón de actividad se observa para la enzima GOGAT. En tejidos verdes la mayor parte de la actividad de GS se encuentra localizada en cloroplasto (91,70), aunque siempre existe un cierto porcentaje en el citoplasma (65). La actividad de GOGAT se encuentra localizada exclusivamente en cloroplasto (91), mientras que la GDH se encuentra predominantemente en mitocondria (66).

Tanto en raíz como en nódulos la GS se encuentra igualmente distribuída en plástidos y citoplasma. La actividad de GOGAT se encuentra asociada a plástidos y la de GDH a mitocondria (62).

Recientemente se han encontrado dos GS presentes en arroz y cebada (27,55). En cebada la GS1 se encuentra distribuída en hojas verdes, raíz, semilla y hojas etioladas, en tanto que la GS2 solo se encuentra en las hojas verdes, por lo que se propone que la GS2 es específica de este tejido. En nódulos de frijol también se han descrito 2 GS. Una de ellas (GSn_1) es semejante a la que se encuentra normalmente en raíz en tanto que la otra (GSn_2) parece se específica de nódulos.

Asimilación de Nitrógeno en Hongos.

La asimilación de nitrógeno ha sido extensamente estudiada en los hongos *Neurospora crassa*, *Aspergillus nidulans* y *Saccharomyces cerevisiae*. En estos tres microorganismos, el metabolismo nitrogenado se encuentra regulado por sistemas de control mas o menos generales (12,61,74,83). No obstante que el amonio, el glutamato y la glutamina son las mejores fuentes del nitrógeno, estos microorganismos son capaces de utilizar una gran variedad de otros compuestos, que incluyen: nitratos, nitritos, aminoácidos, proteínas, purinas, etc. El uso de cualquiera de estas fuentes requiere de la presencia de los sistemas específicos que dan lugar a la formación de amonio. Uno de los objetivos generales al estudiar el metabolismo nitrogenado es encontrar los elementos de control que coordinan la expresión de estos sistemas metabólicos.

Una buena parte de la evidencia que existe acerca de la regulación del metabolismo nitrogenado en estos hongos se deriva del análisis genético de las mutantes estructurales y regulatorias. Este análisis genético deberá ser complementado, siempre que sea posible con estudios bioquímicos y de biología molecular sobre la estructura y función de los productos de los genes bajo estudio, con objeto de definir los genes estructurales y establecer el nivel de acción de genes reguladores. Mutaciones en los genes reguladores pueden causar cualquiera de dos fenotipos: constitutividad (parcial o total) o una disminución (en ocasiones total) de la concentración de la enzima blanco. En la mayoría de los sistemas de regulación positiva (mediados por proteínas activadoras), el tipo de mutantes regulatorias mas frecuentemente halladas son las que suprimen a la enzima regulada. En el

caso de los sistemas de control negativo, las mutantes constitutivas son las más abundantes. Existen ejemplos de ambos sistemas de control en los microorganismos mencionados (33,75,82). Hay mecanismos de control generales que integran la expresión de varias vías y sistemas de control que solo afectan caminos metabólicos específicos. Debido a lo anterior, las mutantes con cambios en la actividad de una enzima, reflejan alteraciones en la expresión de un gene, afectando cualquiera de los siguientes niveles de regulación: a) A nivel de transcripción y en algunos casos procesamiento y transporte del RNA mensajero, b) Traducción, c) Estabilidad del RNA mensajero, d) Maduración de la enzima y e) Recambio de la enzima e inhibición y/o modificación. Con el uso exclusivo del análisis genético no es posible definir en cual de estos niveles ocurrió la alteración.

Un sistema que participa en el proceso de la asimilación del nitrógeno en hongos es la reducción del nitrato a amonio. El nitrato es una excelente fuente de nitrógeno para *Neurospora* y *Aspergillus*. Este compuesto es inicialmente reducido a nitrito por la enzima nitrato reductasa (2) en una reacción donde se transfieren dos electrones. Subsecuentemente el nitrito es reducido a amonio por la nitrito reductasa en una reacción compleja que involucra la transferencia de seis electrones. La síntesis de estas dos enzimas es inducida por nitrato y reprimida por amonio. En *Neurospora* los genes *nit-3* y *nit-6* son los genes estructurales para la nitrato y nitrito reductasa respectivamente (88), en tanto que *niaD* y *niiA*, que se encuentran genéticamente ligados, son los genes equivalente en *Aspergillus nidulans*.

En *Neurospora* se han aislado mutantes regulatorias en el sistema de

asimilación de nitrato. Las mutaciones mapean en uno de dos *loci*: las primeras se encuentran en el gene *nit-4* y solo afectan la expresión de la nitrato y nitrito reductasa (88) en tanto que otras localizadas en el gene *nit-2*, alteran la expresión de estas enzimas y además tienen un efecto pleiotrópico sobre la regulación de otras enzimas del metabolismo nitrogenado (54). La evidencia de que los productos de estos dos genes controlan la transcripción de la nitrato reductasa se desprende de los elegantes experimentos realizados por Premakumar y col. (76).

En *Aspergillus nidulans* el producto del gene *nira*A parece ser un regulador positivo de la transcripción de la nitrato reductasa. Asimismo se ha propuesto que en este microorganismo existe un fenómeno de autoregulación mediante el cual la nitrato reductasa impide su propia síntesis a través de inactivar al producto del gene *nir*A. Solo en presencia del inductor (nitrato en este caso) la nitrato reductasa no se uniría al regulador positivo, procediendo la transcripción del locus *niaD* (8).

Cuando la fuente nitrogenada es amonio y se encuentra en concentraciones relativamente altas (mayor a 1mM) la GDH biosintética es la enzima responsable de su asimilación. En *Neurospora*, *Aspergillus* y *Saccharomyces* existe una segunda GDH (74), dependiente de NAD, que cataboliza el glutamato para liberar amonio. El gene estructural para la GDH-biosintética ha sido identificado tanto en *Neurospora* (*am*), como en *Aspergillus* (*gdh*A) y en *Saccharomyces* (*gdh-1*). El gene estructural para la GDH catabólica solo ha sido identificado en *Aspergillus* (*gdh*B) (52).

De todas las GDH biosintéticas, la de *N. crassa* es la mejor caracterizada. La enzima es un hexámero de subunidades idênticas, cada una con

de 452 aminoácidos, para la cual se conoce su secuencia (30). Con esta enzima se han realizado estudios pioneros en el campo de la regulación alostérica y de la complementación intragéncia (21,36). En tanto que la caracterización de la enzima se encuentra muy avanzada, la regulación de su expresión es limitada. Ambas enzimas parecen estar reguladas por los circuitos nitrogenado e hidrocarbonado. La actividad de la GDH-biosintética de *Neurospora crassa* se encuentra elevada en cultivos que contiene una buena fuente de carbono y amonio o nitrato como fuente de nitrógeno (10,29,35). En cambio, cuando se cultiva en concentraciones bajas de glucosa y una fuente orgánica de nitrógeno, la actividad de GDH-NADPH-dependiente disminuye, en tanto que la actividad de la GDH-NAD-dependiente se incrementa. En *Aspergillus*, mutantes en el gene *gdhB* (GDH-NAD$^-$) crecen muy mal en glutamato y en todos aquellos compuestos que dan este aminoácido como un producto de degradación (35). De lo anterior se desprende que la GDH-biosintética no participa en la degradación de glutamato. De manera similar, estas mutantes tampoco usan estos compuestos como fuente de carbono.

La cepa sin deshidrogenasa biosintética, tanto de *N. crassa* como de *A. nidulans* y *S. cerevisiae*, son capaces de crecer en medios con bajas concentraciones de nitrógeno. Esta capacidad de crecer sin una fuente orgánica de nitrógeno podría deberse a la presencia de la GDH-NAD dependiente, que al invertir su reacción normal fuera capaz de sintetizar glutamato. Sin embargo, el hecho de que esta enzima tenga tan poca afinidad por amonio, que no se encuentre inducida en las condiciones mencionadas y finalmente a que una doble mutante de *Aspergillus gdhA;gdhB* crezca tan bien como la mutante

actividad mediada por modificación química de la enzima. Los resultados experimentales indican que la síntesis *in vivo* en un reflejo directo de la concentración del RNA mensajero específico (73,81), lo que sugiere, que la síntesis de la GS se controla a nivel de la transcripción o del procesamiento del RNA mensajero. Sin embargo, existe cuando menos una condición en donde la actividad de la GS es regulada a un nivel diferente. Cuando *Neurospora* se crece limitada de carbono, la síntesis *de novo* de la GS se reprime completamente y la vida media de la enzima es varias veces menor comparada con la vida media de la enzima cuando la célula se crece en exceso de carbono (69). Se propone que la degradación de la GS durante la limitación en la fuente de carbono sirve para disminuir el gasto de esqueletos de carbono y de ATP en la síntesis de glutamina, cuando la célula esta enfrentando una emergencia energética.

El estado oligomérico de la GS varía en diferentes condiciones de cultivo (49). La cepa silvestre crece en exceso de nitrógeno con una GS octamérica en tanto que en limitación de amonio crece con una GS tetramérica.

Por último, en *Neurospora* existen dos polipéptidos con actividad de GS. Estos polipéptidos, llamados α y β, se encuentran presentes en la cepa silvestre al final de la fase exponencial de crecimiento y la relación entre sus concentraciones varía dependiendo de la calidad de la fuente nitrogenada (79).

Saccharomyces cerevisiae solo presenta una GS. La enzima se ha purificado 130 veces y se ha visto que se encuentra constituída por 10 a 12 monómeros de 43,000 daltones de peso molecular (67). La GS de este microorganismo se encuentra regulada al menos por tres mecanismos diferentes:

a) Represión de su síntesis, mediada por amonio o sus derivados, b) inactivación reversible mediada por glutamina y, c) Degradación proteolítica en respuesta a una deprivación de nitrógeno (44).

En A. *nidulans* la actividad de GS también está regulada por la fuente de nitrógeno:encontrándose la mayor actividad en presencia de glutámico y la menor en glutamina (58).

En conclusión, se puede decir que las enzimas claves en la asimilación de nitrógeno en microorganismos y plantas son la GOGAT, la GDH biosintética y la o las GSs.

Algunos microorganismos utilizan alternativamente las enzimas que sintetizan glutamato (GDH o GAGAT) en respuesta a diferentes condiciones nitrogenadas (*Klebsiella*, *N. crassa*). En cambio en otros ambas se encuentran activas, independientemente de la condición nitrogenada (*E. coli*, *Salmonella*, *Saccharomyces*). Finalmente, algunos organismos biosintetizan glutamato unicamente a través de la GOGAT, ya que carecen de la GDH (*Anabaena*, *B. subtilis*) y otros sintetizan glutamato a traves de la GDH y no utilizan la GOGAT, a pesar de que esta última enzima está presente (nódulos, plantas). La presencia de dos enzimas con actividad de GS ha sido descrita en una serie de organismos (*Rhizobium p*, *B. brevis*, *B. caldolyticus*, *N. crassa*, plantas y nódulos). En los enterobacterias, aún teniendo un solo polipéptido con actividad de GS se puede pensar en dos GS, con propiedades diferentes, en función del estado de adenilación de la enzima.

La descripición de dos enzimas con actividad de GS en un gran número de organismos ha llevado a la generación de una serie de hipótesis para explicar la función fisiológica de estas dos enzimas. Esta función se ha relacionado con eventos celulares muy diferentes, tales como: relaciones

regulatorias con otras vías de asimilación (nif) y capacidad de nodulación en *Rhizobium*, la capacidad de esporulación en B. *brevis*, las propiedades catalíticas complementarias de las enzimas en B. *caldolyticus* y con la expresión diferencial de las enzimas en función de las condiciones nitrogenadas del medio de cultivo en N. *crassa*.

ENFOQUES Y OBJETIVOS

Los enfoques iniciales utilizados para abordar el estudio de la regulación de la asimilación de amonio en *Neurospora crassa*, fueron los siguientes: a) El fisiológico, que trata de establecer la participación de las enzimas en la asimilación de amonio y la regulación que ejerce la fuente de nitrógeno sobre ellas, b) el enfoque bioquímico, que trata de purificar estas enzimas y analizar sus propiedades fisicoquímicas, cinéticas e inmunológicas, y c) el de la biología molecular, que pretende el aislamiento de las moléculas informacionales (RNA mensajero y genes estructurales) de cada una de estas enzimas.

El presente trabajo versa sobre el análisis genético del sistema de asimilación de amonio, centrándose principalmente en el de la glutamino sintetasa de *N. crassa*. Debido a la escases de mutantes auxótrofas de glutamina en eucariotes sencillos; solo una en *Neurospora crassa* (78) y algunas en *Saccharomyces cerevisiae* (18), de las cuales ninguna había sido caracterizada, se decidió aislar un mayor número de estas mutantes en *N. crassa*. El método de selección empleado consistió en aislar cepas que crecían óptimamente en glutamina como fuente de nitrógeno, pero eran incapaces de crecer en glutamto como única fuente de nitrógeno. Para crecer en glutamato como única fuente de nitrógeno, *N. crassa* requiere una alta actividad de GS (90), por lo que las cepas que no crezcan en ese medio podrían tener una alteración en la actividad de GS o en el mecanismo de inducción de la enzima.

Una vez incrementado el número de auxótrofos se inició el análisis genético de los mismos. En primer lugar las cepas *gln*⁻ aisladas, se cruzaron por la cepa silvestre para clonar las mutaciones y simultaneamente determinar el número de locus involucrados en la auxotrofía por glutamina. Con las cepas clonadas, se procedió a hacer un análisis por complementación de la recesividad o dominancia de las mutaciones y cruzas entre las mutantes para determinar la posición relativa de las mutaciones responsables del fenotipo Gln^-.

Las cepas aisladas y clonadas se analizaron en cuanto a su capacidad de crecimiento en medios con diferentes condiciones nitrogenadas desde la deprivación hasta el exceso de nitrógeno. Este tipo de estudio permite conocer si existe una actividad residual de síntesis de glutamina *in vivo*. Para determinar el nivel metabólico del bloqueo, se cuantificaron las pozas intracelulares de substratos y producto de la enzima, en este caso de glutamato y glutamina. La medición de las pozas se realizó en condiciones de crecimiento en exceso de amonio, ya que, si el bloqueo se encuentra en la síntesis de glutamina, se espera un incremento en la poza intracelular de glutamato y un decremento en la poza de glutamina.

El análisis bioquímico de GS incluyó: la medición *in vitro* de la actividad de esta enzima para compararla con la actividad de la enzima de la cepa silvestre, una caracterización inmunologica de la GS de los auxótrofos aislados para determinar si existen alteraciones en el número de recambio de la enzima o si se encuentra alterada la concentración de la misma (80).

Se realizó también un análisis de los estados oligoméricos de la GS

de las mutantes para determinar si alguna de las mutaciones alteraba la estructura cuaternaria de la enzima. Estos estudios estuvieron encaminados a buscar si alguna de las mutaciones se localizaban en el gene estructural de la GS de *Neurospora*.

La presencia en *N. crassa* de dos polipéptidos (α y β) con actividad de GS, nos llevó a buscar la relación entre estos polipéptidos y los estados oligoméricos (tetrámero y octámero) de la enzima. Estos experimentos trataron de definir si existen dos GS distintas en este microorganismo y que papel juega cada una de ellas en las vías de asimilación del amonio.

Finalmente se propuso realizar una caracterización fisicoquímica de los polipéptidos presentes en las cepas Gln^-, por medio de electroforesis en dos dimensiones de los productos sintetizados *in vivo* o *in vitro*, (en un sistema de traducción derivado de reticulocitos) inmunoprecipitados con anticuerpos específicos dirigidos contra la GS de la cepa silvestre. Este análisis se extendió a las cepas revertantes aisladas de estos auxotrofos. Los resultados que se derivaron de este enfoque, permitiron conocer la interrelación entre los monómeros α y β de la GS de *N. crassa* explicando el requerimiento por glutamina de los auxótrofos y el fenotipo Gln^+ de las revertantes.

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Genetics and Physiology of *Neurospora crassa* Glutamine Auxotrophs

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This work reports on the isolation and characterization of two glutamine auxotrophs in *Neurospora crassa*. The mutations responsible for the glutamine-requiring phenotype were very closely linked, and one of them proved to be recessive to wild type. The mutations impaired the conversion of glutamic acid to glutamine and resulted in changes of both the activity and oligomeric structure of the enzyme glutamine synthetase.

Recently, the role of glutamine synthetase (EC 6.3.1.2) in the regulation of nitrogen metabolism as well as in its own regulation has been established in procaryotes by the detailed studies of Magasanik and co-workers (6, 9, 11, 14). The isolation and characterization of glutamine auxotrophs in eucaryotes should help to elucidate whether the enzyme plays a similar role in these organisms. Glutamine auxotrophs have been isolated from two eucaryotes, namely, *Saccharomyces cerevisiae* (2, 3) and *Neurospora crassa* (E. Reich and S. Silagi, Abstr. Proc. Int. Congr. Genet., 11th, Abstr. no. 1, p. 49–50, 1963). However, characterization of the enzyme products of these mutations is lacking.

Studies from our laboratory have established that *N. crassa* accumulates glutamine and arginine as carbon and nitrogen reservoirs and that a metabolic link exists between the synthesis of glutamine and the catabolism of arginine (15; Y. Mora, G. Espín, K. Willms, and J. Mora, J. Gen. Microbiol., in press). In addition, we have purified and partially characterized glutamine synthetase from the wild-type 74-A strain of *N. crassa* (10) and studied the regulation of enzyme concentration (12; I. Vichido, Y. Mora, C. Quinto, R. Palacios, and J. Mora, J. Gen. Microbiol., in press). In this paper we report the isolation and partial characterization of two glutamine auxotrophs from *N. crassa*.

MATERIALS AND METHODS

Stocks. All stocks came from the Fungal Genetics Stock Center at Humboldt State University Foundation, Arcata, Calif. or from the collection of J. Mora. The basic stocks were wild-type strains 74-A and 73-a, glutamine auxotroph *gln-1a* (Reich and Silagi, Abstr. Proc. Int. Congr. Genet., 11th, Abstr. no. 1, p. 49–50, 1963), arginine auxotroph *arg-5*, tryptophan auxotroph *tryp-2*, and proline auxotroph *prol-3*. All double mutants were obtained from appropriate crosses of the mentioned stocks.

Growth conditions. *N. crassa* was grown on liquid minimal medium (N medium) of Vogel (16) supplemented with 1.5% sucrose. Conidia were harvested from slants of N medium supplemented with 1.5% sucrose that had been incubated in the dark for 3 days at 29°C followed by 2 days under incandescent light at 25°C. Cultures were started by inoculating conidia into 2-liter Florence flasks containing 1 liter of N medium. The initial optical density of the culture at 540 nm was 0.05. Incubation was for 12 to 36 h at 25°C with continuous bubbling with hydrated air. Other nitrogen sources in place of or in addition to NH_4NO_3 were employed as indicated in the text.

Fed-batch cultures of *Neurospora* were incubated at 25°C in 6-liter Florence flasks containing 5 liters of N medium without nitrogen source. After inoculation with conidia, the cultures were fed with 9.2 mM NH_4Cl at a dilution rate of 3.2×10^{-3} h^{-1} (7).

Protein determination. Samples of mycelium were collected on membrane filters (Millipore Corp., Bedford, Mass., type HA, 0.45 μm) and washed with 2 volumes of distilled water. The suspended samples were then precipitated in 2 ml of 5% trichloroacetic acid and centrifuged for 5 min at 2,000 rpm, and the pellets were suspended in 1.0 N NaOH. Protein was determined by the method of Lowry et al. (8), using bovine serum albumin as a standard.

Mutagenesis and mutant selection. A conidial suspension (2×10^7) in twice-distilled water was incubated with 1.36 mM N-methyl-N'-nitro-N-nitrosoguanidine (Sigma Chemical Co., St. Louis, Mo.) for 1 h in the dark at 25°C with shaking. Conidia were then concentrated by filtration on a membrane filter (Millipore Corp., type RAWP, 0.47 μm) and washed with cold water. After mutagenesis, conidia were incubated in a 500-ml Erlenmeyer flask with 250 ml of N medium supplemented with 1.5% sucrose and 5 mM glutamate as the sole nitrogen source. The flask was incubated with shaking at 37°C. Every 6 h the entire culture was filtered through cheesecloth into an Erlenmeyer flask containing fresh medium, the process being repeated eight times. The enriched spore population was concentrated by filtration, washed, resuspended in twice-distilled water, and plated on N agar medium supplemented with glucose and fructose (0.05% each) in place

of sucrose and containing 1% sorbose and 1.35 mM glutamine as the sole nitrogen source. Plates were incubated at 29°C, and the colonies that appeared after 2 to 5 days of incubation were transferred to slants of N agar medium supplemented with 1.5% sucrose and 1.36 mM glutamine.

Spot testing, crosses, and progeny analysis were carried out as previously reported (1). Complementation tests were performed in tubes with N medium by incubating conidia of both mutant strains at a final concentration of 5 × 10^4 each per ml for 5 days at 29°C. Mutants were purified by crosses with the wild-type strain.

Glutamate and glutamine pools. Glutamate and glutamine were extracted as described by Vaca and Mora (15) and separated as reported by Ferguson and Simms (4). Both amino acids were measured by an isotopic dilution method after addition of [^{14}C]glutamine and [^{14}C]glutamic acid (New England Nuclear Corp., Boston, Mass.) to the crude extract.

Determination of glutamine synthetase activity. Cell-free extracts were prepared as previously described (10). Glutamine synthetase was measured by its transferase and synthetase activities by the methods of Ferguson and Sims (5). Units of activity were expressed as micromoles of γ-glutamyl hydroxamate produced per minute at 30°C.

Sucrose gradient sedimentation. Samples of 0.5 ml of the cell-free extracts were layered over a 5 to 20% continuous sucrose gradient in either buffer A (5 mM phosphate-0.5 mM ethylenediaminetetraacetic acid-50 mM K_2SO_4 [pH 7.2]) or buffer B (50 mM imidazole-0.5 mM ethylenediaminetetraacetic acid-50 mM K_2SO_4-5 mM 2-mercaptoethanol-80 mM MgCl-50 mM glutamate [pH 7.1]) as indicated and centrifuged for 12 h at 4°C in a Spinco L5-75 ultracentrifuge at 40,000 rpm with a SW40 rotor. After centrifugation, 0.3-ml fractions were collected from the top of the tubes, and glutamine synthetase activity was determined in each fraction. Globular proteins were used as markers of sedimentation as previously described (10).

Purification of in vivo-labeled glutamine synthetase. Batch cultures of the wild-type strain grown on N medium with 5 mM glutamate as the sole nitrogen source received a 60-min pulse of [^{3}H]methionine (New England Nuclear Corp., 1 μCi/ml). From these cultures, glutamine synthetase was purified as previously described (10).

RESULTS

Spot testing, complementation, and allelism of glutamine auxotrophs. Table 1 shows the growth patterns on plates of the two different glutamine auxotrophs. Mutant *gln-1a* was not able to grow on ammonia and/or glutamic acid at 29°C. It grew at 37°C with glutamate as the sole nitrogen source, but not when both glutamate and ammonia were present in the medium; only glutamine was used as a nitrogen source at both temperatures.

TABLE 1. *Growth response of N. crassa glutamine auxotrophs to different nitrogen sources and temperatures*

Strain	Temp (°C)	Growth[a]			
		NH_4NO_3	Glu	NH_4NO_3 + Glu	Gln
74-A	29	+	+	+	+
	37	+	+	+	+
gln 1a	29	−	−	−	+
	37	−	+	−	+
gln 1b	29	−	−	−	+
	37	−	−	−	+

[a] Plates were incubated for 36 h before growth was scored as + (good growth) or − (no growth). Glu and Gln were added at a final concentration of 200 μg/ml, and NH_4NO_3 was added at a final concentration of 25 mM.

The selection procedure used to obtain mutant *gln-1a* is unknown (Reich and Silagi, Abstr. Proc. Int. Congr. Genet., 11th, Abstr. no. 1, p. 49–50, 1963). The mutant strain *gln-1b*, which was selected because of its inability to grow on glutamate at 37°C, also did not utilize ammonia as a nitrogen source at either 29 or 37°C, and only glutamine supported growth.

The recessiveness of the *gln 1b* mutation was established by complementation analysis. Heterocaryons were produced when conidia of the double mutant *gln-1b trp-2* plus conidia of the mutant strain *arg-5* were cultured together in N medium, in which neither strain alone was able to grow. We were not able to perform the complementation analysis of the *gln-1a* strain because of its leakiness after prolonged incubation in N medium.

The mutant *gln-1b* segregated as a monogenic mutation. When this mutant was crossed with the 73-a wild-type strain, dissection of two asci demonstrated that half of the ascospores were glutamine auxotrophs, whereas the remainder were prototrophs. The cross between *gln-1a* and *gln-1b* only gave one prototroph out of 5,000 viable ascospores. Thus, the two mutations map very close to one another and are probably allelic.

Physiological and biochemical characterization of the mutants. As shown in Fig. 1, the mutant strains *gln-1a* and *gln-1b* started growing slowly only after 18 h of incubation in N medium.

The conidia used as the inoculum in these experiments were obtained from slants of N medium in which the NH_4NO_3 was substituted by 10 mM glutamine. A different situation held when conidia were harvested from slants of N medium plus 200 μg of glutamine per ml. In this case, growth started after 6 to 8 h of incubation and at a rate higher than that above (Fig. 1A).

In fed-batch ammonium-limited cultures, the

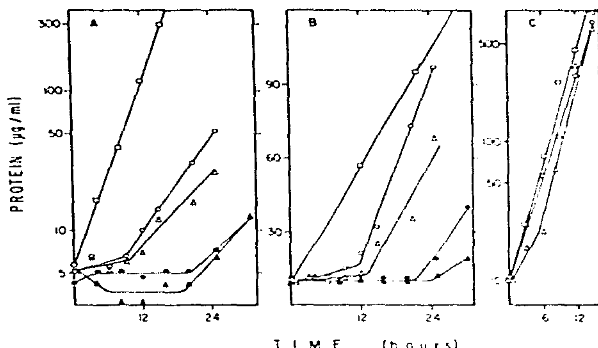

FIG. 1. *Growth curves of wild type (○) and mutants gln 1a (○, ●) and gln 1b (△, ▲) on 25 mM NH_4NO_3 (A), on fed-batch ammonium limited cultures (B), and on 5 mM glutamine (C). Open symbols indicate conidia harvested from slants containing 25 mM NH_4NO_3 plus 1.26 mM glutamine; closed symbols indicate conidia harvested from slants containing 10 mM glutamine as the sole nitrogen source.*

mutants grew linearly after a lag similar to that observed in an excess of ammonium (Fig. 1B). Optimal growth of both mutants was observed when 5 mM glutamine was present as the sole nitrogen source (Fig. 1C).

In the presence of either low or high concentrations of ammonium, as the sole nitrogen source, i.e., under nongrowing conditions, the mutants accumulated a high glutamate pool, indicating a block in the synthesis of glutamine. Accordingly, the glutamine pool was found to be very low under this condition (Table 2). A lowering of the accumulated glutamate was observed after prolonged incubation when the mutants started to grow on ammonium (data not shown). The *prol-3* mutant was included as a control in these experiments to compare the pool size of glutamic acid in a different amino acid auxotroph under nongrowing conditions.

Glutamine synthetase activity as judged by the synthetase assay (see above) was 20- to 30-fold lower than the wild-type activity when the mutants were grown in the presence of glutamine as the sole nitrogen source (Table 3). Enzyme activities, when measured by the transferase assay, were about 10-fold lower in the mutants than in the wild type. The ratios of synthetase to transferase activities were, therefore, lower in the mutants than in the wild type.

Previous studies in our laboratory have established that *N. crassa* glutamine synthetase can exist in two different oligomeric states. When the organism is grown exponentially in the presence of glutamate, glutamine, or ammonia as the sole nitrogen source, the enzyme is found as an octamer (Vichido et al., J. Gen. Microbiol., in press). On the other hand, when limiting ammonia is present as the nitrogen source, the enzyme is found as a tetramer (7).

Instead of the octameric structure of the enzyme present in the wild-type mycelium grown on ammonium excess, the mutants had lower oligomeric forms under similar conditions (Fig.

TABLE 2. *Glutamate and glutamine pools of glutamine auxotrophs under different growth conditions*

Strain	Ammonium condition	Glutamate[a]	Glutamine[a]
74 A	Limiting	0.094	0.036
	Excess	0.170	0.265
gln 1a	Limiting	0.595	0.042
	Excess	0.702	0.029
gln 1b	Limiting	0.424	0.043
	Excess	0.565	0.061
prol 3	Excess	0.170	0.330

[a] Amino acid pools were determined at 12 h after inoculation of conidia in liquid medium and are expressed as micromoles per milligram of extracted protein.

2A). Figure 2B shows the sedimentation profiles of glutamine synthetase from wild-type and mutant strains *gln-1a* and *gln-1b* grown with limited ammonia. Both showed the presence of glutamine synthetase as a tetramer. In addition, the *gln-1b* mutant also showed an oligomeric form corresponding to a dimer. An abnormal oligomeric pattern was also observed in the mutant strains when they were grown on glutamine at 37°C, and extracts were prepared in the presence of glutamate, magnesium, and 2-mercaptoethanol (Fig. 2C). This experiment was carried out at 37°C since growth is optimal and glutamine synthetase activities are higher than those found in cultures grown in glutamine at 25°C. In this case, wild-type glutamine synthetase was present as an octamer, whereas in the mutants the enzyme was found in oligomeric structures of lower molecular weight.

The extracts of mutant strains did not seem to alter significantly the purified octameric form of the wild type as evidenced by the presence of both the mutant tetramer and the wild-type octamer when they were centrifuged together in the same gradient (Fig. 3).

TABLE 3. *Glutamine synthetase activity in glutamine auxotrophs*

Strain	Glutamine synthetase activity[a]		
	S	T	S/T ratio
74-A	0.0200	0.060	0.33
gln 1a	0.0010	0.005	0.20
gln 1b	0.0006	0.006	0.10

[a] Glutamine synthetase specific activity was determined after 12 h of growth at 25°C on glutamine as the sole nitrogen source. Specific activity is expressed as micromoles of γ-glutamyl hydroxamate produced per minute per milligram of protein at 30°C. S, Synthetase activity; T, transferase activity.

DISCUSSION

Of the two glutamine auxotrophs partially characterized, one of them, *gln-1b*, was selected because of its inability to synthesize glutamine at 37°C in the presence of glutamic acid as the sole nitrogen source. The rationale for this was to maintain conditions whereby mutant strains would show a negative phenotype while the wild-type strain has a fully induced glutamine synthetase.

The other mutant strain included in this study, *gln-1a*, isolated some years ago (Reich and Silagi, Abstr. Proc. Int. Congr. Genet., 11th, Abstr. no. 1, p. 40–50, 1963), showed somewhat different phenotypic characteristics. In particular, it was able to grow at 37°C with glutamate as the sole nitrogen source, whereas mutant *gln-1b* was not.

The low frequency of prototrophs obtained after crossing the two mutant strains indicates

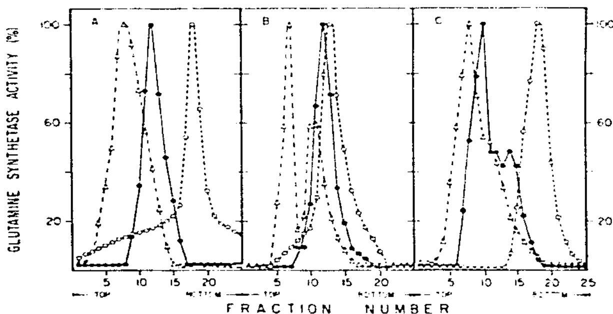

FIG. 2. *Sucrose gradient sedimentation of glutamine synthetase activity from crude extracts of wild type (○) and gln-1a (●) and gln-1b (△) mutants. (A) Wild-type strain grown for 12 h at 25°C and mutant strains grown for 24 h at 25°C on 25 mM NH_4NO_3; extracts and sucrose gradients prepared in buffer A (see text). (B) Three strains grown for 24 h at 25°C on fed-batch ammonium limited cultures; extracts and sucrose gradients prepared in buffer A. (C) Three strains grown for 12 h at 37°C on 5 mM glutamine; extracts and sucrose gradients prepared in buffer B. Glutamine synthetase activity was normalized to the peak fraction in each gradient.*

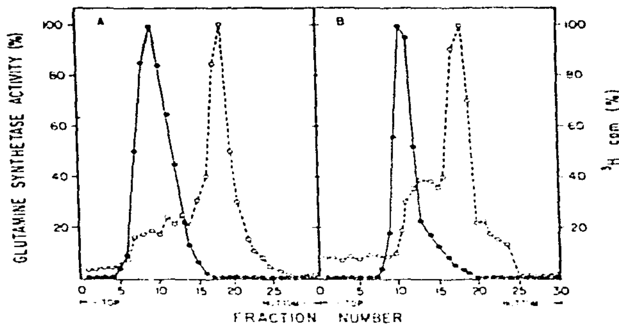

FIG. 3. *Sucrose gradient sedimentation of crude extracts from mutant strains (●) mixed with ^{3}H-labeled purified glutamine synthetase (○) from the wild type strain. Mutant strains were grown for 12 h at 37°C on 5 mM glutamine as the nitrogen source. Extracts were prepared in buffer A and mixed with purified in vivo-labeled glutamine synthetase. (A) gln 1a strain, (B) gln 1b strain. Glutamine synthetase activity and radioactivity were normalized to the peak fraction in each gradient.*

that both mutations are very closely linked in chromosome VR, where mutant *gln-1a* has already been mapped (Reich and Silagi, Abstr. Proc. Int. Congr. Genet., 11th, Abstr. no. 1, p. 49–50, 1963). The complementation analysis, possible only in mutant *gln-1b*, showed that this mutation is recessive to the wild type.

The fact that glutamate was accumulated by the mutant strains in either excess or limiting ammonium during the lag period and the decrease of this amino acid pool after growth resumed indicate that these mutations impair the synthesis of glutamine. The adaptation of the mutants to grow on ammonium after a lag, as well as the relationship between the nitrogen source present in the inoculum slants and the duration of this lag phase, is being studied.

The ability of the mutant strains to grow on ammonium after a prolonged lag phase, together with the presence of the tetramer in mutant *gln-1a* and of the tetramer and dimer in the mutant *gln-1b*, suggests a physiological role for these oligomeric structures of glutamine synthetase in the fixation of ammonium. These data reinforce the contention that the prevalence of the tetrameric over the octameric forms of the enzyme in wild-type *N. crassa* is a regulatory response to the presence in the medium of a limiting concentration of ammonium, and vice versa with ammonium excess (Fig. 2) (7).

The differences in glutamine synthetase activity of the mutants as compared with those found in the wild type growing under a variety of nitrogen conditions (Table 3) could be due to either structural or regulatory alterations in the mutant strains. The differences in behavior of the mutants in regard to the oligomeric structure of glutamine synthetase, namely, (i) the absence of the octameric form, (ii) the impairment of the in vitro conversion of the mutant tetramer to the octameric form as occurs in the wild-type strain (unpublished data), and (iii) the presence of dimeric forms, indicate alterations in the structure of the enzyme. This view is further supported by an immunochemical study of enzyme activity (Sánchez, Dávila, Mora, and Palacios, submitted for publication). The possibility that these mutant enzymes could alter the regulation of the enzyme during its biosynthesis is now being studied. The autogenous regulation of glutamine synthetase has been well documented in *Escherichia coli* (13, 17) and *Klebsiella aerogenes* (6, 9, 14).

The isolation of mutants unable to grow on limited amounts of ammonia and deficient of glutamine synthetase activity will help to correlate the structure and function of eucaryotic glutamine synthetase as well as to better understand the genetics of the stucture and regulation of this enzyme.

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RELATION BETWEEN STRUCTURE AND FUNCTION OF *Neurospora crassa* GLUTAMINE SYNTHETASE

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Summary

Two distinct monomers, α and β participate in the structures of different oligomers of *Neurospora crassa* glutamine synthetase (EC 6.3.1.2). In ammonium-limited cultures a tetrameric form composed mainly of α monomers was found. In excess of nitrogen an octameric form composed mainly from β monomers is the predominant oligomeric state. The presence of both monomers was observed in intermediate oligomeric forms.

Introduction

The nitrogen source regulates the concentration (1) and the *de novo* synthesis of glutamine synthetase (GS) in *Neurospora crassa* (2). On glutamate as nitrogen source the rate of synthesis of GS is 10-fold higher than in glutamine and corresponds to a similar difference in the specific mRNA levels of the enzyme (3). *N. crassa* GS purified from mycelia grown on glutamate is structured in an octameric form composed of monomers with a molecular weight of 48,000 (4). In fed-batch ammonium-limited cultures the activity of GS increased and instead of an octamer, mainly a tetrameric form of GS was found (5). This lower oligomeric state of GS was also found in glutamine auxotrophs of *Neurospora* (6). Since these mutants grow in limited ammonium, it is possible that the two oligomeric states of GS correspond to different gene products (6), and have a different function in ammonia fixation.

Recently it has been found that two different monomers contribute to the activity of GS (Sánchez, *et al*; submitted for publication, 1979). These

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monomers can be separated in acrylamide gel electrophoresis in SDS-urea where one of them (α) runs slightly slower than the other (β).

In this paper we report the relation that exists between these monomers with the oligomeric state and the function of GS in *Neurospora crassa*.

Material and Methods

Strains and Chemicals. - *Neurospora crassa* wild-type strain 74-A, the glutamic acid dehydrogenase deficient mutant am-1 and the glutamine auxotroph gln-1a, were obtained from the Fungal Genetics Stock Center at the Humboldt State University Foundation, Arcata, Calif. U.S.A. The glutamine auxotroph gln-1c was obtained in our laboratory and is an allele of the auxotrophs previously reported (6). All chemicals used were analytical grade.

Growth Conditions. - Batch cultures of *N. crassa* were grown after inoculating conidia in Vogel's minimal medium (7) with 1.5% sucrose. The nitrogen source was glutamate at 25°C, or 37°C and glutamine at 37°C. Fed-batch ammonium-limited cultures at 25°C with a constant growth rate, were achieved as reported (5), except that the conidia were not previously incubated in the absence of nitrogen. Growth was determined as described (1).

Determination of Glutamine Synthetase Activity. - Glutamine synthetase measured as transferase activity was assayed as described by Ferguson and Sims (8) in cell-free extracts of *Neurospora* prepared as in a previous work (1).

Immunoprecipitation of in vivo-labeled Glutamine Synthetase. - The cultures were pulsed with [^{3}H] leucine for one hour before harvesting. The cell-free extracts were sedimented in sucrose gradients (4) and fractions of the principal peaks of activity were precipitated with $(NH_4)_2SO_4$ at 70% saturation, resuspended and dialyzed overnight. Aliquots were immunoprecipitated with antibody against GS as described (2).

Electrophoresis and Fluorography. - The immunoprecipitates were subjected to acrylamide slab gel electrophoresis in the presence of SDS and 7 M urea (Sánchez, *et al*; submitted for publication, 1979), stained with Coomassie blue (9) and treated for fluorography (10).

Purification of Glutamine Synthetase. - Conidia obtained from slants with glutamine (10 mM) as nitrogen source, were used to inoculate cultures with glutamate at 25°C. After 3 hs the germinated conidia were filtered, washed and dried with acetone (1). From this powder the octameric GS was purified as previously reported (4). The tetrameric GS from the glutamine auxotroph gln-1c was purified as reported (4) from cultures grown on glutamate at 37°C, except for the following modifications. The cell-free extract was prepared in buffer A diluted 10-fold, this buffer was used to equilibrate the DEAE-cellulose column, which was eluted with Buffer A (Buffer A contains 50 mM imidazole, 50 mM glutamic acid, 80

mM $MgSO_4$, 50 mM K_2SO_4, 0.5 mM EDTA, 5 mM 2-mercaptoethanol, 25 mM $NaHSO_3$, pH 7.2). The fractions with activity were pooled and made 2.25 mM $MnCl_2$ and after added to the sepharose-anthranilic acid column previously equilibrated with Buffer A 2.25 mM $MnCl_2$, the enzyme was eluted with 40 mM AMP in this buffer. The protein was precipitated with $(NH_4)_2SO_4$ at 70% saturation and resuspended and dialyzed against Buffer A.

Results and Discussion

To study the distribution of the two different monomers of GS in the different oligomeric forms of this enzyme, the wild-type 74-A strain was grown on batch cultures at 25°C with glutamate as nitrogen source and in Fed-batch cultures ammonium-limited. In these conditions the GS is mainly found as an octamer (1) and tetramer (5), respectively. In addition the 74-A strain and the gln-1c mutant strain were grown in glutamine

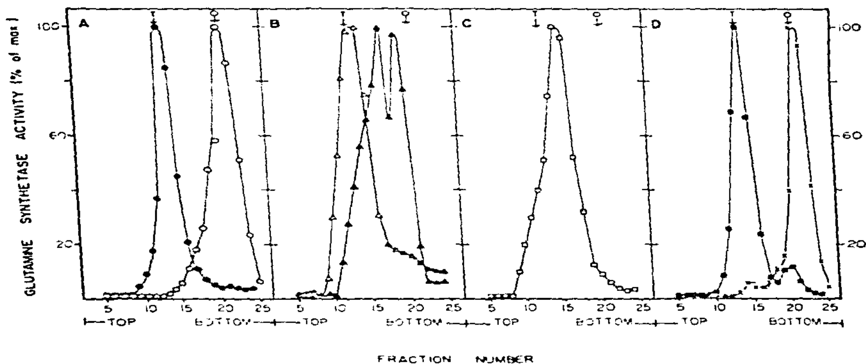

Fig. 1. Sucrose gradient sedimentation of glutamine synthetase from crude extracts: A, wild-type grown at 25°C on 5 mM glutamate (o) or ammonium-limited (●) B, at 37°C on 5 mM glutamine the wild-type (▲) and the mutant strain gln-1c (△) and C, the am-1 strain at 25°C ammonium-limited (□). D, purified GS from cultures grown on glutamate at 25°C from the wild-type (x) and from the gln-1c at 37°C (■). T tetramer, O octamer.

at 37°C and the am-1 mutant strain in limited-ammonium. The glutamine auxotrophs were grown on glutamine at 37°C since in this condition they had a sizable activity of GS structured in a tetramer (6). All the cultures were pulsed with [^{3}H]leucine as described in methods.

In Fig. 1 is presented the transferase activity in sucrose gradients from extracts of these cultures. As expected the tetrameric GS was present in extracts of the wild-type ammonium-limited and in the gln-1c (Fig. 1 A,B), the octamer was found in the 74-A strain grown on glutamate (Fig. 1 A). On the other hand in the wild-type grown on glutamine at 37°C, in addition to the octamer, a well defined peak of activity was found in the sucrose gradient left and next to this oligomer (Fig. 1 B). A different oligomer of GS, that banded next and right to the tetramer, was also found in the am-1 mutant strain ammonium-limited (Fig. 1C). The bands of activity immunoprecipitated, stained with Coomassie blue after acrylamide gel electrophoresis in SDS-urea, are shown in the top of Fig. 2. It is clear that the octamer is composed mainly by β monomers (Fig. 2c) and the tetramer by α monomers (Fig. 2d). Both monomers were found in the intermediate oligomeric forms that banded in sucrose gradient between the tetramer and the octamer (Fig. 2a,b). As these oligomers (am-1 strain and wild-type grown on glutamine at 37°C) band closer to the octameric form, an increase in the β monomers and a decrease in the α monomers was apparent. Only in the case of the wild-type strain grown in glutamine at 37°C the monomers corresponded to the pool of two peaks of activity of the sucrose gradient.

The fluorography of the gel (Fig. 2 bottom) shows that the distribution of α and β monomers resembles very closely what is seen after staining the gel (Fig. 2 top), and emphasizes that the newly synthesized

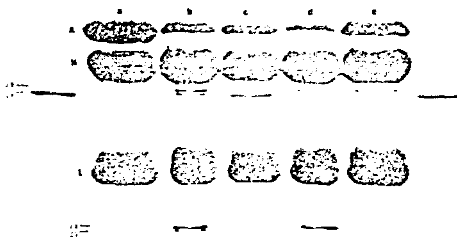

Fig. 2. Top panel Gel after coomassie-blue staining: a) wild-type on glu tamine, b) am-1 strain ammonium-limited, c) wild-type on glu tamate, d) wild-type ammonium-limited and e) gln-1c strain on glutamine. In the first and the last tracks of the gel purified non-labeled GS from glutamate grown cultures was run as a marker. For other conditions see Fig. 1 and methods. Bottom panel: gel after fluorography. A albumin, H γ-globulin heavy chain, L γ-globulin light chain, α and β monomers of GS.

α's are incorporated almost exclusively in tetramers and the β's in octamers. In contrast, hybrid monomers were found in the oligomers intermediate between the tetramer and the octamer.

It has been possible to obtain growth conditions in which the octamer can be purified with only traces of α monomers (compare Fig. 2 and 3). These data indicate that the α monomers are not required for the structure and activity of the octamer. The tetramer purified from gln-1c mutant strain is composed only of α monomers (Fig. 3).

The presence of some α monomers in the octamer and of some β monomers in the tetramer, of the wild-type strain may be the result of monomer hybridization, or that the equilibrium of α's favors more the arrangement in tetramers than in octamers, and viceversa for the β's. The fact that the α monomers of the mutants strain gln-1a and gln-1c in

Fig. 3. Gel after coomassie-blue staining of the purified preparations of GS obtained from gln-1c strain (a,b and c) and from wild-type (d,e and f). The cultures were grown as described in methods. In the first and the last traks a mixture of α and β monomers were run as markers.

addition to the tetramer, are able to form octamers in a low proportion, and that the monomers of the wild-type strain behave oppositively (Fig. 1d), is in favour of the equilibrium hypothesis. This equilibrium would also depend of the intracellular conditions. It is in intermediate oligomeric forms of GS where both monomers are found in an important proportion, then it is possible that these intermediate states are composed by hybrids of α and β monomers, which appear when the nitrogen content is neither limiting nor in excess. Experiments are in progress to purify the wild-type tetramer and test *in vitro* if the presence of this form in low ammonium and the octamer in nitrogen excess, are related with a different affinity for ammonium of these oligomers. Recent evidence has demonstrated that glutamate synthase (GOGAT) is present in ammonium-limited cultures (Hummelt, *et al*; submitted for publication, 1979). This enzyme together with the tetrameric GS participates in the assimilation of low ammonium concentrations.

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TITLE: Genetic and Biochemical Characterization of Glutamine Synthetase from *Neurospora crassa* Glutamine Auxotrophs and their Revertants.

RUNNING TITLE: Glutamine Synthetase Mutants and Revertants.

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SUMMARY

In this paper we present the isolation and characterization of glutamine auxotrophs of _Neurospora crassa_ and their revertants.

The results show that in spite of the fact that various enrichment procedures were used we found only two types of auxotrophs.

Genetic crosses performed between the different mutants showed that the mutations responsible for their phenotypes are highly linked and probably affect the same gene.

The biochemical characterization of the glutamine synthetase (GS) polypeptides of the different mutants showed that both types contain the α monomer. However in place of the normal β monomer, each type has a new polypeptide differing from normal β either in its molecular weight or in its isoelectric point.

On the other hand, the revertants have only the α monomer and are capable of growing without glutamine. On the basis of these data we propose that the lack of GS activity of the auxotrophs is due to the interaction of the altered β with the α monomer, and as a consequence, the α monomer, of the revertants regains its activity because of the absence of the altered β.

INTRODUCTION.

The regulation of glutamine synthetase (GS) (EC 6.3.1.2.) of *Neurospora crassa* by nitrogen and carbon sources has been reported (9,20). Nitrogen source regulates GS concentration at the level of specific enzyme synthesis (15), which correlates with the amount of mRNA as indicated by an *in vitro* protein synthesizing system (14,17). On the other hand, carbon source also participates in the regulation of GS degradation (11).

It has been found that GS is composed of two polypeptides (α and β) which show different electrophoretic mobilities (16). These polypeptides are arranged in different oligomeric forms; the tetrameric GS is composed mainly of the α polypeptide, whereas the octameric form has mainly the β polypeptide (3). We have proposed that the assimilation of ammonia occurs by two different pathways. One operates in low ammonia with the participation of tetrameric GS and glutamate synthase (EC. 1.4.7.1.), while in the other the biosynthetic glutamic acid dehydrogenase (EC 1.4.1.4.) and octameric GS are the enzymes responsible for the assimilation to ammonium in a high ammonia environment (3).

Central to understanding the role of GS in nitrogen metabolism is the isolation of glutamine auxotrophs and the characterization of the genes and gene products involved. In this regard, few reports exist on glutamine auxotrophs in eucaryotic microorganisms. This may be a consequence of this involvement of two GS polypeptides, as reported in *Neurospora* and in plants (8, 16, 19) in *Saccharomyces cerevisiae* the isolation of glutamine auxotrophs has been reported; however, no characterization has been presented (5,6).

Neurospora crassa glutamine auxotrophs have been obtained and partially characterized. The physiological behavior, the enzymatic activity, and the oligomeric structure of their GS have been previously reported (2).

In this work we describe the isolation of several glutamine auxotrophs using various selection procedures. The genetic characterization of these strains reveals the existence of two types of GS mutants. Based on biochemical analysis of the *in vivo* and *in vitro* synthesized GS polypeptides of the auxotrophs and their revertants, we propose a mechanism by which the interactions between the GS polypeptides, present in the mutant strains, causes the inactivation of both isozymes.

MATERIAL AND METHODS.

Strains. *Neurospora crassa* wild-type strain *74-A*, the inositolless strain *inl* (890601), and the glutamine auxotroph *gln-1a* came from the Fungal Genetics Stock Center at The Humboldt State University Foundation, Arcata, Calif., U.S.A. The other glutamine auxotrophs, *gln-1b* and *gln-1c*, came from the collection of J. Mora.

Growth Conditions. All the strains were grown for 12 h at 25°C or 37°C on Vogel's minimal medium N (21) supplemented with 1.5% sucrose. Other nitrogen sources in place of, or in addition to ammonium nitrate were used as stated in the text. Mycelium was grown from a conidia inoculum in liquid medium sparged with hydrated air (18).

Determination of glutamine synthetase activity. Cell-free extracts were prepared from mycelium as previously described (13). Glutamine synthetase was measured by its transferase and synthetase activities, as reported (17). Protein was determined by the method of Lowry et al (10).

Mutagenesis and mutant selection. A conidial supension of the 74-A or *inl* strain was prepared and adjusted to $2x10^7$ conidia per ml, and 20 ml of each suspension was incubated with 3 mg of N-methyl-N'-nitro-N-nitroso-guanidine (Sigma Chemical Co., St. Louis, Mo.) for 1 h at room temperature with occasional shaking. Conidia were then concentrated by centrifugation and washed twice with 20 ml of cold water. After the mutagenic treatment, spores of the *inl* strain were enriched by incubation in a 500 ml Florence flask with 200 ml of N medium supplemented with 1.5% sucrose, with 25 mM KNO_3 at 37°C or with 25 mM NH_4NO_3 at 25°C. The flasks were incubated for 64 h with shaking. The spores of the *74-A* strain were enriched on 200 ml of N medium supplemented with 1.5% sucrose, 5 mM KNO_3, and 5 mM glutamate at 37°C for 72 h sparged with moist air. Every 12 h the entire culture was filtered through cheesecloth into an Erlenmeyer flask containing fresh medium. The enriched population of both strains was concentrated by filtration, washed, resuspended in distilled water, and plated on N agar medium supplemented with glucose and fructose (0.02% each), sorbose (2%), and 10 mM glutamine as the sole nitrogen source. Plates were incubated at 29°C until colonies appeared (2 to 5 days) and then transferred to slants of N agar medium supplemented with 1.5% sucrose and 10 mM glutamine. Crosses were made in 2X corn meal agar (Difco). Spot testing and progeny analysis were carried out as described (4). All the mutants were transferred to standard genetic background by 3 succesive crosses with the wild type strian.

Immunoprecipitation of *in vivo* labeled glutamine synthetase. The cultures were pulse-labelled with ^3H leucine at 5µCi/ml for 2 h before harvesting, and cell-free extracts were immunoprecipitated with purified polyclonal antibodies against α and β wild type GS as described (15). The cell-free extracts were also sedimented through a 5 to 20% continuous sucrose gradient prepared in buffer A (50 mM imidazole, 50 mM glutamic acid, 80 mM $MgSO_4$, 50 mM K_2HSO_4, 0.5 mM EDTA, 5 mM 2-mercaptoethanol, 25 mM $NaHSO_3$ pH 7.2) or buffer B (5 mM KH_2PO_4, 0.5 mM EDTA, 50 mM K_2SO_4 pH 7.2) as indicated in text. The fractions collected were precipitated with $(NH_4)_2SO_4$ at 70% saturation, resuspended in and dialyzed against buffer A, and immunoprecipitated (15).

Electrophoresis and Fluorography. The immunoprecipitates were subjected to polyacrylamide slab gel electrophoresis in the presence of SDS and 7 M urea (16), stained with coomassie blue, and processed for fluorography (1).

Two-dimensional electrophoresis. Two dimensional electrophoresis of the glutamine synthetase immunoprecipitates were done as described by O'Farrell (12) with the following modifications: Electrofocusing gels were run at 400 volts for 18 h omitting the final high voltage pulse, and the second dimension electrophoresis was carried out as previously reported (16).

RNA preparation and cell-free-protein-synthesizing system. Polysomal RNA prepared as described (17) was chromatographed on a column of oligodeoxythymidylic acid cellulose and used to direct the *in vitro* translation system (14). The translated GS was immunoprecipitated by a direct procedure (17) and analyzed by two dimensional gel electrophoresis.

RESULTS.

Phenotypic characterization of glutamine auxotrophs.

The isolation and partial characterization of strains gln-1a and gln-1b was reported prviously (2,16). The gln-1c, gln-1d, gln-1e gln-1f, gln-1g and gln-1h strains were obtained after mutagenesis of the inl strain. When the enrichment of this strain was done on nitrate at 37°C, all the glutamine auxotrophs obtained were inositol-plus (gln-1d, gln-1e, gln-1f, gln-1g, gln-1h); this was not the case when the enrichment was done on ammonium at 25°C (gln-1c). The reversion of the inl mutant may be related to the fact that these glutamine auxotrophs are leaky on nitrate at 37°C while they do no grow on ammonium at 25°C, the gln-1i and gln-1j mutants were obtained by filtration enrichment of the 74-A strain after mutagenesis (see material and methods).

Although three different selection procedures were used, the auxotrophs obtained showed only two distinct phenotypes (Table 1). The gln-1i strain shows a phenotype similar to that of the gln-1b strain; these two mutants could not grow without supplementation by glutamine at either 25°C or 37°C (2). All the other mutants showed a phenotype similar to the gln-1a; they were capable of growing at 37°C on glutamate as the sole nitrogen source or on glutamine at either temperature (Table 1). Both types of mutants, gln-1i and gln-1j, were obtained during the same enrichment procedure.

The glutamine synthetase specific activity of the mutants was measured in comparison with the wild type GS in cultures grown for 12 h on glutamine as nitrogen source at 25°C or 37°C. The results show that the synthetase activity is approximately thirty-fold lower in the mutants than in the wild type, when grown either at 25 or 37°C (Table 2).

Since the glutamine synthetase activity is ten-fold higher in glutamine cultures grown at 37°C versus 25°C, either inthe wild type or in the mutant strains, the characterization of enzyme polypeptides was performed on cultures grown at 37°C.

Isolation of revertants from glutamine auxotrophs.

The ability of the different mutant strains to regain the capacity to grow on ammonium was explored by plating conidia in this substrate as sole nitrogen source and without any mutagenic treatment.

The number of revertants obtained from any of the mutant strains increases with the number of vegetative growth cycles performed on

glutamine. After the first passage the reversion frequency was 1×10^{-7}. The number of revertants could be as high as 1×10^{-4} after five growth cycles. Revertants were distinguished from the wild type strain by the presence of a lag phase of about 5 h on cultures containing ammonium as sole nitrogen source and by a higher sensitivity to L-methionine sulfoximine, a GS inhibitor (22).

Genetic analysis of glutamine auxotrophs.

In order to determine the linkage between the mutations responsible for the two different phenotypes, we performed genetic crosses between the two different kinds of strains and obtained the following results:

In the corss between strains *gln-1a* and *gln-1b*, we found a recombination frequency of 1×10^{-5} after analyzing 1.5×10^{6} viable ascospores. Meanwhile, in crosses between mutants that presente the same phenotype (*gln-1a* X *gln-1c*) no recombinants were obtained out of 0.5×10^{6} viable ascospores. Also, these recombinants did not appear in self crosses, where 0.5×10^{6} viable ascospores were analyzed.

In all the crosses performed, a large number of revertants was obtained. The recombinants and revertants were distinguished by the following criteria. The revertants appeared only after 5 days of incubation in the selective media while the wild type recombinants appeared after 12 h of incubation.

Revertants were more sensitive to L-methionine sulfoximine than the wild type strain, and appeared when self crosses were performed.

The previously reported recombination frequency of 1 in 5000 between *gln-1a* and *gln-1b* (2), can now be explained by the high reversion rate of these mutations.

Characterization of GS polypeptides synthesized *in vivo* and *in vitro*.

Cell-free extracts, from cultures grown for 12 h on glutamine at 37°C were immunoprecipitated with polyclonal antibodies and subjected to acrylamide gel electrophoresis in the presence of SDS and urea. Under these conditions the wild type strain as well as the *gln-1b* and *gln-1i* strains show both α and β polypeptides, while the others show only the α polypeptide (Fig. 1A).

When *de novo* synthesis of GS was analysed (Fig. 1B), the β polypeptide was absent from all the mutants except the *gln-1b* and *gln-1i*. A new polypeptide (γ) of molecular weight of 30 K was synthesized in all the mutants that lacked the β polypeptide. Another polypeptide of molecular weight of 40K was present in all the strains including the wild type. In view of the fact that this polypeptide precipitates also in the absence of specific antibody (data not shown) we think that its precipitation is due to its insolubility in the immunoprecipitation buffer.

For further characterization of the polypeptides, immunoprecipitates from *in vivo* labeled cultures were subjected to two dimensional analysis using isoelectrofocusing in the first dimension and electrophoresis in the presence of SDS and urea in the second dimension (Fig. 2A). Mutant *gln-1b* contains an altered β polypeptide, which has the same molecular weight but a more basic isoelectric point than that of the wild type. The *gln-1i* strain presents the same pattern as strain *gln-1b*, since immunoprecipitates of mixtures of extracts from both strains show a single spot for the β altered band (data not shown). All the other mutants show the γ polypeptide which has a lower molecular weight (30K) but focuses in a similar pH range to the α and β polypeptides of the wild type strain. The band with molecular weight of 40K is not observed since its isoelectric point is more basic than the range of pH used in this analysis. The α polypeptide of all the mutants is indistinguishable from that of the wild type strain.

To gain insight into the origin of the mutations obtained, RNA from the different strains was extracted from polysomes and fractionated in oligodeoxythymidylic acid columns to separate the poly A enriched fraction, and translated in a cell-free protein synthesizing system derived from rabbit reticulocytes. The *in vitro* synthesized polypeptides were immunoprecipitated and analysed on two dimensional gels. Fig. 2B shows that the polypeptides synthesized *in vitro* have the same mobility as those synthesized *in vivo*, further evidence for this identity, is that when a mixture of immunoprecipitates from *in vivo* and *in vitro* synthesized polypeptides from strain *gln-1a* was analysed in double dimension gels, it showed a single spot for the γ polypeptide (data not shown). The difference in the relative position of α and altered β polypeptides form strain *gln-1b* seen in Fig. 2A and B is the result of small differences in the pH range of the gels.

Biochemical characterization of revertants of the glutamine auxotrophs.

Polypeptides from revertants of the two different types of mutants, were characterized by immunoprecipitation of extracts, followed by analysis in one and two dimensional gels. Interestingly, revertants of the gln-1a or gln-1c mutants do not synthesize the γ polypeptide, and revertants of the gln-1b strain do not synthesize the altered β polypeptide, (Fig. 3A). Analysis of 6 revertants of strain gln-1b, 4 of strain gln-1a, and 2 from strain gln-1c, indicated that none regain the β polypeptide.

RNA from some of the revertants was translated in vitro and the immunoprecipitates of the synthesized polypeptides were analyzed in one (data not shown) and two dimensional gels (Fig. 3B). In accordance with the data obtained from in vivo synthesized polypeptides, the in vitro translated products showed only the α polypeptide.

Oligomeric state of GS from the glutamine auxotrophs and their revertants.

We have previously reported that strain gln-1a has a GS that bands as a tetramer in sucorse gradients, instead of the octameric enzyme of the wild type. We have found that the mutants gln-1c through gln-1h present an oligomeric pattern of GS that is similar to that of the gln-1a strain.

When sucrose gradients of crude extracts from the auxotrophs were run in buffer A (see methods), they showed an activity peak with a shoulder toward the heavier zone of the gradient, this shoulder did not appear when the experiment was carried out in buffer B (gln-1c, Fig. 4). When the pure α GS was subjected to sucrose gradient sedimentation in either buffer A or B, the activity peak profile was unaltered. Similarly, the sedimentation pattern of the GS from strain gln-1cR6 showed a tetramer which behaved as the pure α enzyme (Fig. 4) (3). Other auxotrophs of this type and their revertants show the same sedimentation profile (data not shown). Therefore the abnormal oligomeric pattern found in the glutamine auxotrophs could be explained by the presence of the altered β polypeptide.

Searching for a relationship between the deficient GS biosynthetic activity and the presence of the altered β polypeptide in the glutamine auxotrophs, we incubated the gln-1a strain in minimal medium and pulsed labelled it with ^{3}H leucine; in this condition this strain does not grow due to its barely detectable GS activity. Cell-free extracts from this

culture, were sedimentated through a sucrose gradient with buffer A, fractions were collected for immunoprecipitation, and subjected to gel electrophoresis. As shown in Fig. 5 the γ polypeptide was found in the same pooled fractions as the α polypeptide.

The gln-1b strain showed two different oligomers when cell-free extracts were rapidly prepared in buffer B for sucrose sedimentation analysis. This oligomeric state was different from the one found when the extract was prepared and run in buffer A (2), where it only showed one broad peak with low activity. In Fig. 6 it can be appreciated that α and β monomers consedimented in both oligomers. It is interesting that both oligomers present good transferase activity (Fig. 6), but no synthetase activity (Not shown).

DISCUSSION.

In *Neurospora crassa* two GS monomers (α and β) are responsible for ammonium assimilation. Each one participates in the synthesis of glutamine, depending on the ammonium concentration (3).

The results presented inthis paper show that independent of the selection method used, only two types of glutamine auxotrophs have been isolated (Table 1). The phenotype of one is the ability to grow on glutamate at 37°C but not a 25°C. These mutants have an α GS monomer with the same molec-lar weight and charge as the α GS monomers from the wild type strain. In contrast, these mutants do not synthesize *in vivo* nor *in vitro* the β GS monomer, but instead, synthesize a polypeptide (γ) with a lower molecular weight that cross-reacts with antibodies against α and β GS monomers (Fig. 2). We have previously reported the synthesis of this polypeptide by a cell-free translation system primed with mRNA from one of the mutants (16). The absence of the β polypeptide and the presence of the γ polypeptide in these mutants can be explained as the result of a mutation in the gene for the β polypeptide.

A second and less frequent auxotroph grows only on glutamine. Analysis of these mutants showed the synthesis *in vivo* of a β polypeptide with an altered isoelectric point (Fig. 2A). This abnormal momomer was also synthesized *in vitro* (Fig. 2B).

The low frequency of recombinants found in crosses between these two types of glutamine auxotrophs indicates that these mutations are very close and possibly map in the structural gene for the β polypeptide.

We propose that the altered β polypeptide in the auxotrophs blocks the GS synthetase activity of the α polypeptide by participating in the formation of an abnormal, nonfunctional oligomer, (Fig. 5 and 6). This proposal is supported by the fact that revertants, which are capable of growing in ammonium, do so using only the α monomer assembled as a tetramer (Fig. 4). We have found that the glutamine auxotrophs that grow on glutamate at 37°C have some GS biosynthetic activity in this condition. Perhaps this GS activity is due to the fact that there is not enough γ to titrate (inactivate) all of the α polypeptide. However other factors may contribute to the inactivation of α monomers. For instance glutamine auxotrophs accumulate more glycine in ammonium than in glutamate, and glycine is an inhibitor of the tetrameric α GS. (G. Hernández and J. Mora, in preparation).

As the revertants from glutamine auxotrophs only synthesize *in vivo* and *in vitro* the α GS monomer (Fig. 3), we propose that these strains have a regulatory mutation that turns off the expression of β GS monomers.

Crosses of these revertants do not yield glutamine auxotrophic progeny, indicating that this new mutation is closely linked to the original mutation.

The attainment of only two types of glutamine auxotrophs in *Neurospora crassa*, one having an altered weight and the other altered charge of the β monomer of GS, may be the result of the presence of two monomers of GS that form two different GS isozymes (16). In order that a single mutation results in auxotrophy, it must result in the inactivation of both polypeptides. A single mutation in β could result in the inactivation of both (α and β) by the formation of an inactive hybrid enzyme. Presumably very few single mutation in the β gene result in this complex phenotype.

Further support has been obtained from complementation studies in forced heterokaryons between the auxotrophs and their revertants, which sow that the mutation responsible for the glutamine auxotrophy is dominant over the revertant phenotype (unpublished data).

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TABLE 1. Effect of temperature and nitrogen sources on the growth of glutamine auxotrophs.

Strain	Temp (°C)	Growth NH_4NO_3	Glu	Gln
74-A	25	+	+	+
	37	+	+	+
gln-1c	25	–	–	+
	37	–	+	+
gln-1d	25	–	–	+
	37	–	+	+
gln-1e	25	–	–	+
	37	–	+	+
gln-1f	25	–	–	+
	37	–	+	+
gln-1g	25	–	–	+
	37	–	+	+
gln-1h	25	–	–	+
	37	–	+	+
gln-1i	25	–	–	+
	37	–	–	+
gln-1j	25	–	–	+
	37	–	+	+

Spores of the different strains were spotted on plates of minimal medium containing 0.8% sorbose and 0.4% sucrose as carbon source and the indicated nitrogen source at a concentration of 25mM NH_4NO_3, 5mM glutamate or 5mM glutamine. Plates were incubated at either 25°C or 37°C and growth was scored 24 hrs later, as + good growth or – no growth.

TABLE 2. Glutamine synthetase activity in glutamine auxotrophs.

Strain	Synthetase activity [a] 25°C[b]	Synthetase activity [a] 37°C
74-A	13.90	137.0
gln-1c	ND[c]	3.8
gln-1d	0.30	3.6
gln-1e	0.50	5.0
gln-1f	0.49	5.0
gln-1g	0.42	4.0
gln-1h	0.49	5.0
gln-1i	ND	5.8
gln-1j	ND	0.9

[a]Synthetase specific activity is expressed as namomoles of γ-glutamil hydroxamate produced per minute per milligram of extracted protein at 30°C.

[b]Temperature of the cultures.

[c]ND, not determined.

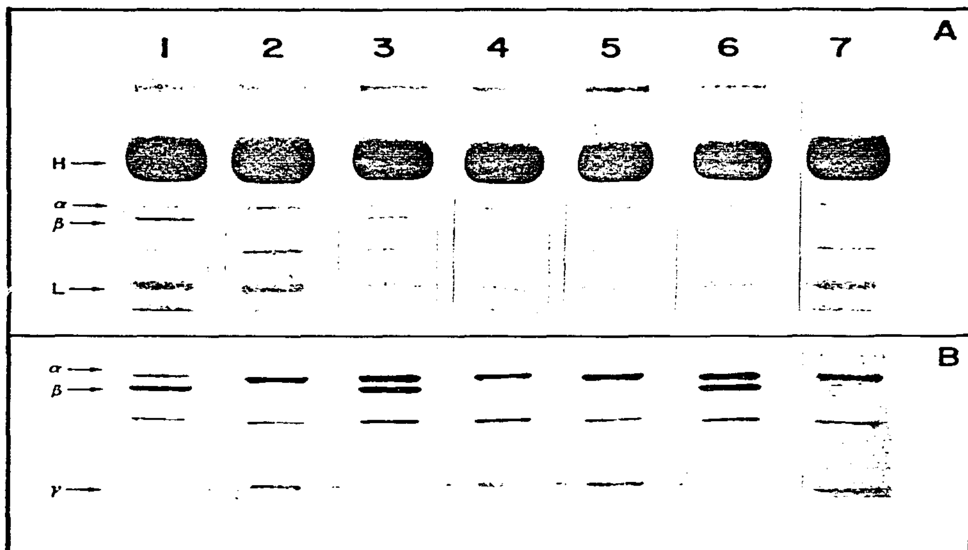

Fig.1. Electrophoretic identification of immunoprecipitated glutamine synthetase polypeptides from wild type and glutamine auxotrophs. Strains grown for 10 hrs at 37°C with glutamine as the sole nitrogen source ived a two hours pulse of H leucine. Cell-free extracts were prepared and immunoprecipitated with anti-GS as indicated in Materials and Methods. Immunoprecipitates were subjected to acrylamide gel electrophoresis in the presence of SDS and 7M urea. Gels were stained with Coomassie blue (A) and then subjected to fluorography (B). The mobility of heavy (H) and light (L) chains of gamma globulin, and of glutamine synthetase polypeptides (α,β,γ) is indicated by arrows.
Strains: 1, 74-A; 2, *gln-1a*; 3, *gln-1b*; 4, *gln-1c*; 5, *gln-1d*; 6, *gln-1i*; 7, *gln-1j*.

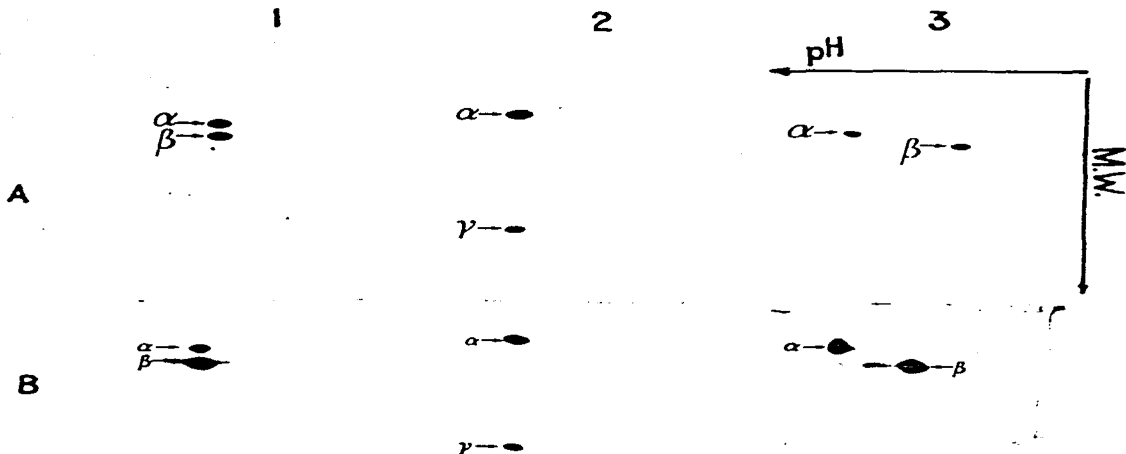

Fig. 2. Electrophoretic characterization of *in vivo* and *in vitro* synthesized glutamine synthetase polypeptides from wild type and glutamine auxotroph strains grown as described in Fig. 1.
Aliquots of the cultures were labeled with H leucine for two hours and cell--free extracts were immunoprecipitated with anti-GS. The rest of the culture was used to extract RNA as described in Material and Methods. RNA was translated in a cell-free system derived from rabbit reticulocytes and the product was immunoprecipitated with anti-GS. Immunoprecipitates of both *in vivo* (A) and *in vitro* (B) synthesized GS were subjected to double dimension electrphoretic analysis followed by fluorography. Strains: 1, 74-A; 2, *gln-1a*; 3, *gln-1b*. Symbols as in Fig. 1; the first dimension (pH) and the second (M.W.) are also indicated.

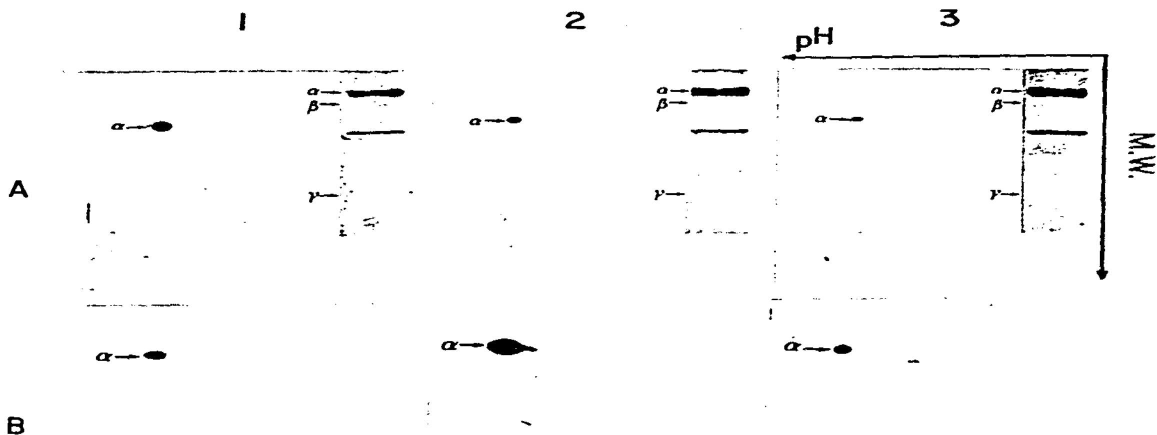

Fig. 3. Electrophoretic characterization of *in vivo* and *in vitro* synthesized glutamine synthetase polypeptides from revertants of glutamine auxotrophs. Strains were grown and samples processed as in Fig. 1. Immunoprecipitates of both *in vivo* (A) and *in vitro* (B) synthesized GS were subjected to double dimension electrophoretic analysis followed by fluorography. Also, immunoprecipitates were subjected to acrylamide gel electrophoresis in the presence of SDS and 7M urea and followed by fluorography (inserts). Strains: 1, *gln-1aR11*; 2, *gln-1bR8*; 3, *gln-1cR6*.

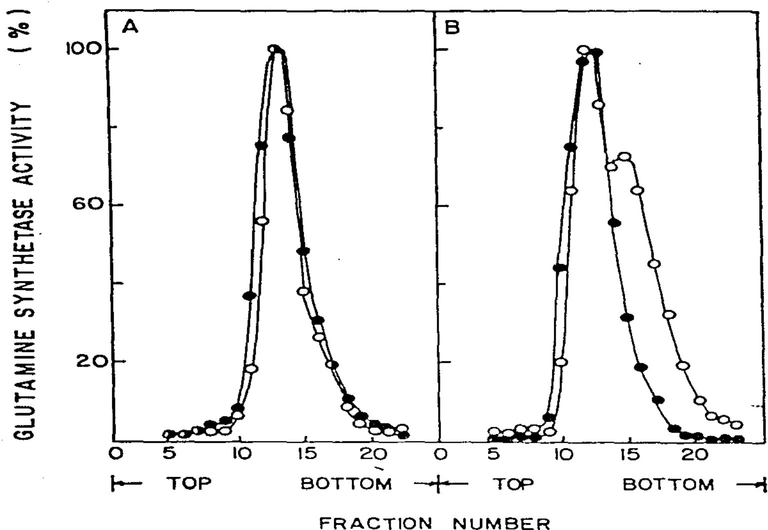

Fig. 4. Sucrose gradient sedimentation of glutamine synthetase from a glutamine auxotroph and a revertant, Strains gln-1c (O-O) and gln-1cR6 (O-O) were grown 12 hours at 37°C in the presence of glutamine as the sole nitrogen source. Cell-free extracts were prepared in either buffer B (A) or buffer A (B) as indicated in Materials and Methods. Samples were overlayed on a 5% to 20% sucrose gradient prepared in the corresponding buffer, and centrifuged 12 hours at 40,000 RPM in the Beckman SW40 rotor. Fractions were collected from the top of the gradient and assayed for GS transferase activity. Activity was normalized to that of the peak fraction.

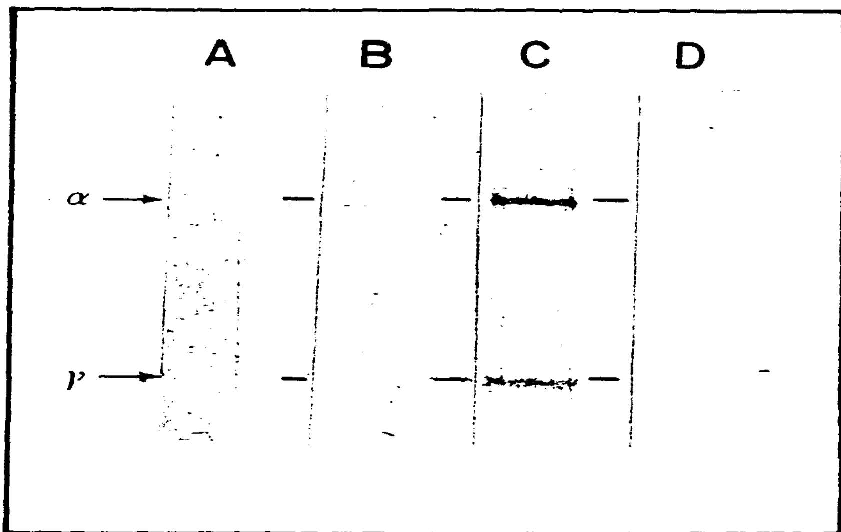

Fig. 5. Sucrose gradient sedimentation of GS polypeptides from the gln-1a strain. Spores from gln-1a strain were incubated in minimal medium at 25°C for 10 hours and labelled for 2 hours with H leucine. A cell-free extract was prepared in buffer A and centrifuged as in Fig. 4. Fractions were collected and assayed for GS transferase activity. Activity was negative in all fractions. Fractions were combined in four pools to the following molecular weigths: A, 65 to 90 K; B, 100 to 130 K; C, 150 to 210 K; D, 220 to 290 K. The tetrameric form of GS corresponds to pool C. Collected fractions were immunoprecipitated with anti-GS and then subjected to acrylamide gel electrophoresis in the presence of SDS and 7M urea followed by fluorography.

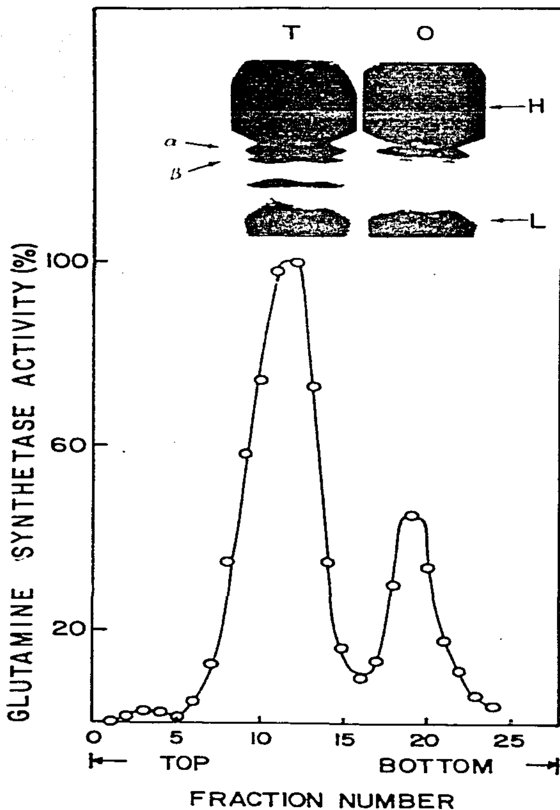

Fig. 6. Sucrose gradient sedimentation of GS polypeptides from gln-1b strain. Cell-free extracts from cultures of gln-1b strain grown for 10 h at 37°C in glutamine as the sole nitrogen source were centrifuged, fractionated, and assayed for GS transferase activity as in Fig. 4. Fractions with maximal activity were immunoprecipitated separately; subjected to acrylamide gel electrophoresis and stained with coomassie blue. T. tetramer; O, octamer; other symbols as in Fig. 1.

DISCUSION

Los resultados presentados en los artículos muestran que, no obstante que se aislaron auxótrofos de glutamina usando distintos métodos de selección, solamente se pudieron obtener dos tipos de cepas Gln⁻. Uno de ellos se caracteriza por su capacidad de crecer en glutamato a 37°C; el otro , que es el fenotipo encontrado, con menor frecuencia, se define por su total dependencia por glutamina para crecer.

Los resultados del análisis genético indicaron que la auxotrofía por glutamina se debe a una sola mutación en cada cepa; que estas mutaciones son posiblemente alélicas, ya que producen recombinantes silvestres a muy baja frecuencia (aproximadamente $1x10^{-5}$); y que cuando menos una de estas mutaciones es recesiva frente al alelo silvestre.

Los auxótrofos incubados en exceso de amonio no crecieron y acumularon pozas elevadas de glutamato, en tanto que la poza de glutamina fué cinco veces más baja que la que se encontró en auxótrofos para otros aminoácidos crecidos en las mismas condiciones. Todas las cepas Gln⁻ estudiadas son capaces de adaptarse a crecer en amonio (en exceso o limitado) después de un período inicial de no crecimiento. Esta adaptación habla de la posibilidad de un segundo sistema de síntesis de glutamina. En la cepa silvestre existen dos estados oligoméricos de la enzima, tetrámero y octámero, con una participación alternativa en la asimilación de amonio (49) en tanto que en las mutantes solo esta presente el tetrámero. Estos datos hablan en favor de que la enzima tetramérica es la responsable de la síntesis de glutamina que

ocurre durante la adaptación de los auxótrofos para crecer en amonio.

La presencia de dos polipéptidos de GS, α y β (79), cuya concentración varía de acuerdo a la fuente nitrogenada del medio de cultivo, nos llevó a buscar la relación entre estos monómeros y el estado oligomérico de la GS. Encontramos que la cepa silvestre en cultivos limitados de amonio, tiene una GS tetramérica compuesta principalmente de los monómeros α, en tanto que exceso de nitrógeno, se encuentra una GS octámerica formada predominantemente de polipéptidos β. Esta relación entre la estructura y la función de las glutamino sintetasa, aunada a la presencia de la enzima GOGAT recientemente descrita en *Neurospora* (31), permite proponer dos vías de asimilación de amonio en este microorganismo: 1) la que funciona en exceso de nitrógeno formada por la GDH-NADPH-dependiente, y la GS β y 2) la que participa cuando el amonio es limitante, constituída por la GS α y la GOGAT.

Estudios cinéticos realizados con preparaciones purificadas de ambas enzimas han demostrado que la GS α tiene más afinidad por amonio que la GS β y que esta última tiene una Vmax 10 veces mayor a la de la primera (Guzmán J. y J. Mora comunicación personal), lo que concuerda con el papel fisiológico que se les ha asignado a estas isozimas.

De la caracterización fisicoquímica de los polipéptidos presentes en las cepas Gln^- destaca el hecho de que conserva el polipéptido α, que es indistinguible al de la cepa silvestre en los dos fenotipos descritos, y se presenta un cambio en el polipéptido β. Las mutantes que crecen en glutamato a 37°C no tienen polipéptido β pero presentan un polipéptido de 30,000 daltones de peso molecular (γ), con el mismo punto isoeléctrico de los monómeros α y β silvestres: las mutantes de dependencia estricta por glutamina presentan

un polipéptido de un peso molecular semejante al de β, pero que tiene un punto isoeléctrico mucho más básico (β'). Estos datos concuerdan con la hipótesis de que las mutaciones se encuentran localizadas en el gene estructural para el polipéptido β.

Contra lo esperado, las cepas revertantes no recuperan el polipéptido β normal y tampoco aumentan sensiblemente la concentración del polipéptido α. Estas cepas son capaces de crecer en exceso de amonio usando únicamente el polipéptido α estructurado en un tetrámero. Debido a lo anterior proponemos que los polipéptidos alterados (α y β'), presentes en los dos tipos de auxótrofos encontrados bloquean la actividad biosintética del polipéptido α. El primero consistió en sedimentar extractos de las cepas mutantes en gradientes de sacarosa e inmunoprecipitar (con anticuerpos anti GS) las fracciones con la máxima actividad de GS, para ver si los polipéptidos alterados (γ ó β') comigraban con los normales (Fig. 5 y 6 del artículo "Genet and Biochem..."). El segundo tipo de experimentos consistió en demostrar que en presencia de un polipéptido alterado, el estado oligomérico de la GS α se modifica, alternando su patrón de migración en gradientes de sacarosa, debido a la formación de enzimas híbridas menos activas? (Fig. 4 del artículo "Genetic and Biochemical..).Los polipéptidos alterados también son sintetizados en un sistema de traducción *in vitro*, lo que implica que no son producto de una modificación post-traduccional.

Las cepas revertantes sintetizan exclusivamente el monómero α, tanto *in vivo* como en un sistema de traducción *in vitro*, lo que sugiere que este fenotipo es el resultado de una mutación regulatoria que impide la expresión del polipéptido β.

Las cruzas realizadas entre las revertantes y la cepa silvestre no generan cepas auxótrofas de glutamina, por lo que se puede concluir que esta segunda mutación se encuentra altamente ligada a la mutación *gln*$^-$ original.

La frecuencia tan baja con la que son obtenidos los auxótrofos de glutamina se debe, en parte, a la presencia de las dos isozimas de GS, ya que para que una mutación sencilla resulte en el fenotipo Gln$^-$, deberá inactivar ambas isozimas.

La presencia del polipéptido γ es una condición necesaria pero no suficiente para inactivar el monómero α, ya que cuando estos auxótrofos son crecidos en glutamato a 37°C, el polipéptido γ se encuentra presente y asociado al α. Esto puede ser debido: a que no existe suficiente polipéptido γ para titular (inactivar) la totalidad de α, a que la vida media de γ sea menor en estas condiciones, o a que se requiera la participación de otros factores como la acumulación diferencial de algunos aminoácidos con efectos inhibitorios sobre la GS α.

La participación de dos vías diferentes para la asimilación de amonio en *N. crassa* se ha fundamentado en estudios fisiológicos con la cepa silvestre (40,68), en estudios cinéticos de las enzimas (J. Guzmán y J. Mora, en preparación) y en estudios fisiológicos y bioquímicos de mutantes auxótrofas de glutamina (este trabajo).

La utilización de estos diferentes enfoques, ha permitido aclarar la función diferencial de los polipéptidos α y β de GS en *N. crassa*. En otros modelos biológicos en los que se han encontrado dos GSs, tales como

Rhizobium, hojas y nódulos de plantas y algunos *Bacilli*, se han generado una serie de hipótesis para explicar su presencia pero aún no se ha obtenido la evidencia requerida para asignar definitivamente una función a cada una de las dos enzimas.

El aislamiento y caracterización de los auxótrofos reportados en el presente trabajo han hecho posible, la purificación del polipéptido α de GS. Asimismo, estas cepas han servido como punto de partida para la búsqueda de nuevas mutantes con alteraciones en el polipéptido α.

Por último, los resultados obtenidos han permitido generar nuevas preguntas acerca de la asimilación de nitrógeno en *Neurospora*:

- Aunque el polipéptido α es el principal responsable de la asimilación de amonio en condiciones de limitación, ¿existe alguna participación de la GS β en estas condiciones?.
- la expresión de los polipéptidos α y β de GS ¿se encuentra coordinada por un regulador común?,
- ¿Cuántos son y en que localización relativa se encuentran los genes estructurales de los polipéptidos α y β?
- ¿hay condiciones de cultivo donde la GS sea una enzima híbrida, constituída por monómeros α y β?,
- los polipéptidos α de las mutantes y revertantes, ¿son idénticos a los de la cepa silvestre?
- ¿la síntesis de α y β se regula a nivel de la transcripción, del procesamiento o del transporte del RNA mensajero?,
- ¿que tanto se parecen entre si la GS α y GS β?.

Para responder a estas preguntas se requiere llevar a cabo una serie de experimentos tales como los siguientes:

- Determinar la composición de aminoácidos de la GS α y GS β. Esto permitirá conocer que tan semejantes son estas dos proteínas. La evidencia conclusiva para determinar la semejanza solo se puede obtener secuenciando ambas proteínas.

- Obtener un mayor número de mutantes afectadas en la actividad de GS β, con el objeto de elaborar un mapa genético fino del (los) gene(s) de GS β, así como para definir la existencia de *loci* regulatorios.

- Aislar cepas con mutaciones en GS α; con el objeto de definir el número y la localización relativa de los genes estructurales que codifican para los polipéptidos α y β. Las mutaciones en α, también serán de utilidad para definir claramente la participación de la GS β en la asimilación de amonio.

- Buscar un mayor número de revertantes Gln^+ a partir de los auxótrofos de glutamina, con el objeto de obtener cepas que recuperen el fenotipo silvestre.

- Estudiar la expresión de los polipéptidos α,β,β' y γ en heterocariones forzados entre cepas mutantes, revertantes y silvestre, para tratar de definir *loci* regulatorios.

- Finalmente, el aislamiento de los genes estructurales de las enzimas que participan en el metabolismo nitrogenado permitirá definir claramente los niveles de regulación de estas enzimas (Transcripción, procesamiento, etc.), así como el aislamiento y caracterización de secuencias regulatorias.

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