UNIVERSIDAD NACIONAL AUTONOMA DE MEXICO

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REGULACION ALOSTERICA Y MECANISMOS DE COOPERATIVIDAD

DE LA ENZIMA GLUCOSAMINA-6-FOSFATO DESAMINASA DE *Escherichia coli.*

Un estudio de correlación estructura-función utilizando modificación química, técnicas fisicoquímicas y mutagénesis dirigida.

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TESIS PARA OBTENER EL GRADO DE

DOCTOR EN INVESTIGACION BIOMEDICA BASICA

(Bioquímica)

El trabajo experimental fue realizado en el laboratorio No. 6 del Dpto. de Bioquímica de la Facultad de Medicina de la UNAM, con la asesoría del Dr. Mario L. Calcagno.

La construcción de las mutantes fue realizada durante una estadía de tres meses en el laboratorio de la Dra. Jacqueline Plumbridge, IBPC, París, Francia.

Este proyecto ha recibido los siguientes apoyos:

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Resumen

La glucosamina-6-fosfato desaminasa (EC 5.3.1.10) cataliza la conversión reversible de la D-glucosamina-6-fosfato (GlcN6P) en D-fructosa-6-fosfato (Fru6P) y amoniaco. Este es un paso clave en la vía de catabolismo de los aminoazúcares en *Escherichia coli*, y está regulada tanto a nivel de la transcripción como de las proteínas. El gen *nagB* que codifica la desaminasa es parte del regulón *nagE-nagBACD*, cuya expresión se induce cuando la bacteria crece en un medio de cultivos que contiene aminoazúcares. La molécula inductora es la N-acetil-D-glucosamina-6-fosfato (GlcNAC6P) que es también el activador alostérico de la desaminasa.

La clonación del gen *nagB* de la desaminasa ha facilitado considerablemente el estudio de esta enzima que se obtiene a partir de cepas sobreproductoras y de la que se han construido mutantes sitio específicas. La enzima puede ser facilmente purificada por cromatografía de afinidad alostérica. La Desaminasa ha sido recientemente cristalizada, los resultados preliminares indican que la enzima posee una estructura cuaternaria, basado en un arreglo hexagonal de las subunidades, con un eje triple de simetría y tres ejes dobles. Esta estructura de trímero de dímeros también se deduce del estudio de los puentes disulfuro de la enzima. Las cisteínas 219 se encuentran oxidadas formando tres disulfuros intercatenarios, este resultado confirmatorio de la estructura cristalográfica y demuestra la existencia de tres contactos isólogos entre las subunidades.

La glucosamina-6-fosfato desaminasa, es una enzima alostérica de tipo K, su cinética cooperativa y la activación por GlcNAc6P pueden ser descritas por el modelo MWC, considerando fijación no exclusiva del sustrato y fijación exclusiva del activador alostérico. La naturaleza concertada de la transición alostérica fue confirmada por estudios con el inhibidor sin salida.

Por medio de diferentes técnicas espectrofotométricas, se ha demostrado la presencia de un residuo de tirosina característicamente ácido (pK = 8.75), localizado en o cerca del sitio alostérico. Este residuo fue identificado en la secuencia como la Tyr 121 y se construyeron las mutantes 121W, y 121T.

En las desaminasas modificadas la afinidad por el activador alostérico es de dos ordenes de magnitud menor. Ambas mutantes presentan una cinética de activación alostérica atípica que evidencia la existencia de un confórmero alostérico intermediario estable. Las mutantes presentan cambios en la Kcat, que a su vez dependen de la concentración del activador alostérico lo que hace que se comporten como un sistema alostérico mixto K/V.

Synopsis

Glucosamine-6-phosphate deaminase (E.C. 5.3.1.10) catalyses the reversible conversion of D-glucosamine-6-phosphate (GlcN6P) into D-fructose-6-phosphate (Fru6P) and ammonia. This is a key step in the pathway of amino sugar utilisation in *Escherichia coli* which is regulated both at the transcriptional and the protein level. The gene *nagB* encoding the deaminase is part of the *nagE-nagBACD* regulon, which expression is induced when the bacterium is grown in D-glucosamine (GlcN) or N-Acetyl-D-glucosamine (GlcNAc) as carbon source. The molecule that induces the regulon is N-Acetyl-D-glucosamine-6-phosphate (GlcNAc6P); this metabolite is also the allosteric activator of deaminase. The activity of the deaminase must be strictly controlled to prevent a "futile cycle" operating within the cell, involving deaminase glucosamine-6-P synthase. The cloning of the gene *nagB* in the last years, together with the other genes of the regulon, has considerably facilitated the study of the deaminase, making available gram amounts for structural studies, and allowing the construction of site-directed mutants. Glucosamine-6-phosphate deaminase was obtained from a strain of *E. coli* transformed with a pUC18 plasmid having a 1095 bp fragment containing the *nagB* gene, which overproduces constitutively the enzyme from the *lac* promoter. The enzyme can be easily purified by allosteric-site affinity-chromatography. The enzyme was recently crystallised; preliminary results with data at 2.1 Å resolution demosntrated that the enzyme has an hexagonal array of its subunits.. Self-rotation function studies suggest that the hexamers have a point group symmetry of the type 32, centered on a 3-fold axis. Crystallographic evidence suggests that the hexamer subunits are arranged as dimers with three local 2-fold axes passing through the center of three dyads that forms the hexamer. This "trimer of dimers" structure is also evident from the study of the sulfhydryl groups of the enzyme. There are four Cys per polypeptide chain, three have their SH reduced and one, corresponding to Cys219, is oxidised forming three interchain disulfide bridges per molecule. This finding demonstrates the existence of three isologous intersubunit contacts in the structure. This structure is also consistent with thermal denaturation curves of the deaminase obtained by differential scanning calorimetry at neutral pH.

Glucosamine-6-phosphate deaminase is an allosteric enzyme of the K-type. It displays intense homotropic cooperative kinetics. It can be described by the non-exclusive substrate binding case of the MWC model. The allosteric activator, GlcNAc6P binds with a K_{dis} of 30-35 ΩM (for the R conformer) and behaves as an exclusive-binding activator, producing Michaelis-Henri kinetics at saturating concentrations. It has been shown by differential UV and differential circular dichroism (CD) spectrophotometry that a distinctly acidic tyrosine residue (hydroxyl pK = 8.75) is involved in the GlcNAc6P binding site. This tyrosyl residue was identified in the sequence as Tyr121, and the site-directed mutants Tyr121→Thr and Tyr121→Trp were constructed. GlcNAc6P binding is impaired but not suppressed in these mutants The allosteric transition to the higher affinity conformer (R) produced by the binding of homotropic (substrate or dead-end inhibitor) or heterotropic (GlcNAc6P) ligands, completely protects these thiols against chemical modification. This property permits the study of the allosteric transition, and the mutant Cys239→Trp was constructed to introduce a "chiral label" for this purpose. The suppression of Cys239 by site-directed mutagenesis produced an enzyme with a lower allosteric interaction energy. Cooperative behaviour of deaminase is strongly dependent on pH; at least three dissociable groups were shown to be inolved, one of these being the thiol group from Cys239→Ser.

I N D I C E

INTRODUCCION.

I. LA ORGANIZACION DE LOS GENES DEL CATABOLISMO DE LOS AMINOAZUCARES Y SU REGULACION EN *Escherichia coli.*

II. GLUCOSAMINA-6-FOSFATO ISOMERASA DESAMINASA DE *Escherichia coli K.*

III. INGENIERIA DE PROTEINAS: Una alternativa para el estudio estructural y funcional de las enzimas.

OBJETIVOS.

RESULTADOS.

I. INTERACCION DE LA GlcNAc6P CON LA ENZIMA GLUCOSAMINA-6-FOSFATO DESAMINASA. Un bello ejemplo de una transición concertada.

II. INFLUENCIA DE LAS MUTACIONES SOBRE EL EQUILIBRIO ALOSTERICO.

III. LA UNIDAD ALOSTERICA MINIMA. Aspectos estructurales de la Glucosamina-6-fosfato desaminasa.

RECAPITULACION

I. LA ORGANIZACION DE LOS GENES DEL CATABOLISMO DE LOS AMINOAZUCARES Y SU REGULACION EN *Escherichia coli.*

a). EL REGULON *nag*

Los aminoazúcares, N-acetil-D-glucosamina (GlcNAc) y D-glucosamina (GlcN) son metabolitos esenciales para el crecimiento bacteriano, ya que son precursores de componentes de la pared celular y de la membrana externa [1]. Cuando al menos uno de estos precursores está presente en el medio de cultivo, el aminoazúcar se capta y se utiliza en la síntesis de la mureína de la pared celular y los lipopolisacáridos de la membrana externa[2]. En ausencia de una fuente externa de aminoazúcares, la bacteria sintetiza glucosamina a partir de la D-fructosa-6-fosfato y la L-glutamina[3,4]. Tanto la GlcN como la GlcNAc son excelentes fuentes de carbono y nitrógeno para la bacteria *Escherichia coli*, y en particular la GlcNAc produce velocidades de crecimiento equivalentes a las que se obtienen con glucosa[5]. Las enzimas necesarias para el transporte y el catabolismo de los aminoazúcares, se encuentran agrupadas en *E. coli* en el regulón *nag*. Éste se localiza en el mapa cromosómico de *Escherichia coli* a los 15.5 min [6,5] y está formado por dos operones divergentes *nagE y nagBACD*[7,8] (Fig. 1). El gen *nagE* codifica para la proteína transportadora específica (EII^{Nag}) de GlcNAc del sistema de la fosfotransferasa dependiente del fosfoenolpiruvato (PTS), y produce

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[1] Mirelman, D. (1979). *In Bacterial outer membranes.* Inouye, M. ed. John Wiley & Sons, Inc. New York.

[2] Raetz, C. R. (1987) . *in Escherichia coli and Salmonella typhimurium: cellular and molecular biology.* American Society for Microbiology. Washington, D.C.

[3] Darvas, M (1971). *J. Bacteriol.* 105: 467-471.

[4] Wu, H. C. y Wu, T. C. (1971). *J. Bacteriol* 105:455-466.

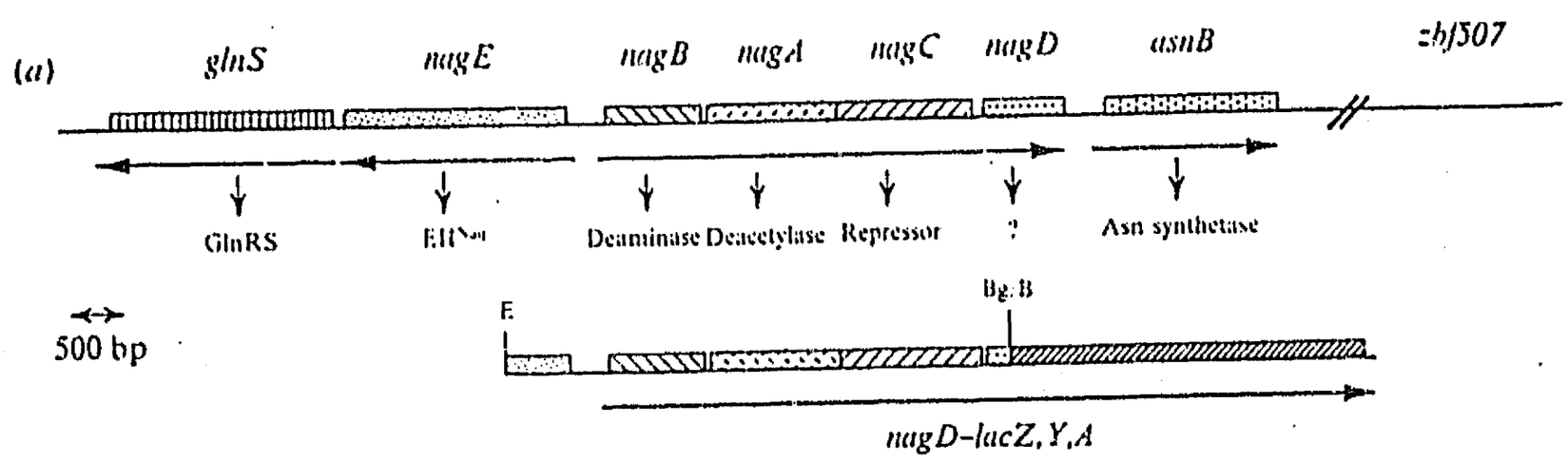
[6] Bachmann, B. J. (1990). *Microbiological Reviews,54*130-197

[5] White, R. J. (1968).*Biochem. J. 106:847-858.*

[7] Rogers, M. J., Ohgi, T., Plumbridge, J. A. & Soll, D. (1988). *Gene, 62*: 197-20.

[8] Plumbridge, J. A. (1989). *Mol. Microbiol* 3: 506-51

GlcNAc6P intracelular[9] [10]. En la dirección opuesta se expresan los genes *nagB* y *nagA*[8] que codifican respectivamente dos enzimas que degradan la GlcNAc6P a D-fructosa-6-fosfato (Fru6P), la N-acetil glucosamina 6 fosfato desacetilasa (*nagA*) y la Glucosamina 6 fosfato desaminasa (*nagB*). El tercer gen, *nagC*, codifica una proteína represora del regulón *nag*[8,11]; al gen *nagD* no se le ha atribuido ninguna función, si bien el alineamiento de secuencias de aminoácidos sugiere que tiene alguna relación con fosfatasas, por la homología que presenta con esta familia de proteínas [12] (Fig. 1).



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[9] Jones-Mortier y Kornberg, (1980). *J. Bacteriol.* 111:290-291

[10] White, R. J. (1970). *Biochem. J.* 106:121-125.

[11] Vogler, A. P. and Lengeler, J. W. (1989). *Mol Gen Genet.* 219: 97-105.

[12] Altamirano, M. M. y Doolittle,R. (1991) *Ejercicios realizados en el taller del curso: Prediction of protein structure*

b. CONTROL DE LA EXPRESION DEL REGULON *nag* EN *Escherichia coli.*

1. La inducción del regulón *nag* .

Los genes agrupados en los operones *nagE-nagBACD* se inducen conjuntamente cuando la bacteria es cultivada en GlcNAc como única fuente de carbono y nitrógeno. Los experimentos de White en 1968[5] aportan la primera evidencia de la inducción de la expresión de las enzimas glucosamina-6-fosfato desaminasa y glucosamina-6-fosfato desacetilasa, producida por el cultivo de la bacteria en un medio con GlcNAc. La actividad de estas enzimas, aumenta aproximadamente 20 veces con respecto al nivel de expresión no inducido. Posteriormente Plumbridge[13], en sus experimentos de fusión de los genes *nagE* y *nagB* con el gen *lacZ*, determinó los niveles de expresión alcanzados cuando la bacteria crece en presencia de diferentes fuentes de carbono: glucosa, glicerol, gluconato, GlcN y GlcNAc. Los resultados muestran que la GlcN y la GlcNAc producen velocidades de crecimiento mayores (2 y 7 veces respectivamente) en relación con los obtenidos con glicerol; así como 5 y 20 veces mayores con respecto a la glucosa. Lo anterior permite concluir que ambos operones del regulón son inducidos en paralelo por GlcNAc y GlcN y que son susceptibles a la represión catabólica, si bien el efecto es más fuerte para el caso del gen nagB. Por otra parte las diferencias observadas en las velocidades de crecimiento y en los niveles de inducción producidos por estos dos aminoazúcares, puede explicarse debido a que la GlcN es un sustrato deficiente para el transportador y por lo tanto no puede acumularse en la célula. También puede considerarse que la GlcNAc no sólo se transporta con mayor eficiencia sino que genera directamente una fuerte señal de inducción o que ésta es diferente. Sin embargo,es concluyente que la inducción no depende de la actividad de un sistema de transporte particular; un *nagE* o ptsM para GlcNAc o ptsM para GlcN es suficiente, lo que sugiere que la señal de indución se recibe en el interior de la célula[13].

Se han aislado bacterias mutantes que son incapaces de crecer en medios con GlcNAc como única fuente de carbono y nitrógeno; se conocen en detalle dos alelos nagB2 y nagA1[5]. La primera de estas mutaciones impide el crecimiento de la bacteria tanto en

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13 Plumbridge, J. A. (1990) *J. Bacteriol* **172** 2720-2735

GlcN como en GlcNAc. La segunda, en cambio, sólo lo hace cuando el medio contiene GlcNAc. La mutación nagA1 es pleyotrópica, aumenta los niveles de la desaminasa e incrementa la actividad del transportador de la GlcNAc, lo que produce una acumulación intracelular de GlcNAc6P[5] [14]. Las mismas característica fenotípicas se encuentran cuando se realizan otras mutaciones en el gen *nagA*, construidas *in vitro* por la inserción de un *cassette* de resistencia a antibióticos[15] o por mutagénesis λp*lac*Mu *in vivo*[11]. Esto a su vez produce la inducción endógena, puesto que la GlcNAc6P es el inductor intracelular del regulón *nag*, que se une a la proteína represora evitando la formación del complejo DNA-represor[15].

2). Papel del complejo funcional cAMP/CAP.

En el centro de la región intergénica del regulón, existe una porción del DNA que tiene una secuencia homóloga, a la secuencia consenso encontrada en otros regulones para el sitio de unión de la proteína CAP, y que es el lugar de interacción del complejo cAMP/CAP. Este sitio es simétrico, el motivo TGTGA altamente conservado esta presente sobre la hebra proximal al operón *nagE*, mientras que la secuencia TGGTGA (con una G insertada) está presente en el lado del operón *nagB*. La expresión de ambos operones es estimulada por la proteína CAP sin embargo el gen nagE es mucho más dependiente[13,15].

3). La formación de una asa de DNA es necesaria para la represión de los genes *nagE y nagB*.

La expresión de los genes *nag* está controlada por un represor, codificado por el gen *nagC*. El represor *nag*, se une a los operadores en el sitio de superposición de los promotores de los dos operones divergentes *nagE* y *nagB*, esta región del DNA que

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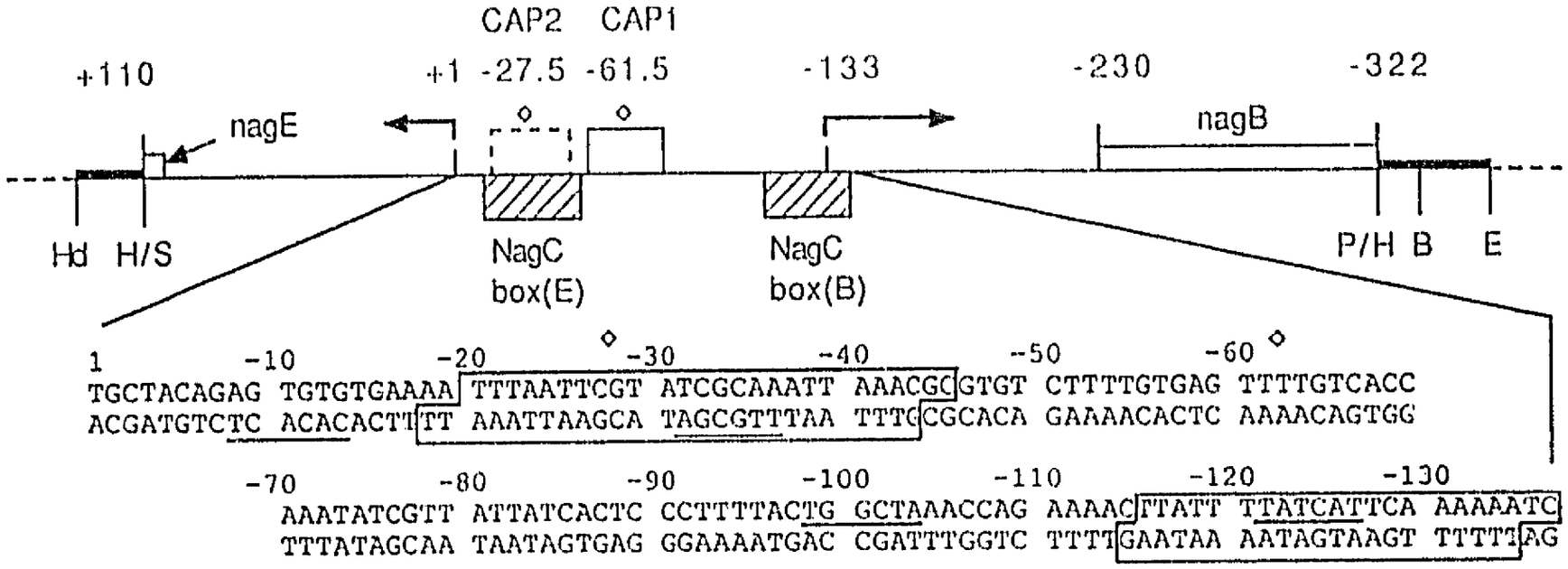
[14] Bornholm, N. J., y Dobrogosz, W. J. (1970). *J. Bacteriol* **101**:384-391.

[15] Plumbridge, J. A. (1991). *Molecular Microbiology* **5**:2053-2062.

es protegida con la unión del represor es llamada NagC CajaB y CajaE[16] (Fig. 2) . En esta región del DNA existe también un sitio de unión para la proteína CAP entre los dos operadores. La presencia del complejo cAMP/CAP estabiliza la interacción del DNA con el represor formando un complejo ternario DNA-Represor nag-cAMP-CAP[13,16].

A *nagE-B*



El análisis de secuencia del DNA en esta región, permite encontrar cierta homología entre las secuencias de otros sitios de unión de represores en procariotes, aunque estos últimos son habitualmente palindrómicos, y en este caso es cuasipalindrómica. Estudios *in vitro* [16] e *in vivo* [17] de la interacción del represor Nag con el operador, demostraron que una mutación de 4 pares de bases sobre la caja B (sitio de fijación del represor sobre *nagB*) provoca una desrrepresión casi total de ambos genes; la inserción de 6 pares de bases, la mitad de un giro helicoidal entre el sitio CAP y la caja B, incrementa 3 veces la expresión de los dos genes. Por el contrario la introducción de un giro helicoidal completo (10 pares de bases) reprime la expresión de los genes.

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[16] Plumbridge, J. A. y Kolb, A. (1991). *J. Mol. Biol.* 217:661-670.

[17] Plumbridge, J. A. y Kolb, A. (1993) Mol. Microbiol. enviado

Estas observaciones constituyen una fuerte evidencia de que la unión del represor al DNA es un fenómeno cooperativo consistente en la interacción de la proteína con estos dos sitios, que se caracterizan por una aparente hipersensibilidad a la enzima DNasa I y cuyos centros están separados entre sí, por 94 pares de bases y por 9 giros de la doble hélice del DNA, lo cual trae como resultado la formación de una asa de DNA que es necesaria para la represión de los dos operones divergentes.

La formación del bucle de DNA, origina ventajas al sistema, como es la inducción simultánea y rápida de los operones divergentes que codifican las proteínas encargadas del transporte y las funciones catabólicas.

c. METABOLISMO DE LA GLUCOSAMINA Y LA N-ACETIL GLUCOSAMINA

La GlcNAc se transporta al interior de la célula por medio de su acarreador específico que forma parte del sistema PTS[9]. La GlcN, en cambio, penetra en la célula principalmente por la acción del complejo EIIM,P/III$^{Man\ 9}$. Durante su transporte ambos azúcares se fosforilan la posición 6; la GlcNAc6P se desacetila por acción de la desacetilasa que la convierte en GlcN6P, la cual a su vez se convierte en Fru6P y amonio por medio de la desaminasa (Fig. 3).

En ausencia de aminoazúcares en el medio intracelular, la bacteria puede sintetizar GlcN6P a partir de Fru6P y glutamina a través de la enzima glucosamina-6-fosfato sintetasa (Glutamina-D-fructosa-6-fosfato amidotransferasa, Fig 3). Esta enzima es codificada por el gen *glmS*, localizado a los 84 min del mapa cromosómico de *E. coli*[18]. Este gen tiene un mecanismo de control muy preciso, que reduce su expresión, cuando el regulón *nag* se encuentra desrreprimido; lo anterior evita la formación de un ciclo improductivo, con gran pérdida de energía, producido por la actividad simultánea de las enzimas glucosamina-6-fosfato desaminasa y glucosamina-6-fosfato sintetasa[5].

Plumbridge y colaboradores[19] utilizaron técnicas de inmunotransferencia y medidas de actividad enzimática, para detectar los niveles de expresión de las proteínas: Glucosamina-6-fosfato sintetasa, Glucosamina-6-fosfato desaminasa y N-acetil

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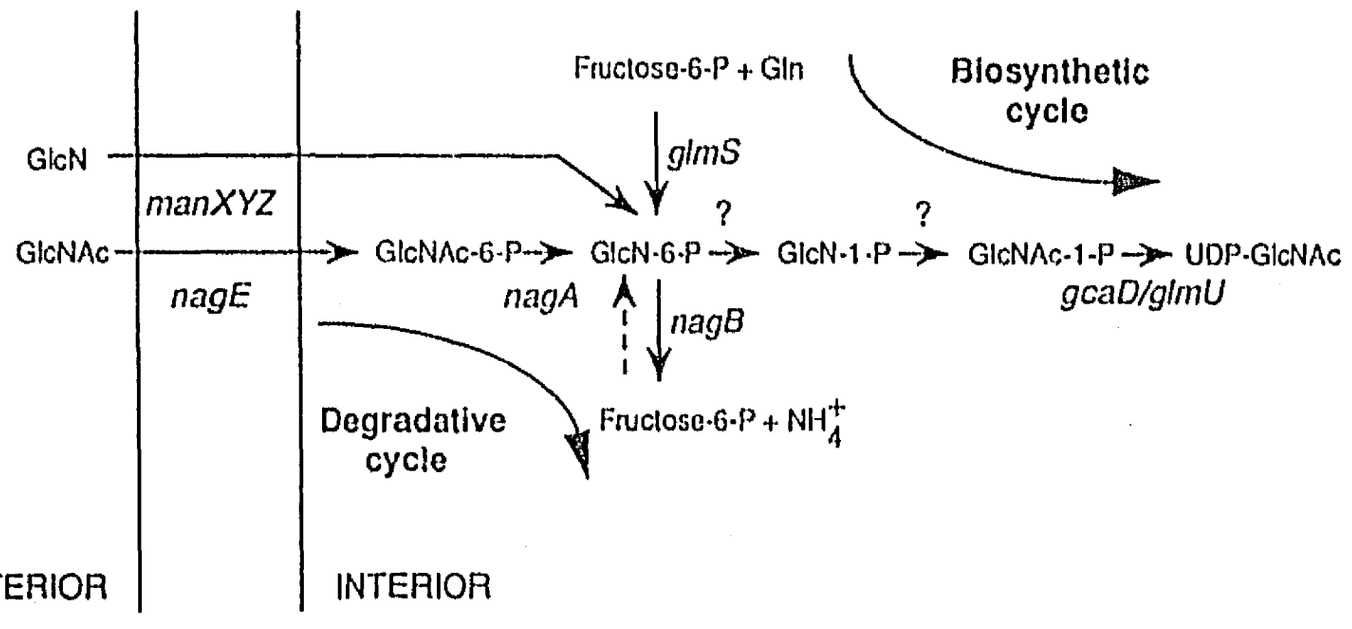
[18] Walker, J. E.,, N. J. Gay, M. Saraste, y Eberle, A. N. (1984). *Biochem, J.* 224:799-815.

[19] Plumbridge, J. A., Cochet, O., Souza, J. M., Altamirano, M. M. y Calcagno, M. *J. Bacteriol. en prensa.*

glucosamina 6 fosfato desacetilasa; con el fin de estudiar la regulación de las enzimas que participan en la síntesis y degradación de los aminoazúcares. Los resultados obtenidos demuestran que la represión de la expresión del gen *glmS* ocurre no sólo cuando la bacteria se cultiva en un medio con GlcN o GlcNAc, sino que también en condiciones en las cuales el represor *nag* presenta mutaciones que lo desrreprimen, o en situaciones experimentales en las cuales existe una acumulación del inductor (GlcNAc6P) como por ejemplo en cepas que tienen interrumpido el gen *nagA* o que producen desacetilasa inactiva. Esto último permite concluir que la concentraciones elevadas en el medio intracelular de la GlcNAc6P, no constituye por sí misma la señal que reprime la expresión de la sintetasa, sino que más bien parece tratarse de un metabolito intermediario de la síntesis de la GlcN6P por ejemplo la GlcN1P o el UDP-GlcNAc (Fig 3).



Es interesante hacer notar que la glucosamina sintetasa en eucariotes se inhibe alostéricamente con la UDP-GlcNAC[20,21], mientras que en la enzima proveniente de *E. coli* este metabolito carece de efecto regulador. Sin embargo sería muy interesante que este metabolito actuara completamente diferente en los dos sistemas; en procariotes como un efector transcripcional y en eucariotes como un inhibidor enzimático. Por otra parte esto también se apoya en el hecho que el gen *urf* que codifica para la proteína N-acetil-glucosamina-1-fosfato uridiltransferasa[22] se localiza inmediatamente corriente arriba del gen *glmS* en el cromosoma de *E. coli*.[23] La distancia intergénica tan pequeña que existe entre ambos genes y la secuencia de DNA que no presenta ninguna homología con la secuencia consenso de otros promotores sugiere que estos genes se transcriben, y podría suceder que el gen *urf* tuviera el mismo mecanismo de regulación que el gen *glmS*[19].

II. GLUCOSAMINA-6-FOSFATO ISOMERASA DESAMINASA DE *Escherichia coli k.*

a). EXPRESION, PURIFICACION Y PROPIEDADES MOLECULARES.

La enzima Glucosamina-6-fosfato desaminasa (D-Glucosa-6-fosfato cetol isomerasa desaminante, E.C. 5.3.1.10) de *E. coli* es un homopolímero hexamérico con subunidades de 29.7 kDa[24]. Esta enzima fue purificada por primera vez en nuestro laboratorio a partir de *E. coli B*, induciendo la expresión del gen *nagB* al cultivar la bacteria en GlcN como única fuente de carbono y nitrógeno[24]. La clonación del regulón *nag* por Plumbridge[6] ha facilitado enormente el estudio cinético y estructural de la desaminasa, ya que nos ha permitido obtener la proteína en grandes cantidades, y con menor tiempo y costo.

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[20] Frisa, P. S. y Sonneborn, D. R. (1982) *Proc. Natl. Acad. Sci. USA.***79:** 6289-6293.

[21] Winterburns,P. J. y Phelps, C. F. (1971). *Biochem. J.* **121:**711-720.

[22] Mengin-Lecreulx, D. Comunicación personal.

[23] Walker, J. E., Saraste M., y Eberle, A. N. (1984) *Biochem. J.* **224:** 799-815.

[24] Calcagno, M. Campos, P., Mulliert, G. Y Suástegui, J. (1984). *Biochim. Biophys. Acta.***787:** 165-173

Construcción de una cepa hiperproductora. El gen *nagB* fue aislado del plásmido pB31-1, previamente construido por Plumbridge[8]. Se aisló como un fragmento *Fsp*I-*Cla*I de 1.095 kb, que tiene interrumpido el promotor *nagB*. Este fragmento fue insertado en el plásmido pUC18 previamente digerido con *Sma*I y *Acc*I, de modo que el gen *nagB* se sitúe corriente abajo del promotor *lac*. En la cepa JM101 (*lac*I^q) se controla la sobreexpresión agregando al medio de cultivo durante en la fase de crecimiento exponencial, isopropil tiogalactosido (IPTG) a la concentración de 1 mM. En las cepas, que hemos utilizado preferentemente en nuestro trabajo que son *Δlac*, la expresión es constitutiva[25].

Producción de la Biomasa. La cepa *E. coli K12* que expresa constitutivamente el gen *nagB*, se cultiva en un medio de Luria B. que contiene 100 μg/ml de ampicilina y se cosecha durante la fase de crecimiento exponencial. La sopreproducción de la desaminasa que se logra con este plásmido es del orden del 30% en relación con el total de proteínas solubles de la bacteria[25].

Purificación por cromatografía de afinidad alostérica. Se construyó una columna de afinidad, utilizando un análogo del activador alostérico (N-ε-aminocaproil-GlcN6P) inmovilizado en agarosa[24].

b. PROPIEDADES MOLECULARES.

En la tabla I se resumen algunas constantes relacionadas con propiedades moleculares de la desaminasa

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[25] Altamirano, M. M., Plumbridge, J. A., Hernández-Arana, A. y Calcagno, M. (1991) *Biochim. Biophys. Acta.* **1076**:266-272.

TABLA I

Algunas constantes fisicoquimicas de la glucosamina-6-P desaminasa de E, coli

Peso Molecular[24] = 179,870	Peso subunidad [24] = 29,780
Coef. Sedimentación[24] = 9.0 S	pH isoeléctrico[24] = 6.1
Volumen parcial específico[24] = 0.737 cm^3/g	ε_{278} (pH 8.0) = 20.0 M^{-1} cm^{-1}
K_{cat} (sentido desaminante) = 1800 s^{-1}	K_{cat} (sentido sintético) = 400 s^{-1}.
Km para GlcN6P = 2 mM	

c. ASPECTOS CINETICOS

La enzima cataliza la conversión reversible de GlcN6P en Fru6P y amonio[26,27,28].

$$\mathbf{GlcN6P + H_2O \rightleftarrows Fru6P + NH_4^+}$$

In vitro se realizan experimentos cinéticos en ambas direcciones, si bien el equilibrio favorece el sentido desaminante (K_{EQ} = 0.22M en Tris-HCl pH 7.7, 30 C)[24]. Sin embargo *in vivo* en condiciones fisiológicas, la desaminasa se comporta como una enzima catabólica. Esto resulta del estudio de las mutantes *GlmS*$^-$, que carecen de la enzima que sintetiza la GlcN6P, y que no crecen salvo que el medio se suplemente con GlcN o GlcNAc[5,10,13]. Sin embargo, Vogler y Lengeler[11] construyeron una cepa en la que la síntesis de aminoazúcares se produce por la reacción inversa de la desaminasa. Es esta cepa el operón nag está desrreprimido por inserción de un *cassette* en el gen *nagC*, que codifica el represor. En esta cepa *GlmS*$^-$, *nagC*$^-$, la bacteria puede crecer en un medio rico en sales de amonio.

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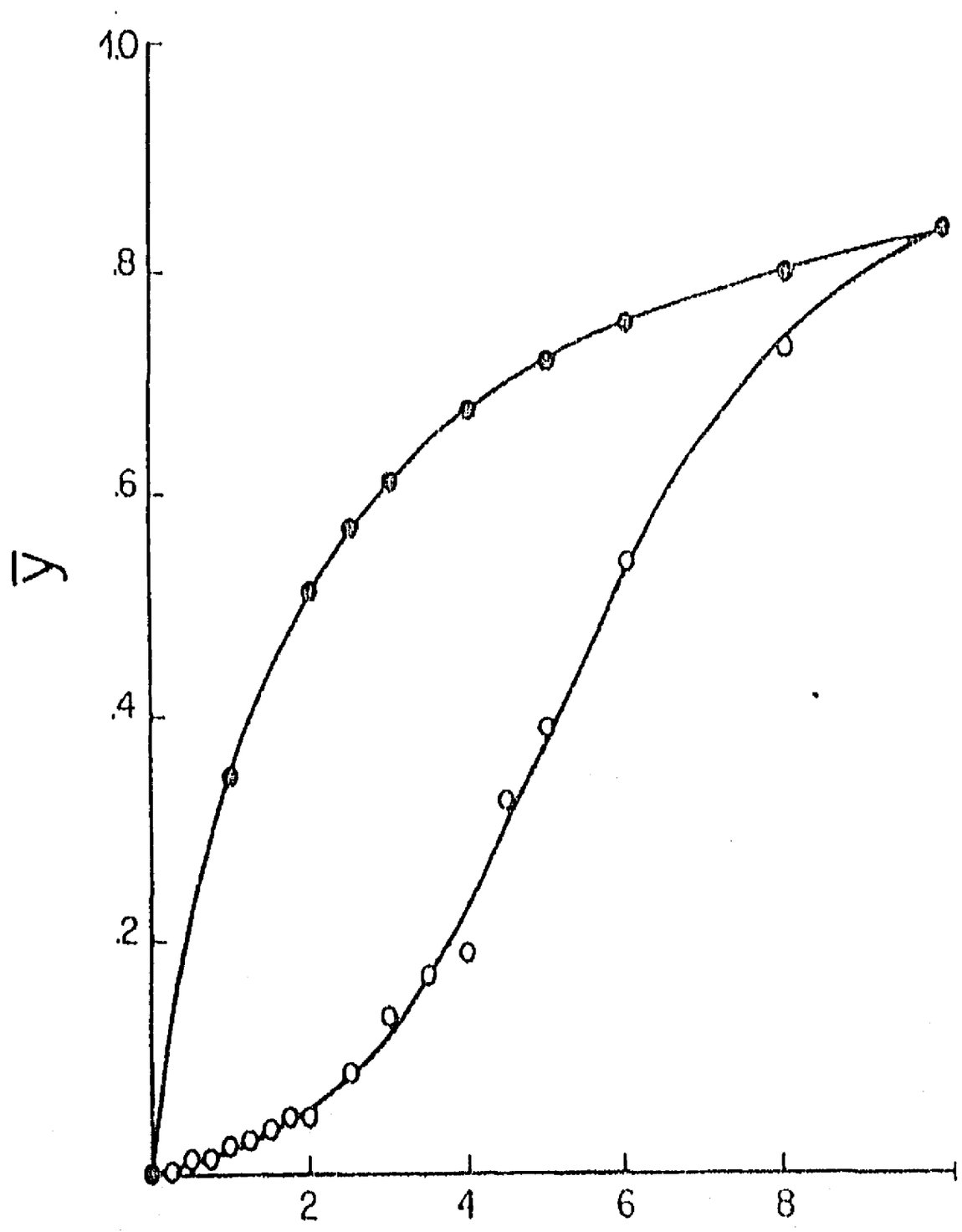
[26] Leloir, L. F. y Cardini, C. E. (1956). *Biochim. Biophys. Acta.* **54**: 273-282.

[27] Comb, D. G. y Roseman, S. (1958). *J. Biol. Chem.* **232** 807-827.

[28] Midelfort, C. y Rose, I. A. (1977) *Biochemistry* **16**: 1590-1596.

La enzima Glucosamina-6-fosfato desaminasa presenta una intensa cooperatividad homotrópica positiva con respecto al sustrato GlcN6P (h_{MAX} = 2.9 ± 0.2 a pH 7.7); en la reacción inversa, cuando el sustrato es la Fru6P la cooperatividad homotrópica es menor (h_{MAX}= 1.4 ± 0.1 a pH 7.7) e independiente de la concentración de ion amonio[24]. Este último no es un ligando alostérico. El activador alostérico, la GlcNAc6P, se comporta como un activador de fijación exclusiva, ya que produce cinética hiperbólica a concentraciones saturantes (por ej. 2 mM). Se trata además de un sistema alostérico de tipo K puro, debido a que al incrementar sucesivamente la concentración del activador, los valores de $S_{0.5}$ disminuyen (efecto K), mientras que los valores de V_{MAX} no se modifican[24]. (Fig. 4)



Los experimentos cinéticos en ambos sentidos de la reacción, en condiciones de cinética de Michaelis-Henri, dan patrones de velocidad inicial y de inhibición por productos consistentes con un mecanismo de equilibrio rápido al azar. Esto valida el empleo de modelos de equilibrio, en el análisis de la cinética alostérica.

d. GRUPOS SH

Los estudios de modificación química de los grupos sulfhidrilo en la glucosamina-6-fosfato desaminasa[29], revelan la existencia de tres grupos sulfhidrilo por cadena polipeptídica, aunque la secuencia de aminoácidos deducida del DNA, revela cuatro cisteínas. Dos grupos SH aparecen expuestos y reactivos en la proteína nativa, mientras que un tercer grupo SH se comporta como oculto al disolvente y sólo reacciona en presencia de desnaturalizantes. El sulfhidrilo faltante corresponde a un residuo de cisteína oxidado.

La titulación con DTNB de los dos sulfhidrilos expuestos, puede ajustarse a una reacción de seudo-primer orden, lo que implica que ambos grupos presentan la misma reactividad, lo que sugiere que se encuentran en un mismo microambiente. La reactividad de estos SH expuestos se modifica de manera importante por la presencia de ligandos homotrópicos o heterotrópicos que inducen la transición alostérica de la proteína[29] .

La enzima con los dos grupos reactivos totalmente modificados por la introducción del grupo 5-tio-2-nitrobenzoato (TNB) o diferentes substituyentes de gran volumen, es totalmente inactiva[29], lo cual puede ser consecuencia de una distorsión conformacional de la estructura de la proteína por la presencia de grupos voluminosos y cargados. Sin embargo la metilación de las mismas cisteínas con yodometano, la enzima es activa y alostérica[29,30], pero presenta una cooperatividad homotrópica menor, con una constante catalítica (k_{cat}) de exactamente la mitad con respecto a la enzima nativa. Estos tioles se encuentran en posición vecinal, debido a que: 1) se modifican con el

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[29] Altamirano, M. M., Mulliert, G y Calcagno, M. (1987) *Arch. Biochim. Biophys.* **268: 95-100.**

[30] Altamirano, M. M., Lara-Lemus, R., Libreros-Minolta, C. y Calcagno, M. (1989)*Arch. Biochim. Biophys.* **269: 555-561.**

arsenito formando un ditioarsenito cíclico, 2) La interacción con el arsenito sólo puede revertirse con un ditiol (etanoditiol); 3) los SH pueden ser oxidados a disulfuro con el oxígeno molecular utilizando como catalizador el complejo (1,10 *orto*-fenantrolina)$_3$-Cu; en estas condiciones ya no se logran titular los SH con DTNB[30].

Todas las modificaciones químicas producidas en los SH reactivos de la proteína tanto con reactivos monofuncionales como bifuncionales, así como el derivado oxidado de estos tioles, tienen el mismo comportamiento cinético y alostérico, lo que sugiere que la región de la proteína en la cual se localizan estos sulfhidrilos vecinales, se mueven en el cambio conformacional manteniendo sus relaciones geométricas.[30]

El carácter vecinal de los SH y los efectos cinéticos producidos cuando se bloquean, los convierte en un probable sitio de regulación de la actividad de la desaminasa, tomando en cuenta esta consideración se realizaron experimentos con metales divalentes especialmente el zinc que podría ser el ligando natural de estos grupos.

El ion zinc tiene una gran afinidad por los SH reactivos de la desaminasa[31], sólo cuando está en la conformación T (menos afín por la GlcN6P). La K_{dis} para el Zn^{++} se ha calculado en 1.5 X 10^{-7} M; la unión de este metal produce los mismos cambios cinéticos descritos previamente para el bloqueo de los SH reactivos, y su efecto desaparece cuando se dializa la enzima-Zn contra un amortiguador con EDTA; sin embargo la proteína no puede ser liberada del Zn^{++} si se dializa bajo la mismas condiciones pero agregando al amortiguador una concentración saturante de GlcNAc6P. Esto es una evidencia clara, que el zinc unido a la proteína es secuestrado por el cambio conformacional T→R[31].

e. ESTRUCTURA SECUNDARIA

La Glucosamina-6-fosfato desaminasa de *Escherichia coli* no presenta ninguna homología con proteínas cuya estructura tridimensional se conoce[25], por lo tanto es un caso típico de proteína no homóloga en la cual la predicción de estructura secundaria a partir de la secuencia, en combinación con métodos fisicoquímicos como el dicroísmo circular, es el camino más adecuado en ausencia de datos cristalográficos, para

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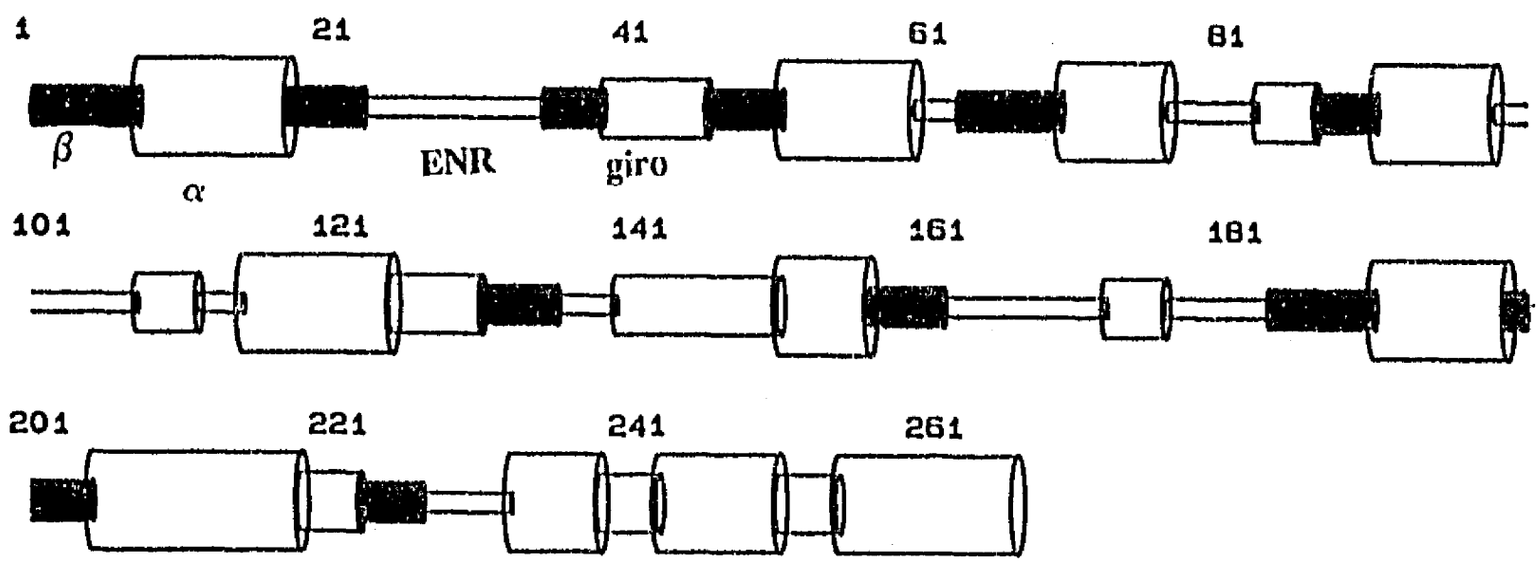
[31] Altamirano, M. M. y Calcagno, M. (1990) *Biochim. Biophys. Acta.* **1038**: 291-294.

conocer la estructura secundaria de la proteína[32]

La estructura secundaria de la desaminasa[25] se investigó simultáneamente por estudios fisicoquímicos de la proteína en solución:dicroísmo circular (CD) en el ultravioleta lejano y análisis de la estructura primaria: métodos de predicción empíricos, utilizando un esquema conjunto que se basa en la combinación de 6 algoritmos, que fueron seleccionados tomando en cuenta su diferente fundamentación teórica[33]. El contenido de hélice α (37% por predicción y 34% CD) y de B plegada (22% predicción y 20% CD) en conjunto con otras características como el marcado grado de alternancia entre segmentos α con β; el carácter anfipático del 75% de las hélices, el predominio de láminas β hidrofóbicas, lo anterior sugiere que desde el punto de vista estructural, la desaminasa pertenece a la familia de proteínas α/β (Fig. 5).



[32] Garnier, J. Levin, J. M., Gibrat, J. F. y Biou, V. (1990) *In Protein Structure, prediction and desing.* pág.11-24. Biochem. Soc. Symp. 56

[33] Deléage, G y Roux, B. (1990) *In Prediction of protein Structure and the principles of protein conformation.* Ed. G. Fasman. pág. 587-597.

Por otra parte existen otros datos que contribuyen a reafirmar esta aseveración, el espectro de Dicroísmo circular de la enzima, es característico de las proteínas α/β[34], con una intensa banda positiva a 192 nm y dos bandas negativas a 219 y 210 nm. El análisis de la composición de aminoácidos de la desaminasa utilizando el algoritmo de Nishikawa[35] que correlaciona ésta con el predominio de estructura supersecundaria da una distancia del centro de la proteína de 2.71 que es un valor compatible con los obtenidos para otras proteínas α/β. Todo este conjunto de evidencias nos permite clasificar a la desaminasa en la familia de proteínas α/β[25].

III. INGENIERIA DE PROTEINAS: Una alternativa para el estudio estructural y funcional de las enzimas.

La facilidad de explorar y extender nuestra compresión de la estructura y función de las proteínas, es un fenómeno de la década de los 80. Esto se debe a la conjunción de la genética molecular, la bioquímica, la computación y la cristalografía, para culminar en la creación de una nueva área de investigación: la ingeniería de proteínas. Esta nueva área combina los conocimientos y las metodologías de estas diferentes áreas de especialización para resolver problemas de Química de Proteínas y Enzimología. Es posible, por ejemplo, analizar de una manera completamente nueva las interacciones proteína-ligando y la estabilidad de las proteínas, disecando la participación individual de los aminoácidos y su contribución a la función, y su importancia estructural.

La ingeniería de proteínas se fundamenta y tiene como punto de partida, el principio central de evolución molecular: Las nuevas propiedades y funciones que se encuentran en las proteínas, son el resultado de la formación y acumulación de mutaciones espontáneas en los genes, a lo largo de grandes períodos de tiempo. La evolución es poderosa e inexorable; es también ciega y lenta. En la actualidad es posible introducir mutaciones en posiciones precisas en los genes usando técnicas *in vitro*, adelantándose a los mecanismos naturales de evolución y acelerando y gobernando

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[34] Manavalan, P. y Johnson, W. C. Jr. (1983) *Nature* 305: 831-832.

[35] Nishikawa, K y Ooi, T (1986) *Biochim. Biophys. Acta* 871: 45-54.

el proceso. Lo anterior ha dado una nueva alternativa en el estudio de las proteínas, y en el diseño *de novo* de proteínas

La ingeniería de proteínas incluye la construcción, análisis y el uso de proteínas modificadas, lo que puede incluir cambios en la especificidad de los sustratos de una enzima o incrementar la estabilidad de la proteína para usos industriales.

Claramente la introducción de técnicas de mutagénesis dirigida, con la posibilidad de introducir, cambiar o quitar un aminoácido deseado dentro de la secuencia de una proteína, una vez que el gen ha sido clonado y secuenciado, representa una gran oportunidad para estudiar y comprobar los mecanismos moleculares propuestos.

OBJETIVO

El proyecto de investigación que he realizado durante el doctorado, tiene como objetivo fundamental desarrollar un estudio de correlación estructura-función de la interacción proteina-ligando alostérico usando como modelo de experimentación a la enzima glucosamina-6-fosfato desaminasa de *E. coli*; con un enfoque multidisciplinario, como un primer paso hacia la ingeniería de proteínas.

ASPECTOS METODOLOGICOS

En los siete articulos que integran esta tesis, se realizaron experimentos multimetodológicos, que se describen en cada uno de los manuscritos que la conforman.

R E S U L T A D O S

Los resultados obtenidos en el desarrollo del proyecto, los he dividido en diferentes capítulos.

I. Interacción de la GlcNAc6P con la enzima glucosamina-6-fosfato desaminasa. Un bello ejemplo de una transición concertada.

1. N-Acetyl-D-glucosamine-6-phosphate binding to the allosteric site of *Escherichia coli* Glucosamine-6-phosphate deaminase.
Myriam M. Altamirano and Mario Calcagno.
Enviado para su consideración a la revista *Eur. J. Biochem.* Junio 10 de 1993.

2. Spectrochemical evidences for the presence of a tyrosyl residue in the allosteric site of Glucosamine-6-phosphate deaminase from *Escherichia coli*
Myriam M. Altamirano., Andrés, Hernández-Arana., Salvador, Tello. and Mario, Calcagno.
Enviado para su consideración a la revista *Eur. J. Biochem.* Julio 7 de 1993.

II. Influencia de las mutaciones sobre el equilibrio alostérico.

1. Identification of two cysteines residues forming a pair of vicinal thiols in Glucosamine-6-phosphate deaminase from *Escherichia coli* and a study of their functional role by site-directed mutagenesis.
Myriam M. Altamirano, Jacqueline A. Plumbridge and Mario Calcagno.
Biochemistry (1992), **32**:1153-1158.

2. Effects of replacement of an allosteric site residue (TYR121) on activity and allosteric properties of Glucosamine-6-Phosphate Deaminase from *Escherichia coli*.
Myriam M. Altamirano, Jacqueline A. Plumbridge and Mario Calcagno.
Manuscrito en su primera versión.

III. La unidad alostérica mínima. Aspectos estructurales de la Glucosamina-6-fosfato desaminasa.

1. Glucosamine-6-phosphate deaminase from *Escherichia coli* has a trimer of dimers structure with three intersubunit disulfides.
Myriam M. Altamirano, Jacqueline A. Plumbridge, Hugo A. Barba and Mario Calcagno.
Biochemical Journal. Aceptado con modificaciones menores Mayo 6 de 1993.
Se anexa carta.

2. Crystallization and preliminary crystallographic studies of glucosamine-6-phosphate deaminase from *Escherichia coli* K12.
E. Horjales, M. M. Altamirano, M. L. Calcagno, Z. Dauter, K. Wilson, R. C. Garratt and G. Oliva.
J. Mol. Biol. (1992), **226**:1283-1286.

RECAPITULACION

1. Allosteric regulation of glucosamine-6-phosphate deaminase from *Escherichia coli*.
Myriam M. Altamirano, Jacqueline A. Plumbridge, and Mario L. Calcagno.
Capítulo en el Libro: CHITIN ENZYMOLOGY, editado por R. A. Muzzarelli, Ancona 1993. Fecha de publicación Julio 1993.

I. Interacción de la GlcNAc6P con la enzima glucosamina-6-fosfato desaminasa. Un bello ejemplo de una transición concertada.

ALLOSTERIC PROPERTIES OF *Escherichia coli* GLUCOSAMINE-6-PHOSPHATE DEAMINASE: KINETIC CONTROL OF COOPERATIVITY BY BINDING OF HOMOTROPIC AND HETEROTROPIC LIGANDS

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Abbreviations: GlcN6P, D-glucosamine-6-phosphate; GlcNAc6P, N-acetyl-D-glucosamine-6-phosphate; GlcN-ol-6P, 2-deoxy-2-amino D-glucitol 6-phosphate; DTNB, 5,5'-dithiobis (2-nitrobenzoate).

Enzyme: Glucosamine-6-phosphate deaminase (D-glucose-6-phosphate ketol isomerase, deaminating, EC 5.3.1.10)

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SUMMARY

Escherichia coli glucosamine-6-phosphate deaminase is a hexameric allosteric enzyme, activated by N-acetyl-D-glucosamine-6-phosphate (GlcNAc6P). The binding of GlcNAc6P to the allosteric site of this enzyme was studied kinetically and by equilibrium dialysis experiments. From equilibrium dialysis, six allosteric sites were found, and the K_d for the allosteric activator was calculated to be 35 μM. Fitting velocity *versus* substrate curves to equations derived from the Monod et al. model, gives similar values for the number of binding sites and allosteric activator dissociation constant. The corresponding equation for an exclusive binding dead-end inhibitor, describes precisely the experimental curves. The competitive inhibitor, D-glucitolamine-6-phosphate produces paradoxical activation at low substrate inhibitor concentration, thereby providing additional evidence for the concerted nature of the allosteric transition and that the entire deaminase oligomer is the functional allosteric unit of glucosamine-6-phosphate deaminase. The protective effect of the T→R transition on the reactivity of thiols from two cysteinyl residues (Cys118 and Cys239) was used as a tool to analyse activator binding, and an equation derived from the allosteric concerted model is proposed to describe this effect.

Glucosamine-6-phosphate deaminase from *Escherichia coli* is an allosteric enzyme of the K-type, activated by N-acetyl-D-glucosamine-6-phosphate (GlcNAc6P). (Comb and Roseman, 1965; Calcagno *et. al*, 1984). GlcNAc6P is a key metabolite involved in the regulation of amino sugar catabolism in *E. coli*; it is the intracellular product of GlcNAc transport and the signal for activating the amino sugar utilisation which induces the expression of the genes clustered in the *nag* regulon (Plumbridge, 1991; Plumbridge *et al.*, 1993), including the *nagB* gene which encodes for glucosamine-6-phosphate deaminase. The allosteric activation of deaminase by GlcNAc6P is known; it acts as an exclusive-binding activator of the enzyme, causing hyperbolic kinetics at saturating concentrations (Midelfort and Rose, 1977, Calcagno *et al.*, 1984).

The deaminase is a hexameric molecule composed by six identical subunits (Calcagno *et al.*, 1984). Preliminary crystallographic data (Horjales *et al*, 1992) and chemical and physico chemical studies (Hernández-Arana et al., 1993; Altamirano et al. 1993) indicate that the enzyme's quaternary structure is organised as a trimer of dimers, linked through three inter-chain disulfide bonds, one for each dimer. Taking into account this information, it is reasonable to speculate about what is the functional allosteric unit of the enzyme, the entire hexamer or a smaller structure, as the disulfide-linked dimer. The studies reported herein were performed to obtain a quantitative description of the binding of GlcNAc6P to glucosamine-6-phosphate deaminase and to better characterise its allosteric equilibrium and its control by homotropic and heterotropic ligands.

EXPERIMENTAL PROCEDURES

Reagents

Biochemicals and most reagents were from Sigma Chemical Co., (St. Louis, MO). GlcNAc6P was prepared by acetylation of GlcN6P and purified by ion-exchange chromatography, according to Leloir and Cardini (Leloir and Cardini, 1962). The same procedure was used to synthesize [^{14}C]-GlcNAc6P isotopically labeled in the acetyl group with [1,1'-^{14}C]-acetic

anhydride, 1 mCi per mmole (Amersham), diluted with non-labeled acetic anhydride, to obtain a specific activity of 1.5 μCi/mmole. The purity of the product was verified by TLC on silica gel precoated plates (Sigma Chemical Co.) using the following solvent systems: a) isopropanol:15M ammonia (6:1); b) ethyl acetate: 17M acetic acid:water:15M ammonia (6:2:2:1) and c) ethanol:1M ammonium acetate:17M acetic acid:water (5:2:1:1). Spots were visualised by means of reagents for organic phosphates and amino sugars using, respectively, Hanes-Isherwood reagent and ninhydrin as described by Churms (1982). As general detection procedures, iodine vapors and 5N H_2SO_4 followed by heating at 110 °C, were used (Churms, 1982). The radiochemical purity

of [^{14}C]-GlcNAc6P was verified by autoradiographic analysis of TLC plates. To calculate the specific radioactivity of the final product (0.73 μCi mmole^{-1}), the GlcNAc6P concentration was determined by a modified Elson-Morgan reaction (Levy and McAllan, 1959), using the pure unlabeled compound as standard. The deaminase dead-end inhibitor, 2-deoxy-2-amino-D-glucitol-6-phosphate (glucitolamine 6-phosphate, GlcN-ol-6P), was synthesised and purified as described by Midelfort and Rose (Midelfort and Rose, 1977); its purity was verified by TLC using cellulose plates and solvent systems *b* and *c* (see above), developed with ninhydrin or general detection procedures.

Bacteria and enzyme

E. coli glucosamine-6-phosphate deaminase was prepared as previously described, from an over-producing strain (Altamirano *et al.* 1991). The concentration of the enzyme was calculated from its absorbance at 278 nm at pH 7.7, using its known molar absorptivity; enzyme assays and data processing were also as described (Altamirano *et al.* 1989). Unless otherwise specified, kinetic data were obtained at pH 8.0 and 30 °C, in the presence of 2.5 mM EDTA. The ENZFITTER program for non-linear regression analysis based on the Marquart algorithm (R.J. Leatherbarrow, *Elsevier Biosoft*, Cambridge, U.K.), was used to fit data to various equations.

Equilibrium dialysis experiments

The binding of GlcNAc6P to deaminase was measured using standard microcentrifuge

polypropylene tubes as dialysis chambers (Reinard and Jacobson, 1989). A dialysis membrane with a cut-off of 14 kDa was used. The tubes with the samples to be equilibrated were shaken for 8 h at 30°C in an adapted "vortex" test tube shaker. The concentration of [^{14}C]-GlcNAc6P was determined by liquid scintillation counting, knowing its specific radioactivity.

Titration of sulfhydryl groups

The kinetics of the reaction of two sulfhydryl groups in the native enzyme (Cys118 and Cys239) with DTNB was performed as described (Altamirano *et. al.*, 1989), using a double-beam spectrophotometer. From the time course of the reaction, the pseudo first-order kinetic constant was calculated at different GlcNAc6P concentrations, including zero.

RESULTS

Kinetics of glucosamine-6-phosphate deaminase activation by GlcNAc6P.

A series of initial velocity-substrate curves were obtained at different constant concentrations of the allosteric activator at pH 7.7. (figure 1A). The behaviors of limit curves (zero and high GlcNAc6P concentration) were the ones expected for a K-system and an exclusive-binding allosteric activator, (Altamirano *et al.* 1989). The fit of the data obtained in the abscence of GlcNAc6P to the general case of the Monod-Wyman-Changeux (MWC) model (Monod *et al.*, 1965), gives a non-exclusive binding constant (c) for GlcN6P of 0.021 $\pm$ 0.002, and a value for the number of sites (n) of 5.42 $\pm$ 0.22 that can be rounded-off to six, and an allosteric constant L, of 15500 $\pm$ 5500. (See also fitted constants for zero inhibitor curve, figure 3). Each curve in the set shown in figure 1A, was fitted to the following form of MWC equation (Monod *el al.* 1965):

$$v/V = \frac{Lc\alpha\,(1+c\alpha)^{n-1} + \alpha(1+\alpha)^{n-1}}{L\,/\,(1+\gamma)^{n} + (1+\alpha)^{n}} \qquad \text{(equation 1)}$$

In this expression α and γ are the specific concentration of GlcN6P and GlcNAc6P, respectively, c is the non-exclusive binding constant for the substrate, and L is the allosteric equilibrium constant. GlcNAc6P binding is assumed as exclusive to the R allosteric conformer, and **n** = 6 was a parameter. The fitted value for K_d for GlcNAc6P was 0.033 $\pm$ 0.002 mM. The plot shown in figure 1B corresponds to the same data, presented in the form of a linear transformation of equation 1. (Blangy *et al.*, 1968). The estimated K_d for the activator was 47 μM. The intercept-replot shown at the inset of figure 1B gives the K_m value for GlcN6P at infinite activator concentration; this value is 2.6 mM, close to the expected value of 2 mM (Altamirano *et al.* 1989). For reference purposes, a similar experimental series in the presence of 2 mM GlcNAc6P gave a K_m for GlcN6P of 1.88 $\pm$ 0.01 mM (not shown).

Effect of the dead-end inhibitor GlcN-ol-6P

GlcN-ol-6P is a dead-end inhibitor of glucosamine-6-phosphate deaminase (Midelfort and Rose, 1977). When assayed with activator-saturated enzyme, it behaves as a typical competitive linear inhibitor, with a K_i of 2 μM (data not shown). In the absence of activator, when deaminase displays a strongly positive homotropic cooperativity, GlcN-ol-6P behaves as an alternative homotropic ligand, causing paradoxical activation at low substrate concentration (figure 2). The data set from this experiment was fitted to the following equation, derived from MWC model, to which was added a competitive inhibitor that binds exclusively to R conformer (Segel, 1975):

$$v/V = \frac{L c\alpha\,(1+c\alpha)^{n-1} + \alpha(1+\alpha+\theta)^{n-1}}{L(1+c\gamma)^n + (1+\alpha+\theta)^n} \qquad \text{(equation 2)}$$

In this expression, θ is the specific concentration of the dead end inhibitor; other symbols have the same meaning as in equation 1.

GlcNAc6P binding to deaminase, studied by equilibrium dialysis.

The saturation curve of glucosamine-6-phosphate deaminase by GlcNAc6P, determined by

equilibrium dialysis, is shown in figure 3, where the moles of GlcNAc6P bound per mole of deaminase hexamer, were plotted versus the concentration of GlcNAc6P. The shape of the curve is sigmoidal and it has a Hill coefficient of 2.5 and a $[GlcNAc6P]_{0.5}$ of 180 μM. When the experiment was performed in the presence of a fixed high concentration of the dead-end inhibitor, GlcN-ol-6P, to shift the allosteric equilibrium in the direction to the R conformer, the curve obtained was virtually hyperbolic, and the K_d for GlcNAc6P was 35 μM. For both curves, the fitted value for the maximal saturation ratio (*i.e.*, the number of allosteric sites per enzyme molecule) was found to be close to the integer six (figure 3).

Kinetics of sulfhydryl protection by GlcNAc6P

Sulfhydryl groups from Cys118 and Cys239 react with several thiol reagents when the enzyme is in the T conformation, but they become completely hindered when the T→R transition is induced by saturation with either homotropic or heterotropic ligands (Altamirano *et al.*, 1989, 1992). The kinetic of the chemical reaction of the sulfhydryl groups in the presence of GlcNAc6P was studied and analysed according to the proposed model. The apparent pseudo first-order rate constant for the reaction of cysteines with DTNB plotted versus GlcNAc6P concentration, is shown in Fig. 4. The protective effect of the allosteric activator, can be described by the following expression (see also the Appendix):

$$v = \frac{Et\, k^* L}{L + (1+\gamma)n} \qquad \text{(equation 3)}$$

The experimental data fit well to this equation. The curve shows the expected cooperative kinetics of the GlcNAc6P protective effect.

DISCUSSION

Our study of the activation of glucosamine-6-phosphate deaminase by GlcNAc6P by non-linear regression analysis confirms the validity of the MWC model to describe the allosteric

properties of this enzyme. We assumed the existence of six allosteric and six active sites, which were the fitted values obtained in the kinetic experiments without activator or inhibitor (Figs. 1 and 3) and the number of GlcNAc6P binding sites obtained from equilibrium dialysis experiments. The fitted model implies exclusive-binding of the activator, assumption which is supported by the observation that the deaminase exhibits hyperbolic kinetics in the presence of saturating concentrations of GlcNAc6P. The quality of the fit to equation 1 can also be shown by means of the elegant plot proposed by Henri Buc in 1967 (c.f. Blangy *et al.* 1967). The value of the Michaelis constant for GlcN6P obtained from the intercept replot shown in the inset of fig. 1B, is close to the expected one, thereby providing an additional support for the validity of using equation 2 to describe the allosteric activation of glucosamine-6-phosphate deaminase by GlcNAc6P.

The precedent analysis presupposes that the allosteric transition of glucosamine-6-phosphate deaminase is concerted. Additional evidence for this assumption is the activation which is observed at low specific concentrations of the inhibitor and substrate, and the satisfactory fit obtained with equation 2, which was derived from the MWC model. We also used the know protective effect that has the T to R conformerisation upon reactivity of cysteines (Cys118 and Cys239) to study the activator-enzyme interaction. Equation 3 may be an useful tool to study the interaction of diverse homotropic or heteropropic ligands with wild-type and mutant forms of the deaminase.

Direct equilibrium binding measurements and model fitting demonstrate that the deaminase has one allosteric site per polypeptide chain. The binding curve obtained in the presence of saturating concentration of the dead-end inhibitor, GlcN-ol-6P, confirms this stoichiometry and allowed us to obtain directly the K_d for GlcNAc6P. This value corresponds to the microscopic dissociation constant of the allosteric activator from the R allosteric conformer. The curve is virtually hyperbolic, as expected if GlcN-ol-6P is an exclusive-binding ligand.

The results reported here prove also that the entire deaminase oligomer is the allosteric unit, excluding the existence of a putative smaller cooperative unit, such as the disulfide-linked dimer and suggest that each pair of subunits forming this threefold repeated structure has equivalent and symmetrical allosteric sites.

REFERENCES

Altamirano, M.M., Lara-Lemus, R., Libreros-Minotta, C.A. and Calcagno, M. (1989) *Arch. Biochim. Biophys.* **269**, 555-561.

Altamirano, M.M., Plumbridge, J.A., and Calcagno, M.L., (1993) *Biochem. J.* In press.

Altamirano, M.M., Plumbridge, J.A., Hernández-Arana, A., and Calcagno, M.L. (1991) *Biochim. Biophys. Acta* **1076**, 266-272.

Blangy, D., Buc, H., and Monod, J. (1968) *J. Mol. Biol.,* **31**, 13-35.

Calcagno, M., Campos, P.J., Mulliert, G., and Suástegui, J. (1984) *Biochim. Biophys. Acta* **787**, 165-173.

Churms, S.C. (1982) Detection reagents for paper and thin layer chromatography, Section II.III in *Handbook of Chromatography*, Vol. 1; Zweig, G. and Sherman, J. Editors-in-Chief, CRC Press Inc., Boca Raton, Florida, 1982

Comb, D.G. and Roseman, S. (1958) *J. Biol. Chem.* **232**, 807-827.

Hernández-Arana, A., Rojo-Domínguez, A., Altamirano, M.M. and Calcagno, M.L. (1993) *Biochemistry*, **32**, 2644-3648

Horjales. E., Altamirano, M.M., Calcagno, M.L., Dauter, Z., Wilson, K, , Garratt. R.C., and Oliva, G. (1992) *J. Mol. Biol.* **226**, 1283-1286

Plumbridge, J.A. (1991). *Mol. Microbiol.* **5**, 2053-2062.

Plumbridge, J.A., Cochet, O., Souza, J.M., Altamirano, M.M., Calcagno, M.L. and Badet, B. J. Bacteriol (1993). In press.

Leloir, L.F. and Cardini, C. (1962) *Methods Enzymol.* **5**, 418-422.

Levy, G.A., and McAllan, A. (1959) *Biochem. J.* **73**, 127-132.

Midelfort, C., and Rose, I.A. (1977) *Biochemistry* **16**, 1590-1596.

Monod, J., Wyman, J. and Changeux, J.P. (1965) *J. Mol. Biol.* **12**, 88-118.

Reinard, T., and Jacobson, H.J., (1989). *Anal. Biochem.*, **176**: 157-160.

Segel, I.H. (1975) *Enzyme Kinetics,* J. Wiley & Sons Inc., New York. Chapter 7, page 450 (equation VII-102)

FOOTNOTES

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LEGEND FOR THE FIGURES

Figure 1.

Activation of glucosamine-6-phosphate deaminase by GlcNAc6P.

A: direct plot of initial velocities versus substrate concentration at the following activator concentrations: (■), none; (○), 5 μM; (●), 10 μM; (▵), 25 μM; (▵), 50 μM; (□) 2.5 mM. Assays were performed using 2 nM deaminase in a final volume of 200 μl, at pH 8.0 and 30 °C. as described elsewhere (Altamirano et al., 1989). Fitted values for the data in abscence of activator were: c = 0.021 $\pm$ 0.002; n = 5.42 $\pm$ 0.22; L = of 15500 $\pm$ 5500, maximum Hill coefficient = 3.02 $\pm$ 0.11.

B. Buc plot of the data from figure 1A. Y is the saturation fraction, estimated as v/V_{max}, at any particular activator concentration; V' is this value for the reference curve obtained in the presence of an excess (1 mM) of GlcNAc6P, corresponding to hyperbolic kinetics. The sixth root of the ratio at the ordinate gives the best fit. The set of lines intersects the abscissa at $-K_d$ for GlcNAc6P. Substrate concentrations are (○), 1 mM; (●), 1.5 mM; (□), 2 mM; (■), 2.5 mM; (▵), 3.0 mM; (▵) 3.5 mM; (▿) 4.0 mM. Inset: replot of ordinate intercepts as a function of substrate concentration. The abscissa intercept of this replot gives the $-K_m$ value for GlcN6P.

Figure 2.

The effect of the dead-end inhibitor GlcN-ol-6P on glucosamine-6-phosphate deaminase. Initial velocities *versus* substrate concentration, in the absence of the allosteric activator. The GlcN-ol-6P concentrations are: (□) none; (■), 5 μM; (●), 15 μM. The assay conditions were similar as

in the experiments described in figure 1.

The curves drawn were given by the fit to equation 2. (Fitted parameters: n = 3.4 ± .9; L = 9050 ± 2550). The fit of the data from the series without GlcN-ol-6P, (□),to equation 1 gives the following values for the variables: L = 10600 ± 1500; c = 0.043 ± 0.002; n = 6.18 ± 0.15. The maximum Hill coefficient for this curve is 2.94 ± 0.16.

Figure 3.

Binding of GlcNAc6P to glucosamine-6-phosphate deaminase, studied by equilibrium dialysis. Microcentrifuge tubes were used as dialysis chambers as described in the text. The enzyme chamber (200 μl) contained a 5 μM deaminase in 0.1 M Tris-HCl buffer, pH 8.0 (20 °C) and 2.5 mM EDTA. The outer chamber (400 μl) contained the same buffer and different amounts of [^{14}C]-GlcNAc6P (1.46 μCi/nmole) to yield at equilibrium the desired concentration range of free allosteric activator. (●) GlcNAc6P binding in the presence of a saturating concentration (0.5 mM) of GlcN-ol-6P; (○) curve in absence of the dead-end inhibitor. Fitted parameters for these curves are: ν_{max} (number of GlcNAc6P molecules bound per deaminase hexamer, at saturation): 6.30 ± 0.28; K_{dis}, 34 ± 4 μM (curve -●-, hyperbolic fit) . From the fit to Hill equation of curve -○-, $[GlcNAc6P]_{0.5}$ is 180 μM; ν_{max}, 6.09 ± 0.84 mM; Hill coefficient 2.51.

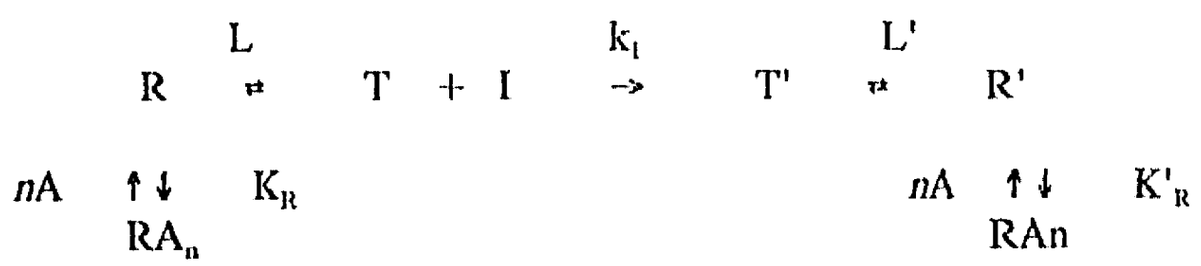
Figure 4.

Effect of GlcNAc6P on the apparent rate constant of the reaction of native deaminase sulfhydryls with DTNB. Samples containing 0.5 μM deaminase in 0.1 M Tris-HCl buffer, and 2.5 mM EDTA (pH 7.7), and variable concentrations of GlcNAc6P, was treated with 2 mM DTNB. The thiol-disulfide exchange reaction was measured with a double beam spectrophotometer using a blank cell without enzyme. These data were fitted to equation 3, with the following parameters: k'_1 = 7.8 x 10^{-4} s^{-1}; n = 6. The fitted values for the variables were: L = 750 ± 120; K_d = 20 ± 6 μM.

APPENDIX

Kinetics of the modification reaction of an allosteric protein in the presence of an exclusive-binding protective ligand.

Scheme 1 shows the general reaction sequence for the chemical modification of a two-states allosteric protein in which only the T-conformer is able to react with the modifying reagent. The ligand A, binds exclusively to the R form. The chemical modification is irreversible, and the modified enzyme is still active and allosteric.



SCHEME 1

The pseudo first-order rate equation for the protein modification is:

$$v = k'_1 E_t [T] \qquad (1)$$

where $k'_1 = k_1 [I]$ and E_t the total concentration of unmodified enzyme; hence, $E_t [T]$ is the fraction of enzyme susceptible to chemical modification. The following expression, derived by Monod *et al.*(1965), gives the fraction of the protein in the R-state.

$$R = \frac{(1+\gamma)^n}{L + (1+\gamma)^n}$$

where γ is the specific concentration of the exclusive-binding ligand A (*i.e.*, $[A]/K_A$), **n** the number of binding sites and L the allosteric constant. The fraction of the enzyme in the T form, 1-R, is:

$$T = \frac{L}{L + (1+\gamma)^n} \qquad (2)$$

Susbtituting for T in equation 1 gives:

$$v = E_t \frac{k'_1 \, L}{L + (1+\gamma)^n}$$

The apparent rate constant for the modification reaction in the presence of the allosteric ligand, is:

$$k_{app} = \frac{k'_1 \; L}{L + (1+\gamma)^n} \qquad (3)$$

The plot of k_{app} versus γ gives a downward S-shaped curve, intercepting the vertical axis in $k_{obs} = k'_1$ (see Results, figure 4). The following expression is a linear transformation of equation 3:

$$\log \frac{k'_1 - k_{obs}}{k'_1 \; k_{obs}} = n \log (1 + \gamma) + \log L$$

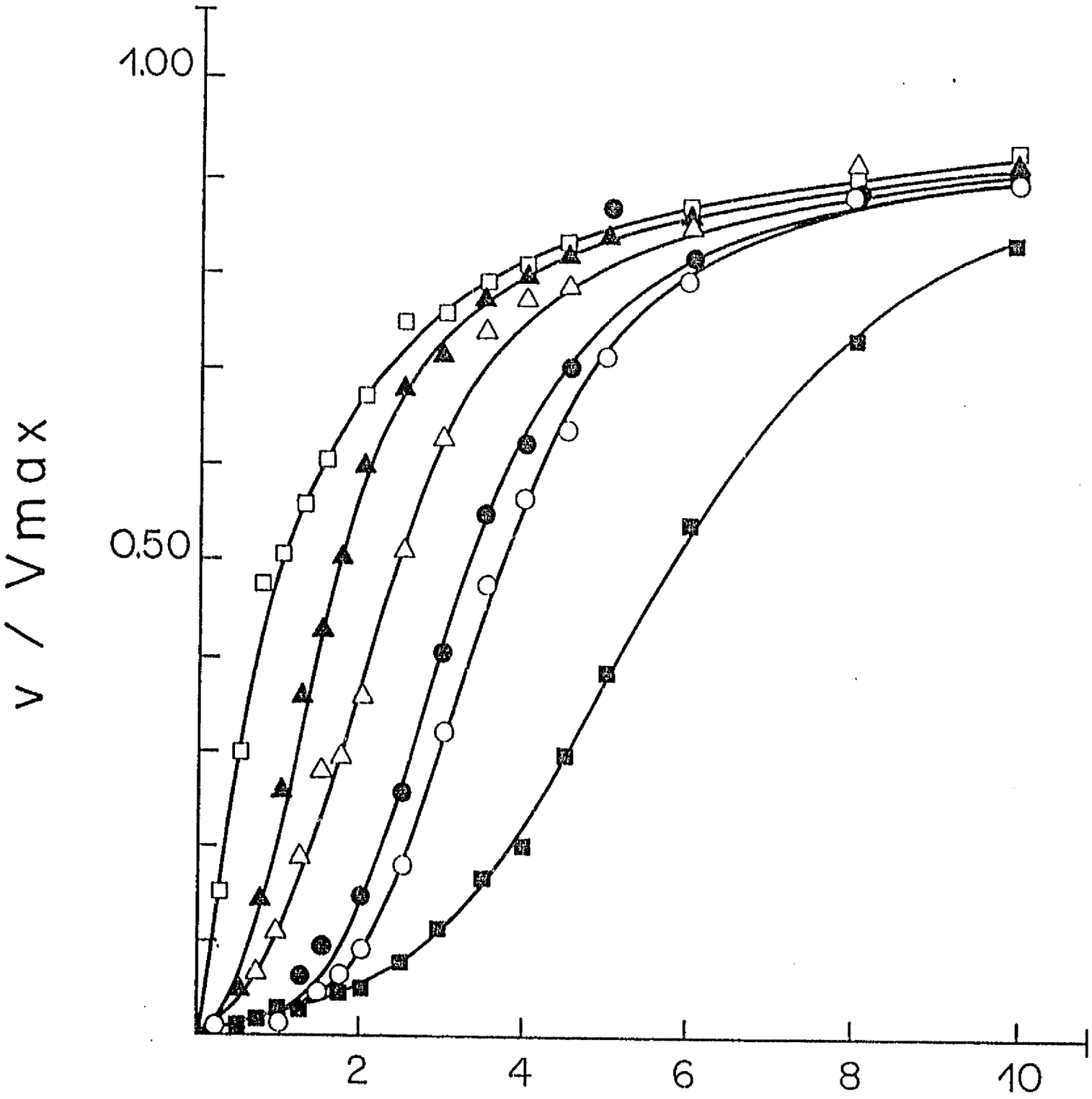


Figure 1

A

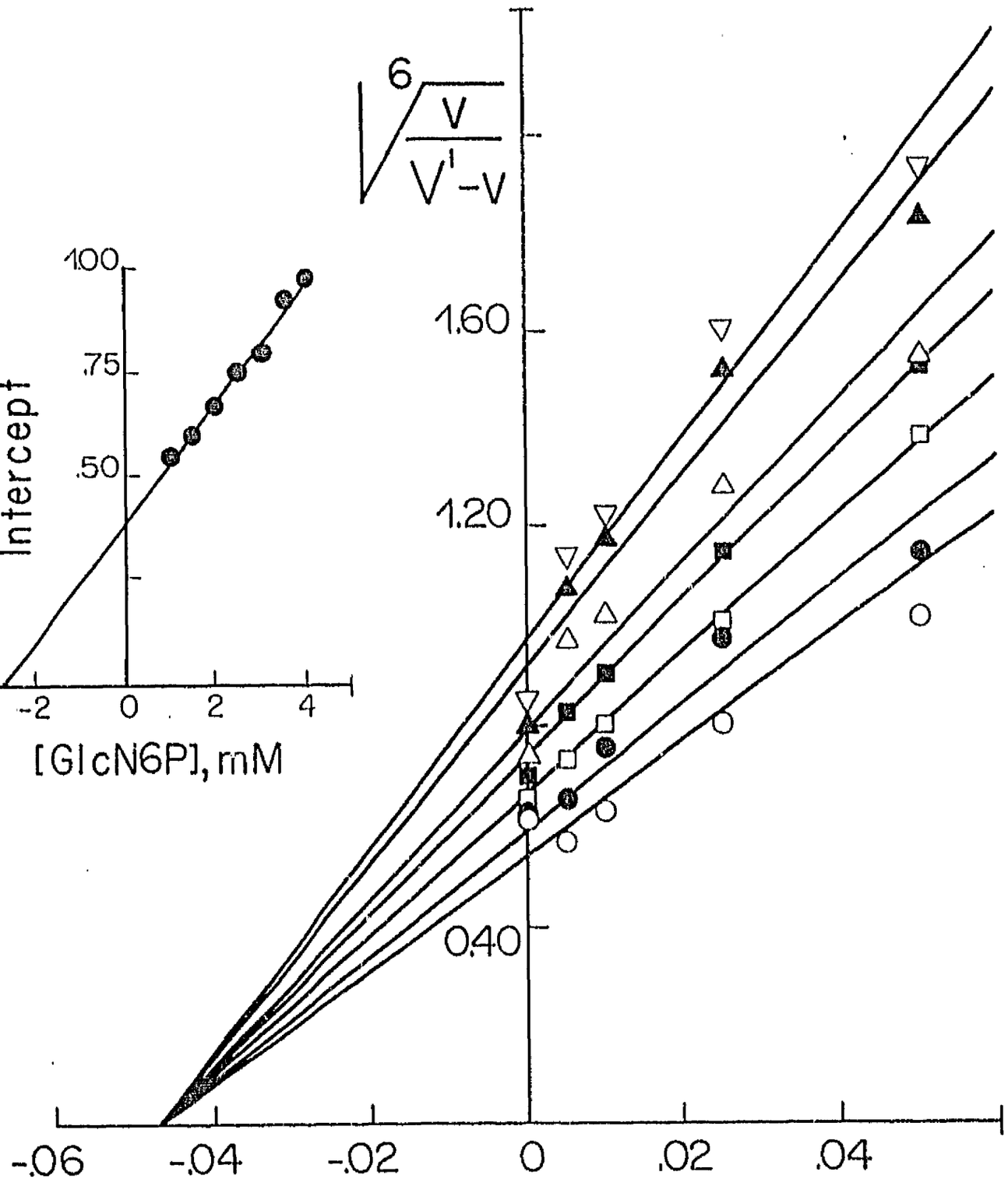

6 V / V'-V
1.60
1.20
0.40
-.06
-.04
-.02
0
.02
.04
[GlcNAc6P], mM
Intercept
100
.75
.50
-2
0
2
4
[GlcN6P], mM

Figure 1

B

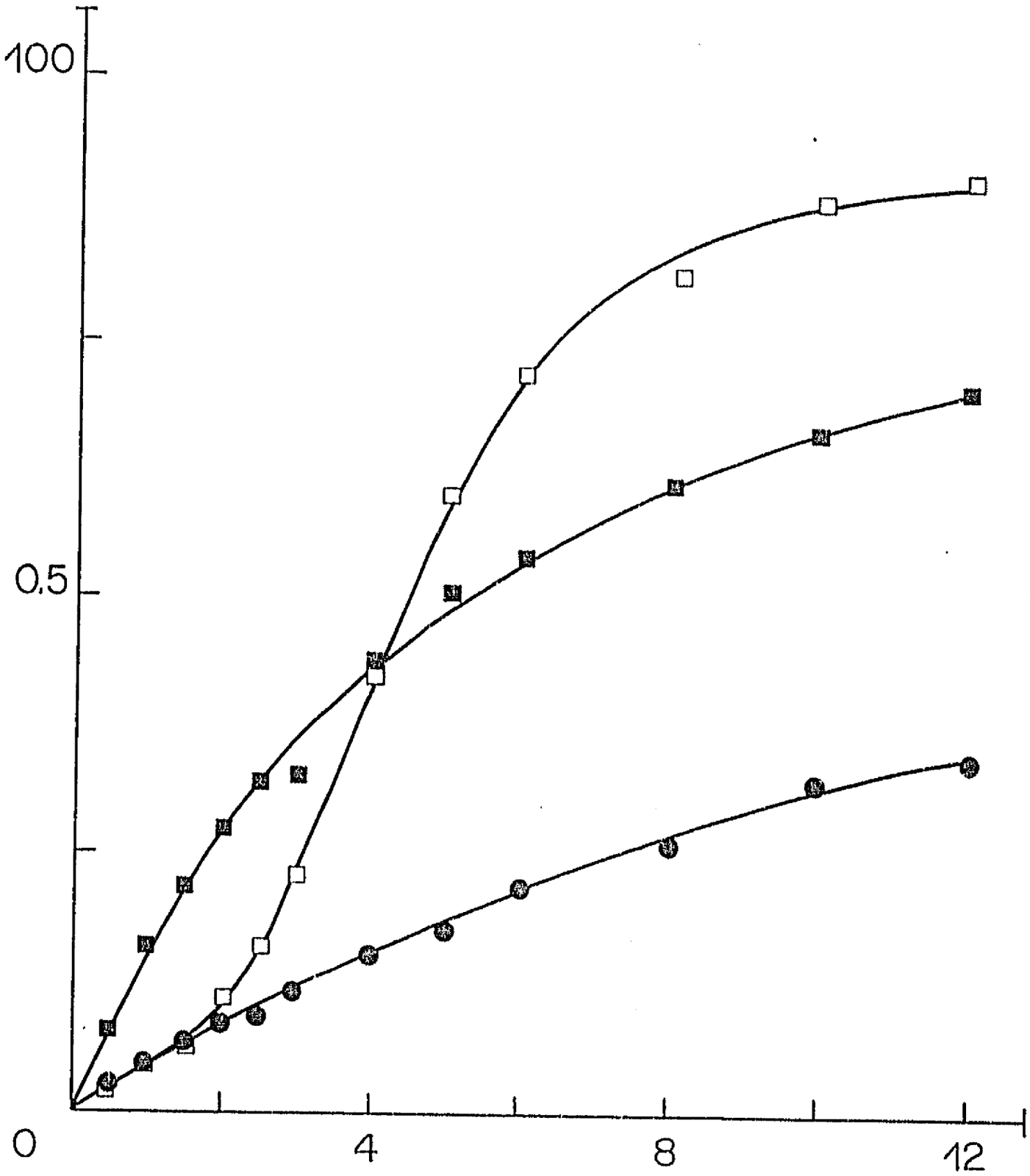


Figure 2

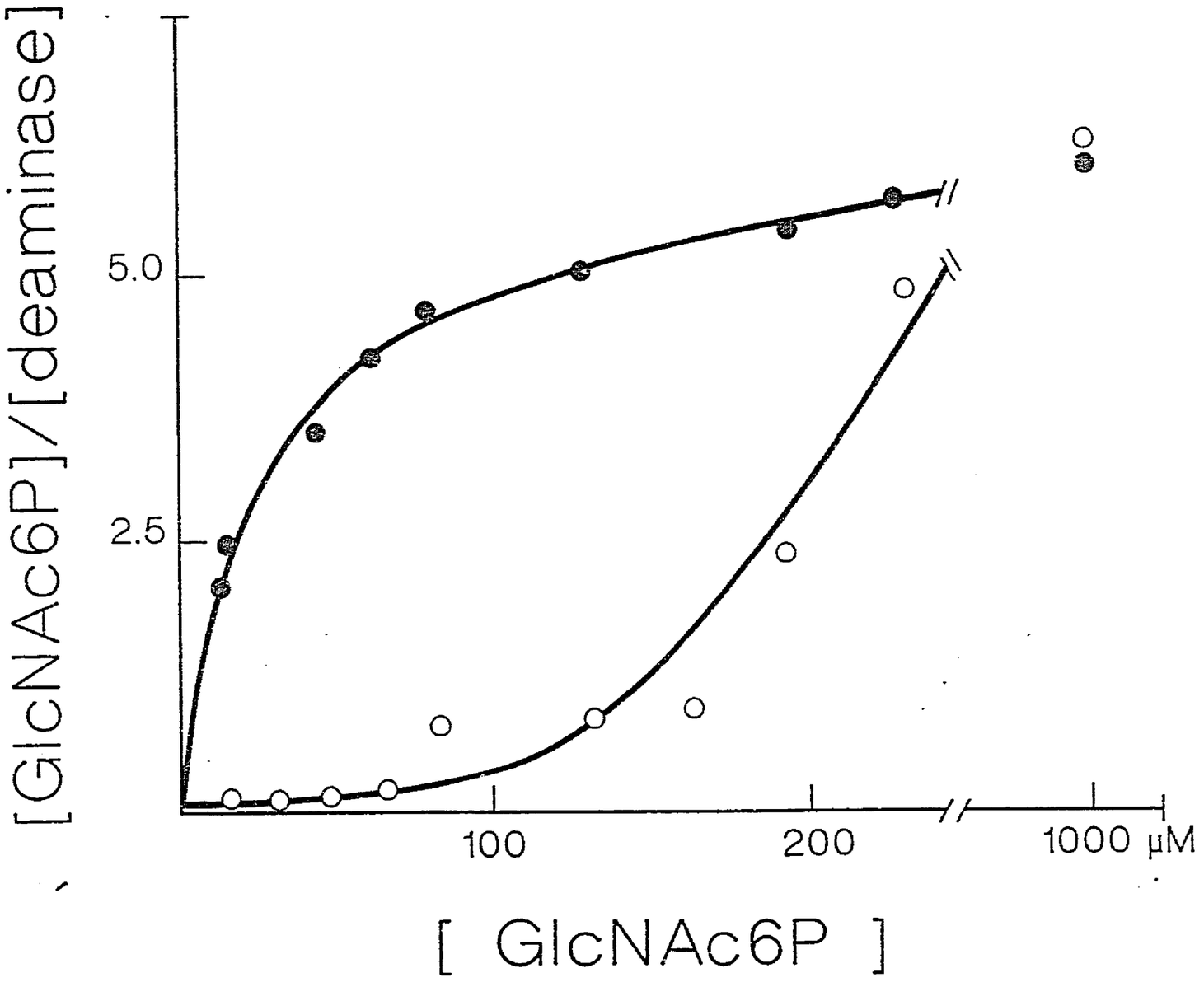


Figure 3

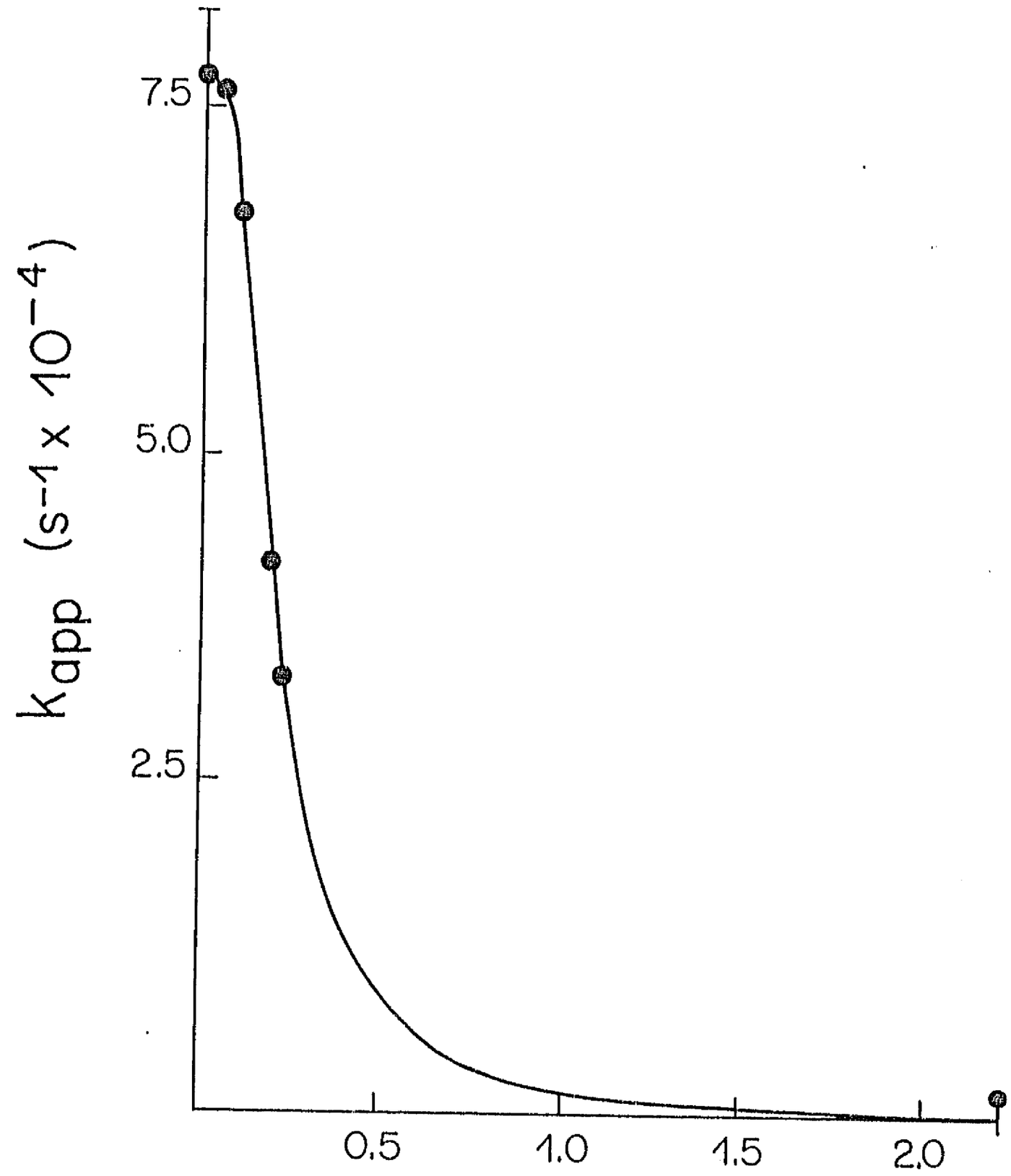


Figure 4

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Allosteric properties of Escherichia coli glucosamine-6-phosphate deaminase: Kinetic control of cooperativity by binding of homotropic and heterotropic ligands

by

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SPECTROCHEMICAL EVIDENCES FOR THE PRESENCE OF A TYROSYL RESIDUE IN THE ALLOSTERIC SITE OF GLUCOSAMINE-6-PHOSPHATE DEAMINASE FROM *ESCHERICHIA COLI*

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Abbreviations: GlcN6P, D-glucosamine-6-phosphate; GlcNAc6P, N-acetyl-D-glucosamine-6-phosphate; GlcN-ol-6P, 2-deoxy-2-amino D-glucitol 6-phosphate.

Enzyme: Glucosamine-6-phosphate deaminase (D-glucose-6-phosphate ketol isomerase, deaminating, EC 5.3.1.10)

SUMMARY:

The interaction of the enzyme glucosamine 6-phosphate deaminase (E.C. 5.3.1.10) from *Escherichia coli*, and its allosteric activator N-acetyl-D-glucosamine 6-phosphate (GlcNAc6P), was studied by different spectrophotometric methods. The analysis of the circular dichroism differential spectra produced by the binding of the activator or the competitive inhibitor 2-deoxy 2-amino-D-glucitol 6-phosphate (an homotropic ligand displacing the allosteric equilibrium to the R-conformer), suggests the presence of tyrosine residues at or near the allosteric site. The involvement of a single tyrosyl residue with a pK of 8.75 in GlcNAc6P binding site of glucosamine 6-phosphate deaminase was further demonstrated by spectrophotometrical pH titration, in the presence or absence of these ligands. In these experiments, a single titrated tyrosyl residue is completely protected by saturation with the allosteric activator. The analysis of the amino acid sequence of the deaminase using a set of indices for the prediction of surface accessibility of amino acid residues, shows that only two tyrosyl residues, Tyr121 and Tyr254, are the most probable candidates for this function.

The enzyme glucosamine-6-phosphate deaminase (D-glucose-6-phosphate ketol isomerase, deaminating, EC 5.3.1.10) catalyses the reversible isomerisation-deamination of D-glucosamine-6-phosphate (GlcN6P) into D-fructose-6-phosphate (Fru6P) and ammonia (Comb y Roseman, 1958; Midelfort and Rose, 1977; Calcagno *et al.* 1984). Deaminase is an hexamer composed of six identical subunits, and has six active and six allosteric sites per molecule (Calcagno *et al.* 1984, Altamirano and Calcagno, 1993, unpublished). This is the single allosterically regulated enzyme in the pathway of amino sugar catabolism in *Escherichia coli*. N-acetyl-D-glucosamine-6-phosphate (GlcNAc6P) is an allosteric modulator of deaminase and its kinetic effects has been well characterised (Altamirano *et al.*, 1989, 1992). Furthermore, deaminase and other proteins involved in amino sugar transport and metabolism are regulated at the level of transcription of their genes, which are clustered in the regulon *nagE-nagBACD*, (Rogers *et al.*, 1988; Plumbridge, 1989; Plumbridge *et al.* 1993). These genes are expressed when the bacterium is cultivated amino sugars as the carbon and nitrogen source; the inducer metabolite is GlcNAc6P (Plumbridge, 1991; Plumbridge and Kolb, 1991). This compound, the allosteric activator of deaminase, is also the product of N-acetyl-D-glucosamine transport and the substrate of other *nag* enzyme, N-acetyl-D-glucosamine-6-phosphate deacetylase. These facts emphasize the key role of GlcNAc6P concentration in the control of amino sugar utilisation in *E. coli*. To evaluate the actual contribution of the allosteric properties of deaminase on the regulation *in vivo* of the entire pathway, the construction of non-allosteric site-directed mutants may be an useful tool. Nevertheless, we do not have information about the structural basis of the allosteric control of the deaminase, as the structures participating in the allosteric conformational changes, or the amino acid side chains involved in GlcNAc6P binding on the allosteric site. The present study is an initial attempt to characterise the critical structural features of the allosteric regulation of glucosamine-6-phosphate deaminase by means of spectrochemical techniques.

EXPERIMENTAL PROCEDURES

Reagents

Biochemicals and most reagents were from Sigma Chemical Co., (St. Louis, MO, U.S.A.); GlcNAc6P was prepared by acetylation of GlcN6P and purified by ion-exchange chromatography, according to Leloir and Cardini (1962). The deaminase dead-end inhibitor, 2-deoxy-2-amino-D-glucitol-6-phosphate (GlcN-ol-6P), was synthesized as described by Midelfort and Rose (1977).

Bacteria and enzyme

E. coli glucosamine-6-phosphate deaminase was prepared as previously described, from an overproducing strain (Altamirano *et al.* 1991). The concentration of the enzyme was calculated from its absorbance at 278 nm at pH 7.7 and its molar absorptivity (ε_{278} = 20.0 x 10^4 M^{-1} cm^{-1}, Altamirano *et al.*, 1987).

Circular dichroism spectroscopy

The circular dichroism (CD) spectra were recorded on both a Jasco J-500A instrument or an AVIV CD Spectrometer, model 62 DS. The spectral changes produced by GlcNAc6P or GlcN-ol-6P binding were studied by running consecutive spectra after the addition of small volumes (0.2 to 2.0 μl) of a 100 mM solution of the ligand. The experiments were made at room temperature, and pH 7.7 in 0.05 M Tris-HCl buffer using cells of 0.2 cm pathlength, unless otherwise specified.

Spectrophotometrical titration of tyrosines

Difference spectra were recorded in a Cary 4 double beam spectrophotometer at 30 °C. Acid-base titration of tyrosyl residues in native glucosamine-6-phosphate deaminase, was performed recording the spectra of a set of 3-5 μM deaminase solutions, prepared in 50 mM ACES, 25 mM MES, 25 mM ethanolamine buffer, at various pH values. As a fully protonated reference sample, the solution at pH 6.0 was used. In this buffer system, the change of ionic strength with pH was minimal (Ellis and Morrison, 1982). The ionisation

of tyrosyl residues was calculated from the absorbance change at 295 nm, using the absorptivity coefficient of 2.33×10^3 M^{-1} cm^{-1} (Donovan, 1973).

Methods of theoretical sequence analysis

A set of four methods for the calculation of propensity scales for each of the 20 amino acids was selected to analyse the sequence of glucosamine-6-phosphate deaminase. These scales describe the tendency of each residue to be associated with physico-chemical properties such as a) hydrophilicity (Hopp and Woods 1981, Parker *et al*, 1986) which are mostly derived from the study of the partition coefficients of amino acids in two non-interacting isotropic phases, and b) surface accessibility (Boger *et al.* 1986, Padlan *et al.* 1975), based on the study of proteins of known three-dimensional structure. All prediction calculations were made with the computer package for protein sequence analysis ANTHEPROT (version 5.5.) for IBM PC computers, by Gilbert Déleage, Institut de Biologie et Chimie des Protéines, Lyon, France. (Déleage *et al.* 1988, 1989).

RESULTS AND DISCUSSION

CD spectral changes produced by ligands.

Spectrochemical techniques are suitable to analyse specific interactions of proteins and their associated structural changes. Our attention was focused on binding of the ligands of glucosamine-6-phosphate deaminase, GlcNAc6P, the physiological allosteric activator, and GlcN-ol-6-P, a dead-end inhibitor of the enzyme (Midelfort and Rose, 1977). This inhibitor is a non-substrate homotropic ligand, which displaces the allosteric equilibrium to the R-conformer (Altamirano and Calcagno, unpublished). The far-UV CD spectrum of the enzyme remains unchanged after the addition of the heterotropic or the homotropic ligand, thus indicating the lack of meaningful changes in the secondary structures of the protein. On the other hand, the addition of GlcNAc6P or GlcN-ol-6P produced marked changes in the near-UV CD spectrum of the enzyme, as shown in Fig. 1. The CD difference spectrum produced by saturating concentration of GlcNAc6P shows a broad distribution of bands over the whole aromatic absorption zone (curve B in Fig. 1). Notwithstanding its complexity, it is possible to gather some information about the

activator-enzyme interaction. The strongest signals at 282-283, 276-277 can be ascribed to ^1L_b bands from tyrosyl residues (Woody, 1985, Horwitz *et al.* 1970). On the other hand, the bands located at 291 and 296 nm can be safely assigned to tryptophanyl residues (Strickland, 1974). Other bands between 282 and 286 originate from tryptophanyl and/or tyrosyl residues and it is not possible to make a precise assignment. (Strickland, 1974, Horwitz *et al.* 1970, Woody, 1985). In contrast, the CD differential spectrum produced by saturation with GlcN-ol-6P (Fig. 1, curve A) is simpler, with the sharp positive bands at 280 and 287 nm. The comparison of both spectra, strongly suggests that the signals characteristic of tyrosine side chains in the 277-283 range are produced by GlcNAc6P binding and may be caused by local interactions at the allosteric site, because the shift to the R conformation induced by an homotropic ligand, GlcN-ol-6P, did not produce bands in the same wavelength range. The stronger positive band at 287 nm observed upon GlcN-ol-6P saturation points out the participation of tryptophanyl residues in the allosteric transition, ligand binding to the active site, or both. This point could be conveniently studied by spectrofluorometric techniques. The far-UV CD spectrum of glucosamine-6-phosphate deaminase (182 to 250 nm) remained unchanged after the addition of GlcN-ol-6P or GlcNAc6P, thus revealing the absence of significant changes in the secondary structure.

GlcNAc6P binding curve, determined by CD spectroscopy.

The CD differential spectrum presented in Fig. 1 shows that GlcNAc6P binding causes an intense negative signal at 275 nm, whereas at this wavelength, the allosteric activation produced by an active site ligand did not produce any significant change. This peak at 275, was then used to obtain the saturation curve of deaminase by GlcNAc6P. The fractional change of the differential CD spectrum was assumed to be proportional to the saturation fraction (Y) of the protein, taking as a reference (Y = 1), the maximal spectral modification produced by a high (2 mM) concentration of GlcNAc6P. Fig. 2 shows the plot of Y versus GlcNAc6P concentration. The binding curve obtained in the presence of a saturating concentration of GlcN-ol-6P was hyperbolic (K_d = 36 $\pm$ 3 μM). In the

absence of the inhibitor, binding is highly cooperative (Hill coefficient, 2.9 ± 0.2, $[GlcNAc6P]_{0.5}$ 64 ± 12 μM). The K_d value obtained for GlcNAc6P coincides with the constant calculated from kinetic experiments or directly measured by equilibrium dialysis (Altamirano *et al.*, submitted). Furthermore, the good fit to hyperbolic saturation reached at high dead-end inhibitor concentration, validates the use of this ligand as a tool to shift the allosteric equilibrium to the high affinity (R) conformer, as discussed in the previous paragraph.

Acid-base titration of tyrosyl residues, and effect of ligands

CD spectral data provided information suggesting that phenolic side chains from tyrosyl residues are involved in GlcNAc6P-enzyme interaction. Tyrosyl residues can be conveniently studied and measured by means of the red shift of its main UV absorption band. The differential spectra of the enzyme (pH 10.5 *minus* pH 6.0) were obtained in absence of ligands or in the presence of a saturating concentration of GlcNAc6P or GlcN-ol-6P; the spectrum at pH 6.0 was used as reference for the full protonated phenolic groups. As shown in Fig. 3, an absorption band with a maximum at 295 nm, corresponding to the dissociation of the phenolic hydroxyl of tyrosine was observed. This peak is absent when pH titration was performed with deaminase previously saturated with GlcNAc6P (Fig. 3), but the spectrum was not modified by a saturating concentration of GlcN-ol-6P (not shown). When this increase in absorbance at 295 nm was measured at different pH values, the titration curve shown in Fig. 4 was obtained. These data were fitted to the Henderson-Hasselbalch equation, giving a pK of 8.75 and 1.16 titrated groups per polypeptide chain. This fit is also presented in the inset of Fig. 4, as a linear transformation of the buffer curve (Pontremoli *et al.* 1969). These titration experiments indicate the participation of one tyrosyl residue per deaminase polypeptide chain, which has a rather low pK phenolic hydroxyl group. This group entirely disappears from titration when the procedure is performed in the presence of the allosteric effector, suggesting that it may be located in or near the allosteric site.

Prediction of the location of the exposed tyrosines.

Amino acid sequence of glucosamine-6-phosphate deaminase is known from the nucleotide sequence of its gene (Rogers et al., 1988), and a secondary structure for this protein has been proposed on basis on CD spectral data and the combined use of diverse prediction methods (Altamirano *et al.*, 1991). The enzyme was recently crystallised and preliminary crystallographic data have been reported (Horjales *et al.*, 1992) but the complete structure from X-ray diffraction data is not yet available. On the other hand, a systematic search for sequence homologies with other proteins, using the data from the GeneBank, and the program FASTA (Pearson, 1990) or progressive alignment methods (Doolittle, 1990), did not revealed significant alignments with any other protein, or with the other *nag* proteins (Altamirano, unpublished results). In the absence of further structural information, we came back to sequence analysis, in an attempt to predict the position of the tyrosines, which according to the foregoing evidences, are involved in GlcNAc6P binding to the allosteric site of the enzyme. The use of a set of indicators of surface probability, presented in Fig. 5, suggests that only two tyrosyl residues of the eight ones which are present in deaminase polypeptide chain, are surface located; these residues are Tyr121 and Tyr254, and the former has a definitely higher score. In a previous study of secondary structure prediction of deaminase (Altamirano *et al.* , 1991) we have also found that these tyrosines appear in sequence segments predicted as highly flexible according to Karplus *et al.* (1985). In the present study, tyrosine 121 appears in segment predicted as a hydrophilic, polar helix (residues 115-124: DAECRQYEEK), and tyrosine 254 in a segment predicted as a highly flexible turn (251-254: TLRY). Based on the consideration that only amino acids which contribute to the "accessible" surface may directly interact with external atoms, tyrosines 121 and 254 are best candidates for the tyrosine residue located at or near the allosteric site. This point can be further examined by means of the construction of the corresponding site directed mutants.

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REFERENCES

Altamirano, M.M., Mulliert, G. and Calcagno, M. (1987) *Arch. Biochim. Biophys.* **258**, 95-100.

Altamirano, M.M., Lara-Lemus, R., Libreros-Minotta, C.A. and Calcagno, M. (1989) *Arch. Biochim. Biophys.* **269**, 555-561.

Altamirano, M.M., Plumbridge, J.A., Hernández-Arana, A., and Calcagno, M.L. (1991) *Biochim. Biophys. Acta* **1076**, 266-272.

Altamirano, M.M., Plumbridge, J.A. and Calcagno, M.L. (1992) *Biochemistry* **31**, 1153-1158.

Boger, J., Emini, E.A., and Schmidt, J.A. (1986). Reports of the VI International Congress of Immunology (Toronto) p. 250.

Calcagno, M., Campos, P.J., Mulliert, G., and Suástegui, J. (1984) *Biochim. Biophys. Acta* **787**, 165-173.

Comb, D.G. and Roseman, S. (1958) *J. Biol. Chem.* **232**, 807-827.

Déleage, G., Clerc, F.F., Roux, B. and Gautheron, D.C. (1988) *Comput. Applic. Biosci.* (CABIOS) **4**, 351-356

Déleage, G., Clerc, F.F. and Roux, B. (1989) *Comput. Applic. Biosci.* (CABIOS) **5**, 159-160

Donovan, J.W. (1973) *Methods Enzymol.* **27**, 525-548.

Ellis, K.J. and Morrison, J.F., (1982) *Methods Enzymol.* **87**, 405-426.

Feng, D. and Doolittle, R.F. *Meth. Enzymol.* **183**, 365-388.

Hopp, T.P. and Woods, K.R. (1981) *Proc. Nat. Acad. Sci. USA* **78**, 3824-3828

Horjales. E., Altamirano, M.M., Calcagno, M.L., Dauter, Z., Wilson, K, , Garratt. R.C., and Oliva, G. (1992) *J. Mol. Biol.* **226**, 1283-1286

Horwitz, J., Strickland, E.H. and Billups, C., (1970), *J. Am. Chem. Soc.*, **92**, 2119-2129

Karplus, P.A. and Schultz, G.E. (1985) *Naturwissenschaften* **72**, 212-213

Leloir, L.F. and Cardini, C. (1962) *Methods Enzymol.* **5**, 418-422.

Midelfort, C., and Rose, I.A. (1977) *Biochemistry* **16**, 1590-1596.

Padlan, E., and Davies, D.R. (1975) *Proc. Nat. Acad. Sci. USA* **72**, 819-823

Parker, J.M.R., Guo, D., and Hodges, S. (1986) *Biochemistry*, **25**, 5425-5432

Pearson W.R., (1990) *Meth. Enzymol.* **183**, 63-97

Plumbridge, J.A. (1989) *Mol. Microbiol.* **3**, 505-515.

Plumbridge, J.A. (1991). *Mol. Microbiol.* **5**, 2053-2062.

Plumbridge, J.A., Cochet, O., Souza, J. M., Altamirano, m.M., Calcagno, M.L. and Badet, B. (1993) *J. Bacteriol.* **175**, in press.

Plumbridge, J.A. and Kolb, A., (1991) *J. Mol. Biol.* **217,** 661-679.

Pontremoli, S., Grazi, E., and Accorsi, A. (1969) *J. Biol. Chem.* **244**, 6177-6181.

Rogers, M.J., Ohgi, T., Plumbridge, J., and Söll, D. (1988) *Gene,* **62**, 197-207.

Strickland, E.H. (1974) *CRC Crit. Rev. Biochem.*, **2**, 113-175

Woody, R.W. (1985) Circular Dichroism of Peptides, in *The Peptides,* vol. 7, (V.J. Hruby, ed.) pp. 15-104, Academic Press, New York.

LEGEND FOR THE FIGURES

Fig. 1.

Change in CD spectrum of glucosamine-6-phosphate deaminase produced by binding of GlcN-ol-6P (curve A) and GlcNAc6P (curve B).

The experimental points correspond to the CD difference spectrum calculated by substraction of data in the absence of ligand from those obtained in the presence of 0.1 mM GlcN-ol-6P (curve A) or 2 mM GlcNAc6P (curve B). The protein concentration was 2.5 μM (0.45 mg ml^{-1}) Scanning conditions: Averaging time: 6 s, plotted data are averages of 10 succesive scans.

Fig. 2.

Saturation curves of glucosamine 6-phosphate deaminase with its allosteric activator, GlcNAc6P, using the ellipticity change at 275 nm as the analytical signal.

Saturation fraction, Y, was calculated from the quotient $\Delta\theta / \Delta\theta_{max}$, where $\Delta\theta_{max}$ is the change in CD differential spectrum produced by the addition of 1 mM GlcNAc6P. Measurements were obtained in the presence and in the absence of 0.5 mM GlcN-ol-6P (curves (●) and (O), respectively). Fitted values: $[GlcNAc6P]_{0.5}$, 64 μM $\pm$ 12; Maximum Hill coefficient, h_{max}, 2.9 $\pm$ 0.2 (curve O); K_d, 36 $\pm$ 3 μM (curve ●).

Fig. 3

Difference UV spectra of glucosamine-6-phosphate deaminase in the pH range 7.0 - 10.5.

A 4.5 μM solution of the enzyme in 50 mM imizadole, 25 mM MES, 25 mM CAPS buffer, pH 10.5 was measured against a reference cell containing the same enzyme concentration, at pH 7.0. The dashed line is the diference spectrum obtained in the absence of ligands (A similar spectrum was obtained after the addition of 0.5 mM GlcN-ol-6P). The continuous line was obtained after the addition of 2.5 mM GlcNAc6P to the sample cell (pH 10.5). The peak of this difference spectrum is at 295 nM. The ΔA_{295} observed is 0.064, which corresponds to 6.2 phenolic groups of tyrosine dissociated per deaminase molecule.

Fig. 4.

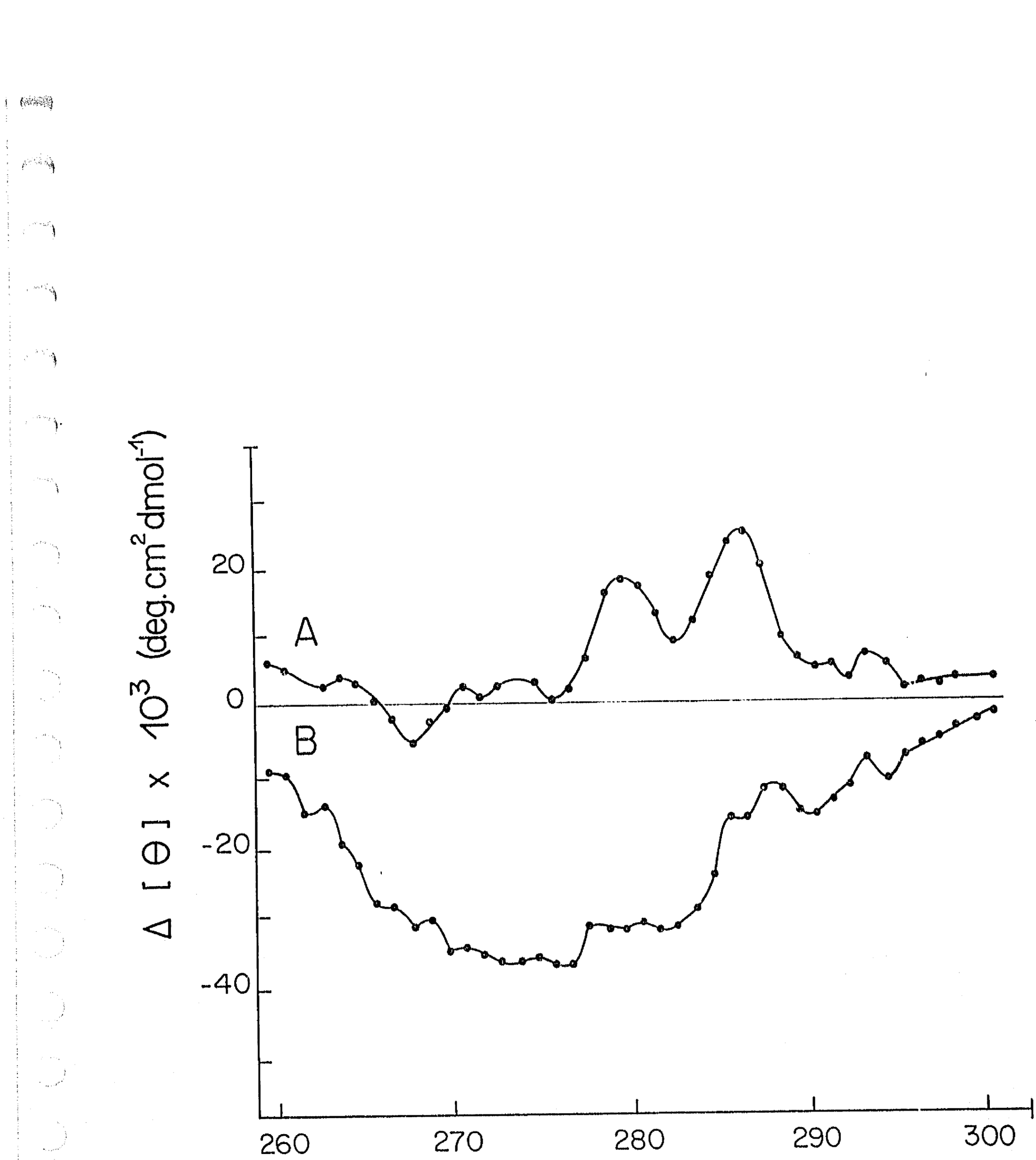
Titration curve of tyrosyl groups of glucosamine-6-phosphate deaminase.

From ΔA_{295} data, obtained in the pH range 7.7 - 10.5, under the same conditions of Fig. 3. The fit to the buffer equation shows a pK of 8.75 and 1.16 titrated groups per deaminase polypeptide chain.

Fig. 5.

Theoretical analysis of glucosamine-6-phosphate deaminase amino acid sequence.

A to D: Plots displaying four indexes for surface accessibility of glucosamine-6-phosphate deaminase. **A**: hydrophilicity scale of Hopp and Woods (1981); **B**, accessibility to the solvent scale of Boger *et al.* (1986); **C**, antigenicity index of Parker *et al.* (1985) and **D**, antigenicity by disymmetry against glycine (Padlan 1975). In all calculations a window of seven residues was employed. The points along the curves, indicate the position of tyrosines 121 and 254. The asterisks on the top of the figure, indicate the position of all the tyrosyl residues of deaminase polypeptide chain (tyrosines 49, 74, 85, 86, 121, 128, 184 and 254).


Δ [θ] x 10³ (deg.cm²dmol⁻¹)
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0
-20
-40
A
B
260
270
280
290
300
Wavelength (nm)

Figure 1

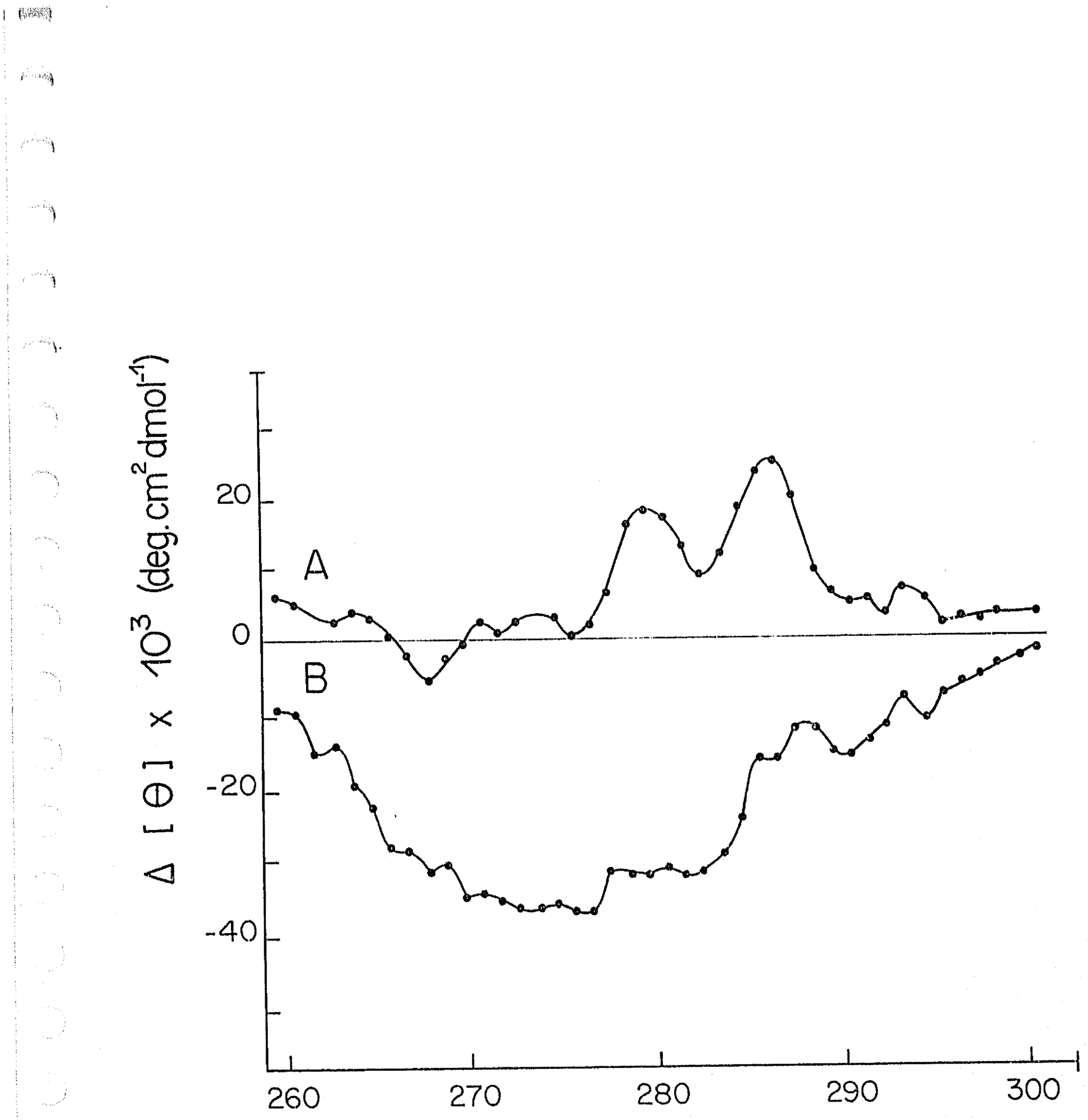
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Figure 1

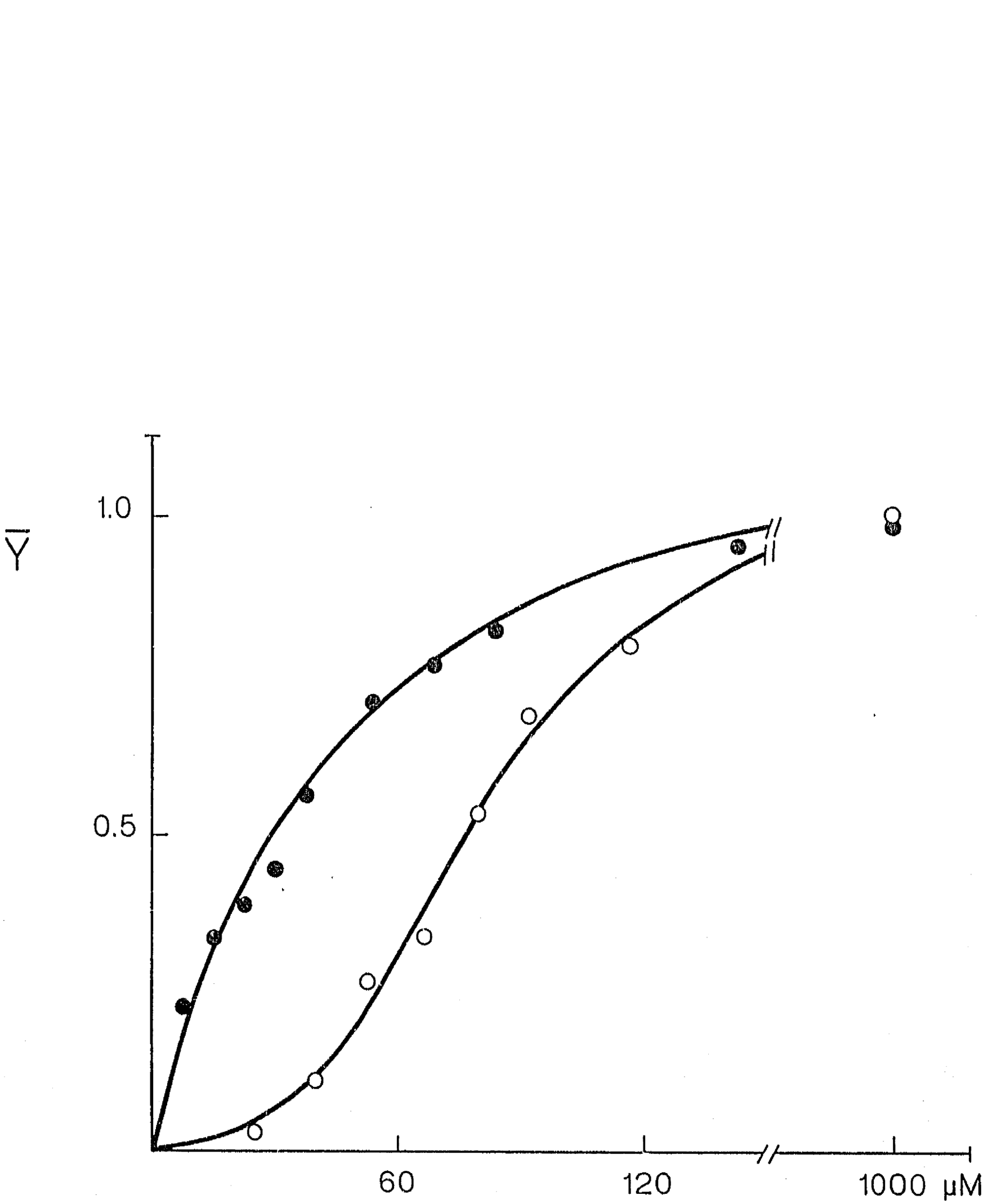

Figure 2

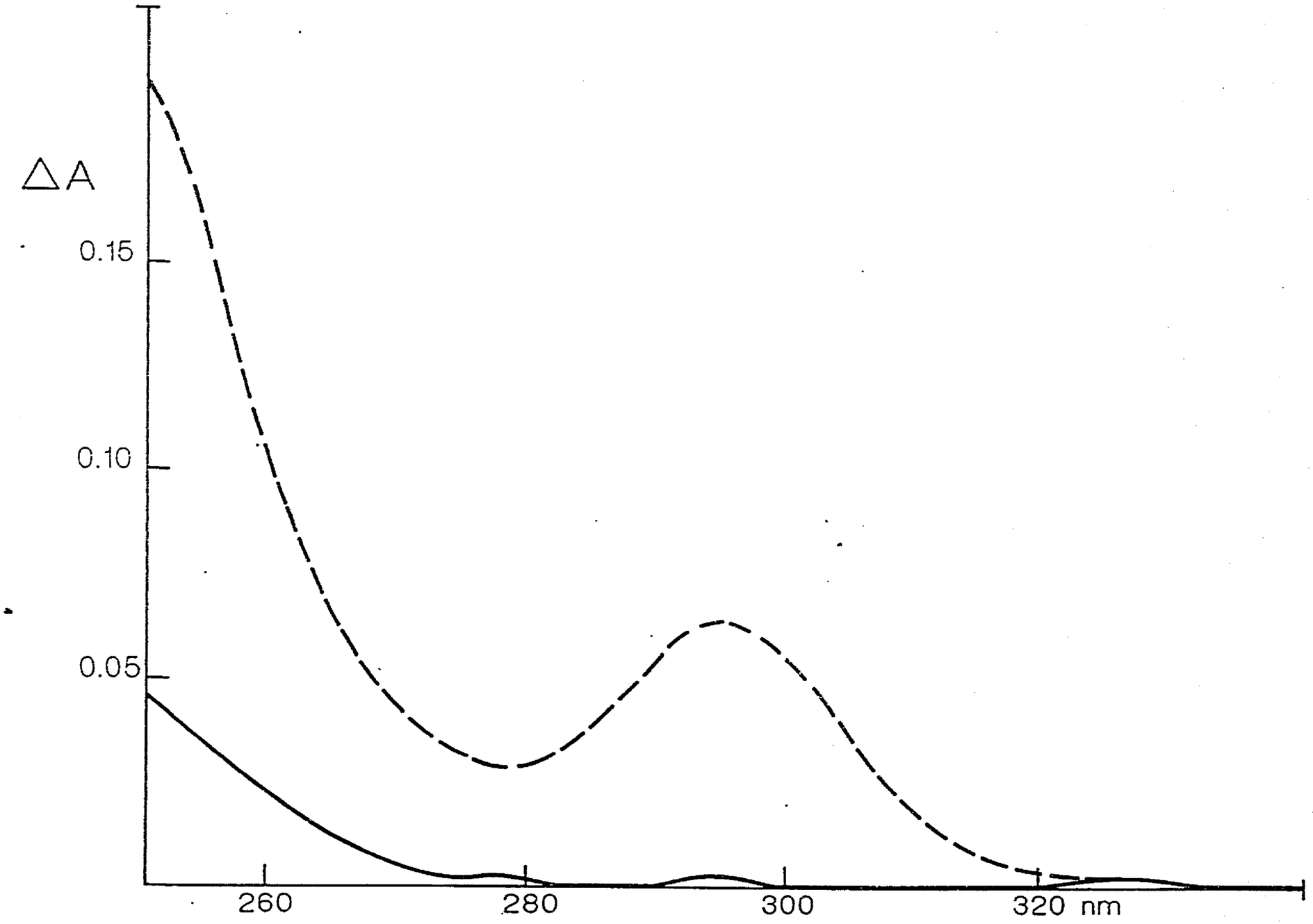

Figure 3

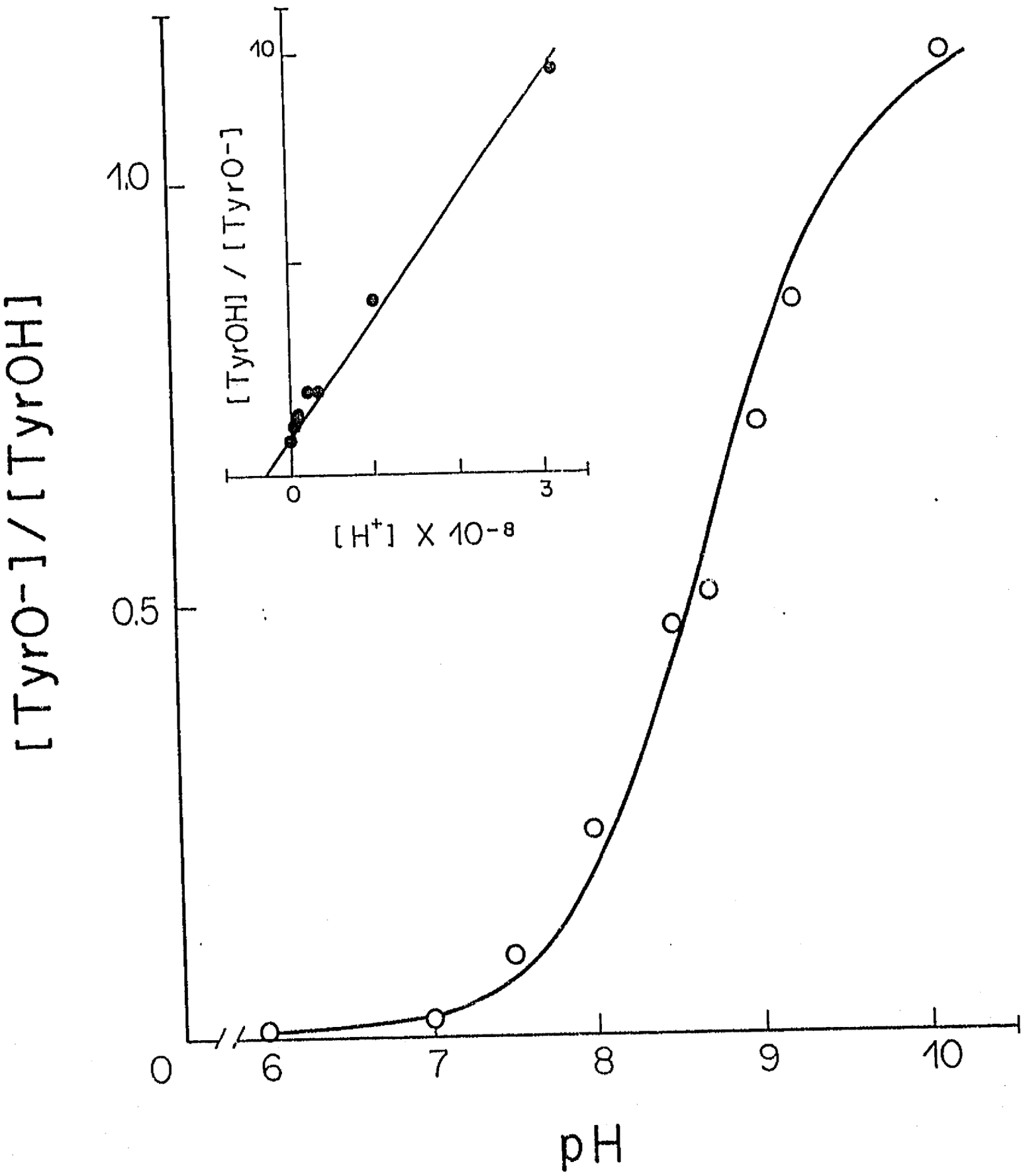

Figure 4

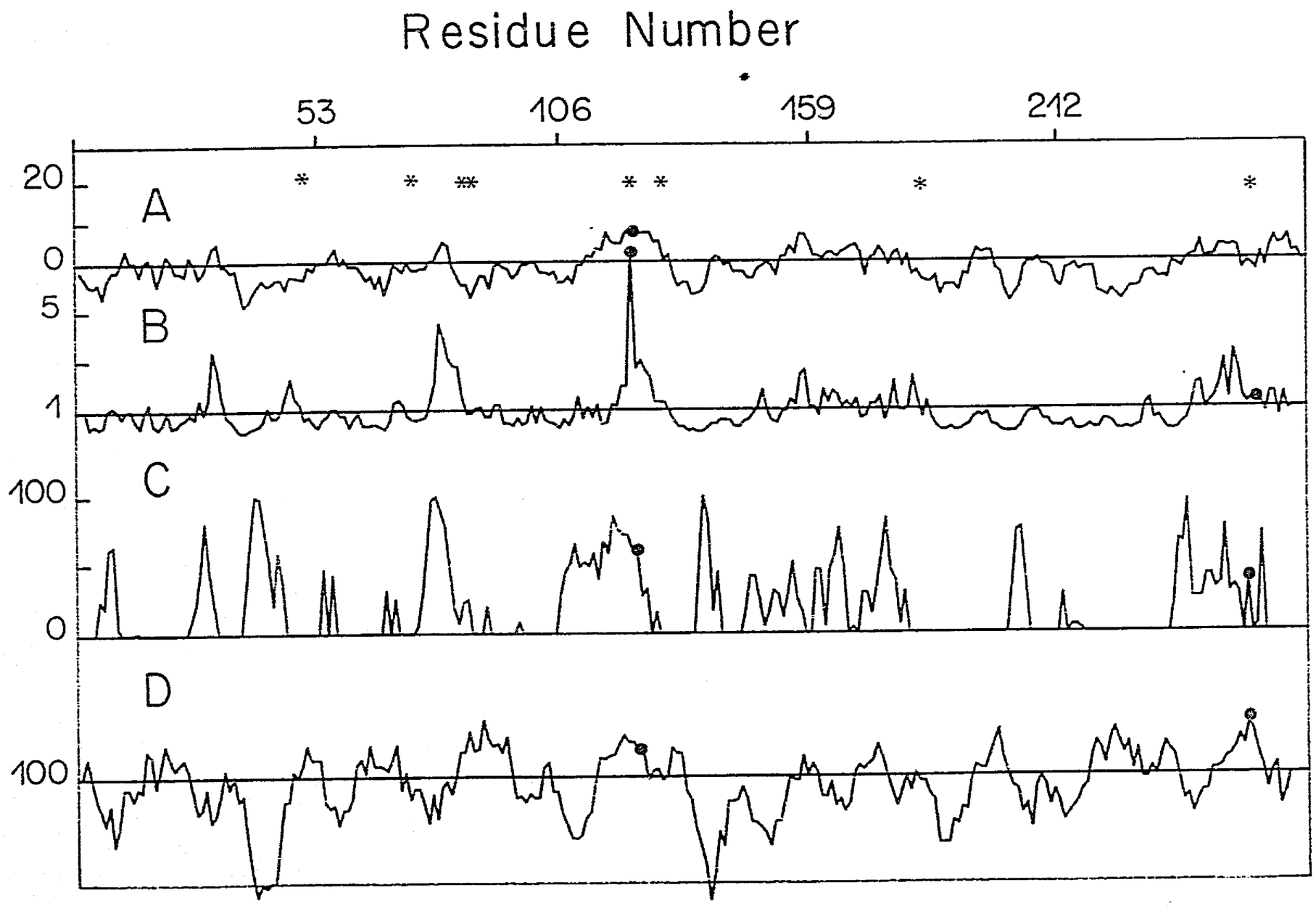

Figure 5

II. Influencia de las mutaciones sobre el equilibrio alostérico.

Identification of Two Cysteine Residues Forming a Pair of Vicinal Thiols in Glucosamine-6-phosphate Deaminase from *Escherichia coli* and a Study of Their Functional Role by Site-Directed Mutagenesis†

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ABSTRACT: The nucleotide sequence of the *nagB* gene in *Escherichia coli*, encoding glucosamine-6-phosphate deaminase, located four cysteinyl residues at positions 118, 219, 228, and 239. Chemical modification studies performed with the purified enzyme had shown that the sulfhydryl groups of two of these residues form a vicinal pair in the enzyme and are easily modified by thiol reagents. The allosteric transition to the more active conformer (R), produced by the binding of homotropic (D-glucosamine 6-phosphate or 2-deoxy-2-amino-D-glucitol 6-phosphate) or heterotropic (*N*-acetyl-D-glucosamine 6-phosphate) ligands, completely protected these thiols against chemical modification. Selective cyanylation of the vicinal thiols with 2-nitro-5-(thiocyanato)benzoate, followed by alkaline hydrolysis to produce chain cleavage at the modified cysteines, gave a pattern of polypeptides which allowed us to identify Cys118 and Cys239 as the residues forming the thiol pair. Subsequently, three mutated forms of the gene were constructed by oligonucleotide-directed mutagenesis, in which one or both of the cysteine codons were changed to serine. The mutant proteins were overexpressed and purified, and their kinetics were studied. The dithiol formed by Cys118 and Cys239 was necessary for maximum catalytic activity. The single replacements and the double mutation affected catalytic efficiency in a similar way, which was also identical to the effect of the chemical block of the thiol pair. However, only one of these cysteinyl residues, Cys239, had a significant role in the allosteric transition, and its substitution for serine reduced the allosteric interaction energy, due to a lower value of K_T.

Glucosamine-6-phosphate deaminase catalyzes the reversible conversion of D-glucosamine 6-phosphate (GlcN6P)[1] into D-fructose 6-phosphate and ammonia. It is allosterically activated by *N*-acetyl-D-glucosamine 6-phosphate (GlcNAc6P) (Comb & Roseman, 1958; Midelfort & Rose, 1977; Calcagno et al., 1984). The enzyme from *Escherichia coli* is an oligomeric protein composed of six identical polypeptide chains (Calcagno et al., 1984) whose primary structure is known from the DNA sequence of the gene *nagB* encoding this enzyme (Rogers et al., 1988). The gene, located at 15.5 min on the *E. coli* chromosome (White, 1968; Holmes & Russell, 1972),

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GlcN6P, D-glucosamine 6-phosphate; GlcNAc6P, *N*-acetyl-D-glucosamine 6-phosphate; NTCB, 2-nitro-5-(thiocyanato)benzoic acid; SDS, sodium dodecyl sulfate; TNB, 2-nitro-5-thiobenzoate.

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is part of the divergent *nagE–nagBACD* operons, which have been recently characterized (Rogers et al., 1988; Plumbridge, 1989; Vogler & Lengeler, 1989). The amino acid sequence of the enzyme (Rogers et al., 1988) shows four cysteinyl residues per enzyme subunit, corresponding to amino acids 118, 219, 228, and 239. Chemical modification studies on the purified deaminase from *E. coli* B had previously identified the presence of two reactive sulfhydryl groups per polypeptide chain in the native enzyme (Altamirano et al., 1987). They were shown to form a vicinal pair and to react with different thiol reagents, but only when the enzyme is in its less active allosteric conformation (Altamirano et al., 1989); upon allosteric activation by homotropic or heterotropic ligands, these thiols become completely protected (Altamirano et al., 1987, 1989). They are also involved in Zn^{2+} binding by the enzyme. When zinc-bound enzyme is allosterically activated, the Zn^{2+} ion is sequestered by the protein and is only released when it returns to its less-active conformation (Altamirano & Calcagno, 1990). This pair of sulfhydryls is essential for full activity of glucosamine-6-phosphate deaminase, affecting both the catalytic constant and allosteric activation. It has been shown that modification of the thiol pair by different reagents (Altamirano et al., 1989), or their participation in Zn^{2+} binding (Altamirano & Calcagno, 1990), produces a decrease of the molecular activity to half of the value for the native enzyme and a diminished cooperativity toward GlcN6P. This latter finding indicates that these thiols are playing some role in the allosteric transition and that the observed changes in their chemical reactivity are not a simple consequence of their passive displacement during the conformational transition. It is also possible that the region of the protein undergoing these structural changes involving vicinal thiols is located in the neighborhood of the active site, as suggested by the k_{cat} alteration in the SH-blocked enzyme.

To better understand the role of these thiols in the mechanism of catalysis and control of the deaminase, we have undertaken their localization along the enzyme's polypeptide chain, and we have used this information to construct the corresponding Cys to Ser mutants. This approach makes it possible to characterize the functional contribution of each thiol group individually. As both sulfhydryls are equally reactive to chemical reagents, their chemical modification does not allow us to evaluate their separate contribution to catalysis and regulation.

Experimental Procedures

Bacteria and Enzyme. Wild-type *E. coli* glucosamine-6-phosphate deaminase was prepared from a strain carrying a plasmid which overproduced the protein from the *lac* promoter, as previously described (Altamirano et al., 1991). Similar plasmids expressing the three mutated forms of the protein (missing one or both of the vicinal cysteines; see below) were constructed. The host strain for expression of these mutated proteins was IBPC546R, where expression of the chromosomal copy of the *nagB* gene is eliminated by insertion of a kanamycin-resistance cassette in the gene (Plumbridge, 1991). This strain is in addition Δ*lac*, so that expression of the deaminase is constitutive. Expression of *nagB* gene was measured by enzyme assays and SDS–polyacrylamide gel electrophoresis in the discontinuous Laemmli system (Laemmli, 1970). Overproduction of both wild-type and mutated proteins was estimated to be in the range 20–25% as judged by Coomassie blue G-250 staining of electrophoresis gels. Bacteria were grown and mutant deaminases were prepared as described for the wild-type enzyme (Altamirano et al., 1991). The *N*-(ε-aminohexanoyl)-D-glucosamine 6-phosphate agarose affinity column, which binds deaminase by the allosteric site, also proved to be useful for the purification of the mutant enzymes. Purity of all enzyme preparations was verified electrophoretically in polyacrylamide gels, both in the denaturing Laemmli system or with the native protein, in 4–30% pore-gradient gels, as described (Calcagno et al., 1984). The concentration of wild-type or genetically modified enzymes was calculated from the absorbance at 278 nm in a solution of 25 mM Tris-HCl buffer, pH 7.8, using the known molar absorptivity for the wild-type protein (Altamirano et al., 1987). Mutations at the two reactive cysteine residues were not expected to change the absorption at 278 nm (Edelhoch, 1967). Enzyme assays and analysis of the kinetic data were performed as described previously (Altamirano et al., 1987, 1989). Unless otherwise specified, kinetic data were obtained at pH 7.7 and 30 °C, in the presence of 2.5 mM EDTA.

Specific Cyanylation of the Reactive Thiols. A 5 μM solution of wild-type deaminase was cyanylated under nondenaturing conditions with 2 mM 2-nitro-5-(thiocyanato)-benzoate (NTCB) in 50 mM potassium phosphate buffer, pH 7.50, containing 2.5 mM disodium EDTA (buffer A). The time course of the reaction was followed spectrophotometrically at 412 nm, with the release of TNB anion measured against an appropriate blank in a double-beam spectrophotometer.

Identification of the Cysteine Residues Reactive with NTCB in the Native Protein. Deaminase polypeptide chain, specifically cyanylated at the reactive thiols, was cleaved at these modified residues by alkaline hydrolysis according to Dégani and Patchornik (1974). Aliquots of 2–3 nmol of deaminase in 100-μL samples were cyanylated overnight under the nondenaturing conditions described above. The reaction mixtures were then extensively dialyzed against buffer A to remove NTCB and TNB and then treated with 50 mM *N*-ethylmaleimide (NEM) for 4 h in 3.5 M guanidinium thiocyanate. This step was introduced in an attempt to block a buried sulfhydryl group, which becomes highly reactive on denaturation (Altamirano et al., 1987) and which would produce undesirable side reactions with the thiocyanoalanine residues. Samples were then dialyzed against water; the precipitated protein was dissolved by the addition of solid guanidinium thiocyanate to give a final concentration of 3.5 M, and the pH was adjusted to 9.5 with 0.1 volume of 0.9 M potassium borate buffer, pH 9.5, and some 0.1 M KOH, if necessary. In other samples, 0.5% SDS was used instead of guanidinium thiocyanate. Samples were incubated at 37 °C for 12–48 h, and then they were dialyzed against 50 mM Tris-HCl buffer, pH 6.8, using a dialysis membrane with a cutoff of 2 kDa (benzoylated dialysis tubing, Sigma Chemical Co.) and stored frozen. Diluted samples were concentrated by lyophilization. Dialyses were performed in microchambers made by cutting 1.4-mL plastic Eppendorf tubes transversely 4 mm below the border and closing the small chamber under the lid with a piece of dialysis membrane. The compartment containing the sample became sealed when the lid was placed in the tube. The position of the membrane is the same used by Reinard and Jacobsen (1989) in their equilibrium dialysis microchambers. Dialysis occurs through the open end of the cut tube; the assembled microchambers were submerged in a flask containing the dialysis buffer and were vigorously stirred.

Electrophoretic Separation of Cleavage Products. Deaminase samples cleaved at cysteinyl residues corresponding to vicinal thiols were fractionated by SDS–polyacrylamide gel electrophoresis using Laemmli discontinuous system and 0.75-mm-thick gel slabs. The stacking gel had 6.5% total

acrylamide concentration with 3.5% cross-linking. The separation gel was 21% with a cross-linkage of 0.5%. Low cross-linking was used to improve gel pliability; both gels contained 10% glycerol, to slow diffusion. These gels yield good separations in the range 30–3 kDa. Protein bands were detected with the silver-staining procedure of Wray et al. (1981). Cyanogen bromide fragments of sperm whale myoglobin, carbonic anhydrase, trypsinogen (phenylmethanesulfonyl fluoride treated), soybean trypsin inhibitor, and glucagon were used as molecular weight standards. Myoglobin BrCN fragments, which were prepared according to Gross and Witkop (1962), gave the five-band pattern described by Kratzin et al. (1989).

Construction of Mutated nagB Genes. The *nagB* gene, on a 1.4-kb *Eco*RI–*Hin*dIII fragment, was cloned from pUC(*nagB*) (Altamirano et al., 1991) into M13mp19. Oligonucleotide mutagenesis was performed by the Kunkel method, as described (Sambrook et al., 1989). In one of the mutants, the Cys(TGC)118 was changed to Ser(AGC); in the other, Cys(TGC)239 was changed to Ser(AGC). Oligonucleotides of lengths of 21 and 23 bases, respectively, were used. They corresponded to the sense strand of the *nagB* gene and were centered around the base to be mutagenized. The double mutation was made by introducing the Cys239 change into the M13 carrying the Cys118 mutation. The mutations were checked by sequencing using oligonucleotides conveniently placed within the *nagB* coding sequence. The changes are to highly expressed serine codons and are not expected to change the expressivity of the protein. The mutagenized genes were excised as *Eco*RI–*Hin*dIII fragments and were recloned back into pUC18 and also pBR322. In the latter plasmid, there is no strong promoter positioned to express the *nagB* gene, which is just expressed at a low level from plasmid transcription. This expression is however sufficient to allow complementation of the classic *nagB2* mutation (Rogers et al., 1988) present in strain IBPC571R (*thi*-1, *argG6*, *argE3*, *his-4*, *mtl-1*, *xyl-5*, *tsx-29*, *rpsL*, Δ*lacX74*, *nagB2*, *zbf507*::Tn10). The three mutated alleles were maintained in *recA* strains to minimize their loss by recombination. For purification purposes, the pUC(*nagB*) mutant plasmids were introduced into IBPC546R (*thi*-1, *argG6*, *argE3*, *his-4*, *mtl-1*, *xyl-5*, *tsx-29*, *rpsL*, Δ*lacX74*, *nagB*::km, *recA1*, *srl*::Tn10), the recombination-deficient strain carrying a null mutation in the chromosomal copy of the *nagB* gene (Plumbridge, 1991).

Measurements of the Reactive and Total SH Groups in Deaminase Mutant Proteins. The number of reactive thiols corresponding to the vicinal pair was determined with 5-5′-dithiobis(2-nitrobenzoic) acid (DTNB), at pH 8.0, 20 °C, in the presence of 5 mM EDTA, by measuring the release of 2-nitro-5-thiobenzoate (TNB), as described previously (Altamirano et al., 1987). After completion of the reaction, 10% SDS was added to give a final concentration of 1%, and the increase in TNB was measured, to determine buried sulfhydryls. Wild-type enzyme was also measured as reference.

Results

Specific Cyanylation of Vicinal, Reactive SH Groups. Wild-type, nondenatured deaminase was cyanylated with NTCB as described under Experimental Procedures. The reaction followed pseudo-first-order kinetics, as expected for two equally reacting groups. The stoichiometry of the reaction, calculated from the amount of TNB released, was 2.07 sulfhydryls/enzyme subunit. A second-order rate constant of 2.1 × 10^2 M^{-1} s^{-1} for the reaction at pH 8.0 and 20 °C was calculated. The kinetic study of the S-cyanylated enzyme at the deaminating direction of the reaction showed a k_{cat} of 820 ± 42 s^{-1} and a K_m for GlcN6P of 1.20 ± 0.07 mM, when assayed in the allosteric activated form in the presence of 1 mM GlcNAc6P. In the absence of the activator the enzyme displayed positive cooperativity, with a maximal Hill coefficient of 1.60 (Figure 1).

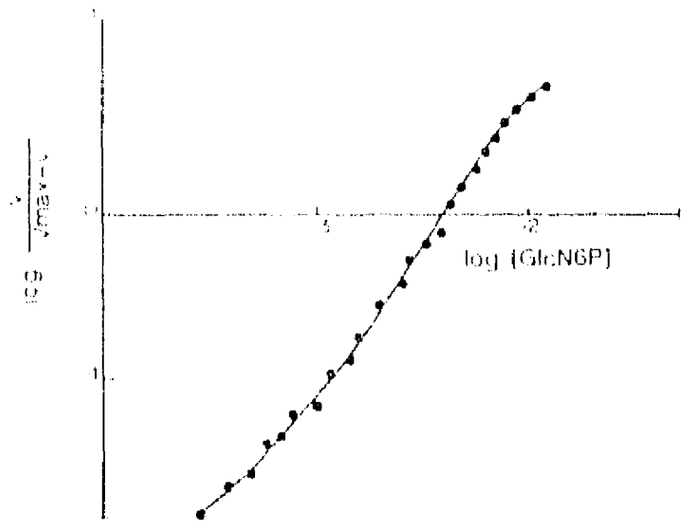


FIGURE 1: Kinetics of glucosamine-6-phosphate deaminase modified by cyanylation at the reactive pair of cysteinyl residues (Hill plot). Data plotted are averages from two independent experimental series. The maximum Hill coefficient for these data is 1.60.

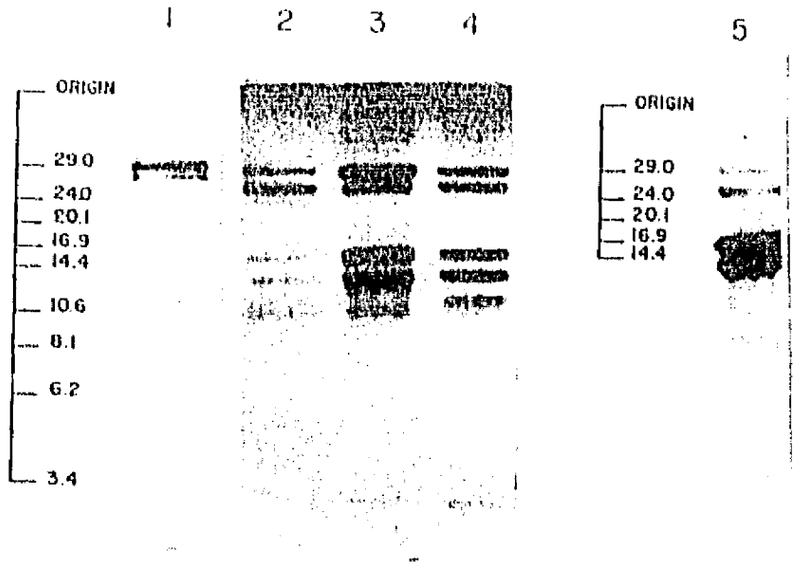
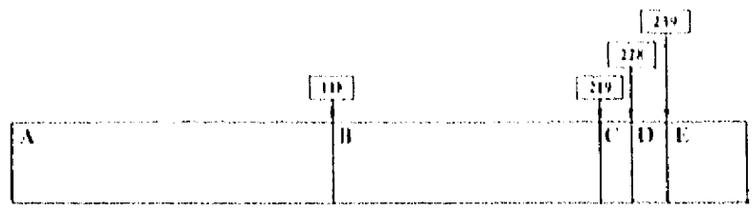


FIGURE 2: Electrophoretic separation of polypeptide chain fragments of *E. coli* glucosamine-6-phosphate deaminase produced by cleavage at the selectively cyanylated sulfhydryl groups. Lanes 2, 3, and 4: alkaline hydrolysis performed for 12, 24, and 48 h, respectively, in the presence of 0.5% SDS. Lane 5: alkaline hydrolysis performed in 3.5 M guanidinium thiocyanate for 24 h. Lane 1: control sample prepared with a noncyanylated enzyme, submitted to alkaline hydrolysis under the same conditions as the sample in lane 5. Total protein samples analyzed were in the range 0.5–2 mg, and the gel was silver stained. Molecular mass markers were carbonic anhydrase (29.0 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa), myoglobin (16.9 kDa), myoglobin chain fragment 1–131 (14.4 kDa), myoglobin chain fragment 56–153 (10.6 kDa), myoglobin chain fragment 56–131 (8.2 kDa), myoglobin chain fragment 1–55 (6.2 kDa), and glucagon (3.5 kDa). Other experimental details are described in the text.

Cleavage at Vicinal Sulfhydryls. Wild-type glucosamine-6-phosphate deaminase, stoichiometrically cyanylated at the reactive vicinal thiols, was cleaved at these residues according to Dēgani and Patchornik (1974). Cleavage fragments were separated by SDS–polyacrylamide gel electrophoresis and identified by their molecular masses (Figure 2, lanes 2–4). A sample of noncyanylated enzyme, submitted to all subsequent steps described under Experimental Procedures, was also prepared and run as a control for nonspecific cleavage (Figure 2, lane 1). The three main fragments observed were found to correspond to molecular masses of 27, 17, and 13 kDa. A



A :	13 210
B :	11 021
C :	1 090
D :	1 250
E :	3 272

Cleavage fragments Theoretical Mr		Fragments found (kDa)
A	13 210	13.0
B C D E	16 636	17.0
A B C D	26 574	27.0
B C D	13 364	13.0
E	3 272	3.3

FIGURE 3: Schematic representation of the polypeptide chain of *E. coli* glucosamine-6-phosphate deaminase. The position of cysteinyl residues is indicated, and the fragments between them are designated with letters from A to E, in order along the protein sequence. Molecular masses of fragments found are the estimated values from electrophoresis gels.

faint band at 3.3 kDa was also observed; probably most of this component is lost in the fixing and staining steps. A considerable amount of enzyme remained uncleaved when the denaturant was 0.5% SDS; the 29.8-kDa band corresponding to the intact protein was more conspicuous in samples hydrolyzed for 12 h (lane 2) than in samples hydrolyzed for 24 and 36 h (lanes 3 and 4). After incubation for 24 h, hydrolysis was more complete when guanidinium thiocyanate was used instead of SDS (lanes 5 and 3, respectively). Under the conditions of incomplete cleavage, 27-, 17-, and 13-kDa fragments were apparent, suggesting that they correspond to the splitting at one or the other of the two possible cleavage sites. When hydrolysis was closer to completion, as judged by the amount of unhydrolyzed enzyme present (Figure 2, lanes 4 and 5), the 27-kDa component diminished and the 13-kDa band increased. The possible cleavage sites, at the four cysteinyl residues of glucosamine-6-phosphate deaminase, are depicted in Figure 3. In this figure, the segments between cysteinyl residues are designated with letters from A to E, and their calculated molecular masses are given. We have found a fragment of 17 kDa, which most likely corresponds to BCDE, with a calculated molecular mass of 16 636 Da. There was, in addition, the appearance of an equivalent amount of a 13-kDa polypeptide which could correspond to fragment A (molecular mass 13 210 Da). The simultaneous appearance of these two bands suggests that Cys118 is a cleavage site. The existence of a 27-kDa peptide is consistent with the other cleavage site being located near the C-terminal end of the chain, where the other three cysteine residues are found. Taking into account the observation of a band at 3.3 kDa, which can only correspond to fragment E (molecular mass 3272 Da), the 27-kDa fragment can be reasonably assigned to fragment ABCD (molecular mass 26 574 Da). Due to this, the other cleavage site corresponds to Cys239. According to this interpretation, the intense 13-kDa band corresponds to the superimposed peptides A (13 210 Da) and BCD (13 364 Da). If cyanylation and hydrolysis occurred at Cys219, fragments of 24 and 5.5 kDa should be seen, while cleavage at Cys228 would generate fragments of 25 and 4.5 kDa. We have not observed peptides corresponding to these molecular masses.

Mutated Forms of Glucosamine-6-phosphate Deaminase at Cys118 and Cys239. After identification of Cys118 and Cys239 as the cysteinyl residues bearing the vicinal thiols, the corresponding mutant genes were constructed, where the cysteine codons were converted to serine. The modified enzymes carrying the mutations Cys118 to Ser, Cys239 to Ser, and the corresponding double mutant involving both residues were overproduced and purified as described under Experimental Procedures. Our initial sequence assignment for the two reactive cysteinyls was confirmed by direct thiol titration with the three genetically modified enzymes under native and denaturing conditions. As shown in Table I, single-mutant enzymes lack one reactive sulfhydryl, and both thiols are absent in the double mutant. Data from the wild-type enzyme are also included for comparison.

Table I: Number of Sulfhydryl Groups Reacting with DTNB, per Deaminase Subunit, in Wild Type and Cys to Ser Mutants[a]

	nondenatured	1% SDS
wild type	2.04 ± 0.09	3.10 ± 0.11
Cys118 to Ser	0.96 ± 0.08	2.02 ± 0.05
Cys239 to Ser	1.050 ± 0.13	1.89 ± 0.07
Cys118 to Ser, Cys239 to Ser	0.11 ± 0.04	0.96 ± 0.06

[a] Experimental conditions are described in the text. Data are averages ± standard errors, from at least five determinations.

Table II: Kinetic Parameters of Wild Type and Cys to Ser Mutants of Glucosamine-6-phosphate Deaminase[a,b]

	K_m (mM)	k_{cat} (s^{-1})	h_{max}
wild type	1.89 ± 0.16	1810 ± 40	3.09 ± 0.07
Cys118 to Ser	0.84 ± 0.07	898 ± 14	3.03 ± 0.08
Cys239 to Ser	0.77 ± 0.03	842 ± 58	1.60 ± 0.10
Cys118 to Ser, Cys239 to Ser	0.81 ± 0.08	887 ± 44	1.45 ± 0.13

[a] Experimental conditions are described in the text. Data are averages ± standard errors, from at least four experiments.

Kinetics of Genetically Modified Glucosamine-6-phosphate Deaminases. The kinetic behavior of the three mutant forms of glucosamine-6-phosphate deaminase, the two single mutants, and the double mutant is summarized in Table II. Data from the wild-type enzyme were also included for comparison. The three mutant enzymes present similar changes in the apparent catalytic constant, $V_{max}/[E_t]$, i.e., the microscopic k_{cat} multiplied by the number of catalytical sites. This value corresponds to half of the value for the native enzyme. The replacement of one or both of the thiols also produced a modification in K_m for GlcN6P, which changed from 2.0 to 0.8 mM. Thus, the catalytic efficiency or specificity constant (k_{cat}/K_m) (Fersht, 1985) remains effectively constant for the wild-type and most mutants (about 10^6 M^{-1} s^{-1}). Since glucosamine-6-phosphate deaminase is an allosteric enzyme of the K-type (Calcagno et al., 1984), these data were obtained in the presence of a saturating concentration of the allosteric activator, GlcNAc6P, to produce hyperbolic kinetics. In the absence of this ligand, the enzyme from the double mutant displays positive cooperativity, but less than the wild-type enzyme, with a maximum Hill coefficient (h_{max}) of nearly 1.5. The enzyme having the mutation Cys118 to Ser displays an unmodified cooperative behavior, similar to that of the wild-type protein. On the other hand, the replacement Cys239 to Ser produces a deaminase distinctly less cooperative, with nearly the same Hill coefficient as that observed for the double-mutant enzyme (Figure 4 and Table II).

Effect of Zn^{2+} on Cys to Ser Glucosamine-6-phosphate Deaminase Mutants. The three genetically modified enzymes prepared were preincubated with zinc acetate, under conditions which result in saturation of the zinc-binding site (Altamirano & Calcagno, 1990); the enzyme was then assayed in the presence and in the absence of the allosteric activator. The kinetic parameters, K_m for GlcN6P and k_{cat} and h_{max} for the

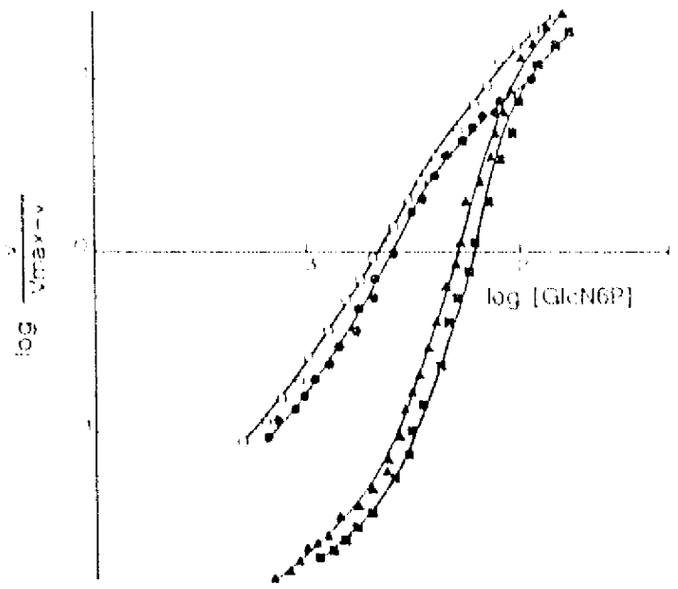


FIGURE 4: Kinetics of the wild type and Cys to Ser mutants of glucosamine-6-phosphate deaminase. Data of initial velocities as a function of GlcN6P concentration are presented as Hill plots. Details are described under Experimental Procedures. The data plotted are averages from two separate experimental series. Squares: wild-type enzyme. Triangles: Cys118 to Ser mutant. Open circles: Cys239 to Ser mutant. Filled circles: Double mutant Cys118 to Ser and Cys239 to Ser. The kinetic parameters derived from these curves are given in Table II.

Table III: Kinetic Changes Produced by Zinc Ion on Cys to Ser Mutants of Glucosamine-6-phosphate Deaminase[a,b]

	K_m (mM)	k_{cat} (s^{-1})	h_{max}
wild type	0.95 ± 0.12	890 ± 65	1.60 ± 0.08
Cys118 to Ser	1.24 ± 0.06	1 030 ± 41	1.57 ± 0.09
Cys239 to Ser	1.04 ± 0.04	1086 ± 50	1.39 ± 0.10
Cys118 to Ser, Cys239 to Ser	2.20 ± 0.06	954 ± 32	1.80 ± 0.10

[a] A 40 nM enzyme solution in 50 mM Tris–MES buffer (pH 8.0) was incubated for 2 h with 1 μM zinc acetate. This incubation time is 10 times the half-life of the Zn^{2+}-binding reaction for the wild-type enzyme (Altamirano & Calcagno, 1990). The enzyme was assayed in the presence of 0.5 mM GlcNAc6P to determine K_m and k_{cat}, and also without activator to calculate h_{max}, as described in the text. Data are averages ± standard errors from at least four experiments. Reference values for the untreated wild-type enzyme are given in the first row of Table II.

three Cys to Ser mutants, are shown in Table III. Results corresponding to the wild-type enzyme are also included. We note that the Zn^{2+} ion did not induce any additional kinetic change on either mutant involving Cys239. On the other hand, the treatment with zinc on the Cys118 to Ser mutant enzyme, produced a change in cooperativity similar to the effect already described for the wild-type protein.

Discussion

Localization of the Reactive, Vicinal Cysteines along the Deaminase Sequence. The two vicinal cysteinyl residues of glucosamine-6-phosphate deaminase, which play an important role in catalysis and allosteric regulation of deaminase activity, have been located in the polypeptide chain of the enzyme. In the absence of ligands, they provide the unique thiols reactive in the native protein. In the presence of either homotropic or heterotropic ligands which shift the allosteric equilibrium to the R conformer, they become unreactive. When this property was taken advantage of, both residues were specifically cyanylated and the polypeptide chain was cleaved at these positions (Dégani & Patchornik, 1974; Jacobson et al., 1973). From the molecular mass of the hydrolysis fragments and the known amino acid sequence of the enzyme, we identified these cysteines as residues 118 and 239. The kinetic study of the deaminase stoichiometrically cyanylated at these cysteines showed changes which are similar to those produced by other chemical reactions involving the same groups (Altamirano et al., 1989, Altamirano & Calcagno, 1990).

Cys to Ser Mutants at Residues 118 and 239. The two single Cys to Ser mutants and the corresponding double mutant involving both residues were constructed, and the modified enzymes were overexpressed and purified. In these variant proteins, serine was chosen to replace cysteine, to introduce a structurally conservative mutation (Gosh et al., 1986). The spectrophotometric titration of thiol groups in these mutant proteins confirmed the sequence assignments made by chemical cleavage (Table I). These data also show the presence of a third sulfhydryl, reactive only under denaturing conditions. Glucosamine-6-phosphate deaminase from *E. coli* K12 has four cysteinyl residues per chain (Rogers et al., 1988), but only three are titrable in the denatured wild-type form, two in both single Cys to Ser mutants, and only one in the double mutant (Table I). This indicates that one of the remaining cysteinyl residues, Cys219 or Cys228, has its thiol group blocked and probably forms a disulfide.

Our previous data and the evidence presented here on the chemical reactivity of Cys118 and Cys239 show that they are specifically and stoichiometrically modified with many sulfhydryl reagents. This is an indication that they are located at the enzyme surface. Indeed, the predicted secondary structure of the enzyme (Altamirano et al., 1991) locates Cys118 at a single hydrophilic α-helical segment, also predicted as a flexibility peak. On the other hand, Cys239 appears in a predicted β-turn with high scores of flexibility and surface probability. The remaining two cysteinyl residues of the enzyme, Cys219 and Cys228, appear in segments predicted to be hydrophobic and are expected to be buried inside the deaminase structure.

Role of Cys118 and Cys239 in Allosteric Transition. From chemical modification experiments, we know that in the three-dimensional structure of the deaminase Cys118 and Cys239 have their thiols in a vicinal position (Altamirano et al., 1989). They are both equally reactive toward a set of sulfhydryl reagents (Altamirano et al., 1987, 1989; Altamirano & Calcagno, 1990), including NTCB (see Results); the chemical blockage of this thiol pair produces a deaminase with modified catalytic and allosteric properties. The present study allowed us to evaluate separately the role of each cysteinyl residue in the function of this enzyme. Data from Table II and Figure 4 show that the role ascribed to the vicinal thiols in the stabilization of the T allosteric conformer (Altamirano et al., 1989) is played only by Cys239. The mutant Cys118 to Ser presented the same cooperativity and has the same ability to be activated by GlcNAc6P as that of the wild-type protein. On the other hand, the single mutant Cys239 to Ser and the double mutant gave similar Hill plots which look like the data reported previously for deaminases having the thiol pair chemically blocked. As the two thiols from the vicinal pair are equally reactive, this was an unexpected result. We must assume that both thiols are involved in thermodynamically equivalent interactions, but with different segments of the polypeptide chain, and that only one of them, Cys239, participates in some kind of bonding having an important role in allosteric transition. Mutants involving Cys239 have a lower interaction energy due to a change in K_T and behaved as if they were in an intermediate position between R and T conformations. Upon saturation with the allosteric activator, the K_m for GlcN6P obtained with all Cys to Ser mutants studied was lower than the value of the wild-type enzyme (Table II); these K_m values can be considered identical to K_R. From Hill

plots, we can estimate a K_I of 38 mM for the wild-type or Cys118 to Ser enzyme. Mutants replacing Cys239 gave lower K_I values, near 9 mM. This indicates that the main allosteric change produced by the Cys239 to Ser mutation is a change in the difference of $K_I - K_R$ and, consequently, in Wyman's apparent interaction energy (Wyman, 1964), mainly at the expense of the K_I.

Catalytic Constants of Cys to Ser Mutants. The replacement of either vicinal cysteinyl residue (Cys118 or Cys239) on the deaminase catalytic constant has the same effect: the apparent k_{cat} for the fully activated enzyme is reduced to nearly half of the value for the wild-type enzyme. This contribution to the catalytic power of the enzyme requires both residues of the thiol pair, and neither Cys118 nor Cys239 alone is sufficient to restore the full catalytic activity. The same 2-fold activity reduction is observed whether the dithiol is suppressed by mutation or by chemical modification. The precise role of this pair in catalysis remains to be established. We can take into account that they retain their vicinal condition in the R conformer (Altamirano et al., 1989) and that their proximity to the active site would modify the proton dissociation constant of a neighboring group involved in the catalytic mechanism. A thorough kinetic study at different pH values of wild-type and the Cys to Ser mutant deaminases may throw some light on this point.

It is interesting to observe that the ratio k_{cat}/K_m (specificity constant) has almost the same value for the wild-type and the mutant enzymes, nearly 10^6 M^{-1} s^{-1}. According to Fersht (1974), a compensatory change in the affinity of the deaminases for GlcN6P is to be expected, if the binding of the transition state of GlcN6P to the protein is not affected by the Cys to Ser mutations.

Concluding Remarks. Modified deaminases in which both cysteines were changed to serine residues (Table II) behaved similarly to the wild-type enzyme in which both thiols have been blocked with different small-sized substituents (Altamirano et al., 1989). This result demonstrates that the kinetic changes described by chemical modification procedures are due to the lack of the free thiol pair and do not depend on steric factors caused by the presence of the chemical groups introduced. The kinetic changes observed in the Cys to Ser mutants point out the different roles of Cys118 and Cys239, in R and T conformers. We have previously discussed that changes in cooperativity and k_{cat} produced by chemical suppression of the dithiol could be accounted for by assuming half-of-the-sites kinetics (Altamirano et al., 1989). The results described here show that both effects are unrelated; only Cys239 is necessary for full cooperative behavior, but both cysteinyls must be present for maximum catalytic activity.

In a previous paper (Altamirano & Calcagno, 1990), we have reported the presence of one zinc-binding site per deaminase subunit. The vicinal thiols, now identified as Cys118 and Cys239, seemed to play an essential role in the Zn^{2+}-binding site. The results, summarized in Table III, show that, as expected, zinc did not produce additional kinetic changes in deaminases lacking the Cys239 thiol. Mutant deaminase lacking only Cys118, which displays an intense cooperativity similar to that of the wild-type enzyme (Table II), was modified by low zinc concentrations, giving the same kinetic pattern as that of the Cys239-lacking enzyme. This observation confirms the important role of Cys239 in the allosteric transition. It also proves that the Zn-binding site is still functional when the Cys118 sulfhydryl is replaced by a hydroxyl group in the Cys to Ser mutation. This demonstrates that Cys118 and Cys239 have different roles in the formation of the Zn-binding site.

Cooperative behavior of glucosamine-6-phosphate deaminase is strongly dependent on pH (Calcagno et al., 1984); it is maximal in the range 7.8–8.2. In the light of the present results, it may be interesting to study the role of sulfhydryl proton dissociation from Cys239 as one of the possible protonic equilibria involved in this pH effect. Ligands involving this group, as Zn^{2+} or H^+, may then play an important role in deaminase regulation.

Registry No. GlcNAc6P, 1746-32-3; GlcN6P, 3616-42-0; GlcN6P deaminase, 9013-10-9; Cys, 52-90-4; Zn, 7440-66-6; Ser, 56-45-1.

References

Altamirano, M. M., & Calcagno, M. (1990) *Biochim. Biophys. Acta 1038*, 291–294.

Altamirano, M. M., Mulliert, G., & Calcagno, M. (1987) *Arch. Biochem. Biophys 258*, 95–100.

Altamirano, M. M., Lara-Lemus, R., Libreros-Minotta, C. A., & Calcagno, M. (1989) *Arch. Biochim. Biophys. 269*, 555-561.

Altamirano, M. M., Plumbridge, J. A., Hernández-Arana, A., & Calcagno, M. L. (1991) *Biochim. Biophys Acta 1076*, 266–272.

Calcagno, M., Campos, P. J., Mulliert, G., & Suástegui, J. (1984) *Biochim. Biophys. Acta 787*, 165-173.

Comb, D. G., & Roseman, S. (1958) *J. Biol. Chem. 232*, 807-827.

Dégani, Y., & Patchornik, A. (1974) *Biochemistry 13*, 1-11.

Edelhoch, H. (1967) *Biochemistry 6*, 1948-1954.

Fersht, A. R. (1974) *Proc. R. Soc. London, B 187*, 397-407.

Fersht, A. R. (1985) *Enzyme Structure and Mechanism*, 2nd ed., W. H. Freeman and Co., New York.

Ghosh, S., Bock, S., Rokita, S., & Kaiser, E. (1986) *Science 231*, 145–148.

Gross, E., & Witkop, B. (1962) *J. Biol. Chem. 237*, 1856–1860.

Holmes, R. P., & Russell, R. R. B. (1972) *J. Bacteriol. 111*, 290–291.

Jacobson, G. R., Schaffer, M. H., Stark, G. R., & Vanaman, T. C. (1973) *J. Biol. Chem. 248*, 6583–6591.

Kratzin, H. D., Wiltfang, J., Karas, M., Neuhoff, V., & Hilschmann, N. (1989) *Anal. Biochem. 183*, 1–8.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Leloir, L. F., & Cardini, C. (1962) in *Methods in Enzymology* (Collowick, S. P., & Kaplan N. O., Eds.) Vol. 5, pp 418–422, Academic Press, New York.

Midelfort, C., & Rose, I. A. (1977) *Biochemistry* 16, 1590–1596.

Plumbridge, J. A. (1989) *Mol. Microbiol. 3*, 506–515.

Plumbridge, J. A. (1991) *Mol. Microbiol. 5*, 2053–2062.

Reinard, T., & Jacobson, H. J. (1989) *Anal. Biochem. 176*, 157–160.

Rogers, N. J., Ohgi, T., Plumbridge, J., & Söll, D. (1988) *Gene 62*, 197–207.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Vogler, A. P., & Lengeler, J. W. (1989) *Mol. Gen. Genet. 219*, 97–105.

White, R. J. (1968) *Biochem. J. 106*, 847–85.

Wray, W., Boulikas, T., Wray, V. P., & Hancock, R. (1981) *Anal. Biochem. 118*, 197–203.

Wyman, J. (1964) *Adv. Protein Chem. 19*, 223–286.

EFFECTS OF REPLACEMENT OF TYROSINE 121 ON ACTIVITY AND ALLOSTERIC PROPERTIES OF *Escherichia coli* GLUCOSAMINE-6-PHOSPHATE DEAMINASE

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Introduction

Glucosamine-6-phosphate deaminase is the only allosterically regulated enzyme of amino sugar catabolism in *Escherichia coli* (Plumbridge *et al*, 1993). The allosteric response of the enzyme to the intracellular levels of N-acetyl-D-glucosamine-6-phosphate (GlcNAc6P) allows for the increase of enzyme activity at the expense of a lowering of the affinity for its substrate, GlcN6P. This is a classical K-effect, and recently we have shown that the kinetics of deaminase can be satisfactorily described by the allosteric concerted model (Monod *et al.*, 1965, Altamirano and Calcagno, *submitted*). The spectrochemical study of the interaction of the enzyme with GlcNAc6P, its allosteric activator, indicated the presence of tyrosyl residues at or near the allosteric site. A specific tyrosyl residue having a pK of 8.75, becomes protected from pH deprotonation by GlcNAc6P saturation (Altamirano *et al.*, 1993, *submitted*). A theoretical analysis of the amino acid sequence of the deaminase using a set of indices for the prediction of surface accessibility of amino acid residues, shows that the most probable tyrosyl residues involved in this function are Y121 and Y254; Y121 , which is located in a segment predicted as a highly polar helical segment, gives the highest scores for surface accessibility (Altamirano *et al.*, submitted). According to these predictions, the site-directed mutants Y121T and Y121W were constructed to assess experimentally the role of this residue in the function of glucosamine-6-phosphate deaminase. The chosen replacement amino acids were Thr, selected as a structural conservative mutation, that does not affect the stability of the secondary structure of the segment, and Trp chosen to introduce an aromatic residue in the same position. This mutation suppresses the phenolic hydroxyl of Y121 but conserves the possible interactions depending on the aromatic side-chain. These two mutant forms of the enzyme were compared to the wild-type enzyme with regard to catalytic activity, substrate and analogue ligands binding, and allosteric properties of the enzyme. As shown here, replacements at position 121 resulted in mutant forms exhibiting striking differences in allosteric activator binding and allosteric properties compared to the wild-type enzyme.

MATERIALS AND METHODS

Bacterial strains and construction of site-directed mutants

E. coli wild-type glucosamine-6-phosphate deaminase was prepared from an overproducing strain, as previously described (Altamirano *et al.*, 1991).

Amino acid replacements at position 121 were performed by the oligonucleotide directed mutagenesis. The synthesis and purification of the oligonucleotides used for site-directed mutagenesis and DNA-sequencing were synthesised on a Beckman DNA synthesiser and purified by 20% urea polyacrylamide gel electrophoresis. The *nagB* gene from puC18 was cloned into PTZ18R plasmid. The *nagB* gene from PTZ18R was then removed by digestion with *Eco*RI and *Hind*III and ligated into M13 for site directed mutagenesis by the method of Kunkel, as described by Sambrook (1989), using the mutagene kit from BioRad. Uracil-containing single-stranded DNA was generated in *a dut- ung-* mutant *E. coli* strain RZ1032 and used as the template to synthesise the mutated strain with the synthetic primer. The oligonucleotide CGAAGTGACGCGACTGG or TCTGAAGAAAAAATCCG for the generation of Y121W or Y121T mutants. The underlined bases are those introduced to produce the mutations. Finally, the obtained double-stranded DNA was transformed into JM101R *E. coli* strain. DNA sequencing by Sanger's dideoxy chain terminator method was used to screen the mutants and to verify the entire sequence of one strand of the mutant DNA. The host strain used for the expression of these mutated proteins, was IBPC590, which is *nagB::kan,recA1*, a recombination-deficient strain carrying a null mutation in the chromosomal copy of the *nagB* gene (Plumbridge, 1991).

Purification of mutant forms of deaminase

Wild-type and genetically-modified forms of glucosamine-6-phosphate deaminase were obtained and assayed using previously reported procedures, using allosteric-site affinity chromatography (Altamirano *et al.*, 1991, 1992).

The concentration of the wild-type enzyme was calculated from its absorbance at 278 nm at pH 7.7, using its known molar absorptivity (20.0×10^4 M^{-1} cm^{-1}, Altamirano *et al.*, 1987). The molar absorptivity for the mutant forms of the enzyme were calculated from the spectra of the proteins in 100 mM Tris-HCl buffer, pH 7.7, and the concentration of the protein in

the solution, measured with the method of Bradford, using the wild-type enzyme as standard. A convenient method to refine the ε_{277} values of the mutants involving aromatic amino acid residues, is the measurement of the number of the reactive thiol groups under native conditions. We have shown that two cysteine residues, C118 and C239 are reactive in the native deaminase, provided that the protein be in the T allosteric conformer. These thiols can be accurately measured with DTNB, and the data used to obtain a good measurement of protein concentration and to refine the molar absorptivity data.

Reagents

Biochemicals and most reagents were from Sigma Chemical Co., (St. Louis, MO). GlcNAc6P was prepared by acetylation of GlcN6P and purified by ion-exchange chromatography, according to Leloir and Cardini (1962). The deaminase dead-end inhibitor, 2-deoxy-2-amino-D-glucitol-6-phosphate (GlcN-ol-6P), was synthesized and purified as described by Midelfort and Rose (1977).

Spectrophotometrical titration of tyrosines

Difference spectra were recorded in a Cary 4 double beam spectrophotometer at 30 °C. Acid-base titrations of tyrosyl residues in native mutant forms of the deaminase, were performed by recording the spectra of a set of 3-5 μM deaminase solutions, prepared in 50 mM ACES, 25 mM MES, 25 mM ethanolamine buffer, at various pH values. This buffer system minimizes the change of ionic strength with pH (Ellis and Morrison, 1982). As a fully protonated protein for reference, the enzyme solution at pH 6.0 was employed. The ionisation of tyrosyl residues was calculated from the absorbance change at 295 nm, using the absorptivity coefficient of 2.33×10^3 M^{-1} cm^{-1} (Donovan, 1973).

RESULTS AND DISCUSSION

Tyrosine 121 is a residue located at or near the allosteric site.

We have previously demonstrated the presence of a tyrosyl residue with a pK of 8.75, detected by spectrophotometrical pH titration, using the red shift of main absorption peak of

the phenolate anion (Altamirano *et al.*, submitted). In Fig. 1 , are shown the near-UV spectra of both mutant forms of deaminase, obtained at pH 10.5 and pH 6.0, and the corresponding difference spectra. All spectra were obtained in the absence of ligands. For both mutant enzymes the spectra did not change in the explored pH range, and the differential spectrum lacks of the peak at 295 nm, corresponding to the dissociation of the phenolic hydroxyl of tyrosine. This result confirms our postulated identification of Tyr121 as the residue whose side chain was protected from titration by the addition of a saturating concentration of the allosteric activator of the enzyme, GlcNAc6P.

Kinetics of mutant deaminases with replacements at position 121: effect on homotropic cooperativity and activity.

Substitution of either Thr or Trp for Tyr at position 121 produced important changes in the allosteric behaviour of glucosamine-6-phosphate deaminase, The curve of initial velocities *versus* GlcN6P concentration, reveal that Y121W deaminase display a more intense homotropic cooperativity than the wild-type enzyme. The magnitude of the cooperativity can be measured by the maximal value of the Hill coefficient (Wyman, 1975). This value is 3.42 $\pm$ 0.2, in contrast with the wild-type or Y121T enzymes, which have a Hill coefficients near 3.0 (Table I). The kinetic data obtained in the absence of the activator give good fits to the MWC model. The observed increase in the homotropic cooperativity of Y121W deaminase, is mainly caused by a decrease of the K_R/K_T ratio, i.e., the **c** parameter of MWC equation (Monod et. al, 1965). This value, usually in the 0.02-0.03 range for the wild-type enzyme, did not change in the Y121T mutant but decreased to 5.7×10^{-4} in Y121W. On the other hand, the parameter L, the allosteric constant, remains almost unmodified in Y121W mutant form of the enzyme (Table I). Assuming that K_R value is identical to the K_m for the R conformer measured under hyperbolic kinetics (in the presence of a saturating concentration of GlcNAc6P), we can calculate a K_T of 1.05 M. On the other hand, Y121T deaminase has practically the same homotropic behaviour as the wild-type enzyme. Nevertheless, the most important kinetic change produced by the amino acid substitution is the significant decrease of the kcat values for both mutant forms (Table I). The k_{cat} for the Y121W enzyme is 7 times lower than the wild-type protein, but the most striking result is the nearly two fold

increase of this parameter by the addition of very low concentrations of the allosteric activator. This unusual V-effect, is also observed with the Y121T mutant, which is less active, with a kcat of 17 s^{-1} in the absence of the activator. This value is two orders of magnitude lower than the corresponding one for the wild-type enzyme, which behaves as a typical K-system.

Heterotropic effects on Y121T and Y121W deaminases. The activation of mutant enzymes by GlcNAc6P.

The kinetics of the activation of the wild-type deaminase by GlcNAc6P can be satisfactorily accounted for by the MWC model, assuming exclusive binding of GlcNAc6P (Altamirano *et al.*, submitted). Although both deaminases with replacements of Y121 are active and allosteric enzymes as shown in Table I (indeed, they were purified by allosteric-site affinity chromatography, *c.f.* Materials and Methods), the kinetics of GlcNAc6P activation appears radically modified, and can not longer be accounted using a simple two-states model (Figures 2 and 3, and Table I). The kinetic changes in GlcNAc6P activation of both mutant deaminases with replacements at position 121 can be better recognized in the plots of h_{max} (the maximal values of the Hill coefficients), *versus* the activator concentration (Fig. 3). The effect of the activator is biphasic, and two distinct activation steps are apparent, suggesting the existence of a stable intermediate conformer of the protein with a halfway structure between the T and R conformations. This stable form seems to predominate in the GlcNAc6P concentration range from 0.1 to 1 mM, in the case of Y121W enzyme.

GlcNAc6P binding curve calculated from the kinetics of the protection of sulfhydryl groups to their reaction with DTNB.

Sulfhydryl groups from C118 and C239 react with DTNB and other thiol reagents when the enzyme is in the T conformation, but they become completely hindered when the T→R transition is induced by saturation with either homotropic or heterotropic ligands (Altamirano *et al.*, 1989, 1992). We studied the kinetics of the sulfhydryl reaction with DTNB and the protective effect of GlcNAc6P to analyse the allosteric conformational change by a different

method, and to estimate the changes in the affinity for GlcNAc6P produced by the amino acid replacements. We have already used this procedure to study the GlcNAc6P-protein interaction in the wild-type enzyme. The apparent pseudo first-order rate constant for the reaction of cysteines with DTNB plotted *versus* GlcNAc6P concentration, is shown in Fig. 4. The protective effect of the allosteric activator, can be described by the following expression (Altamirano and Calcagno, submitted):

$$v = \frac{Et\ k^*\ L}{L + (1+\gamma)^n}$$

where k* is the apparent pseudo-first-order rate constant in the presence of the protective ligand, GlcNAc6P. The experimental data fit well to this equation; the curve shows the expected cooperative kinetics of the GlcNAc6P protective effect (Hill coefficient = -3.01 $\pm$ 0.22, $[GlcNAc6P]_{0.5}$ = 1.5 $\pm$ 0.3 mM). From these data, a Kd of 0.95 $\pm$ 0.35 mM of for GlcNAc6P was estimated. Similar results were obtained with the Y121T mutant form of the deaminase (not shown).

The comparison of these results with those obtained in Figs. 2 and 3, suggests that only the second phase of the biphasic activation curve of the mutant deaminases, is measured by this method, which detects a conformational change associated with the allosteric transition.

Concluding remarks

The results reported here, demonstrate that the tyrosyl residue located at position 121 plays a critical role in the structure and function of glucosamine-6-phosphate deaminase. Tyrosine 121 has been recognized as relevant for GlcNAc6P binding from spectrochemical studies (Altamirano *et al.*, submitted) and its replacement was expected to impair the activation of the enzyme by GlcNAc6P. This change was certainly observed, and both Y121 mutant forms of the deaminase need a higher concentration of the allosteric activator to reach hyperbolic kinetics (Fig. 2 and Table I). However, the kinetic changes produced by both substitutions at the position 121, are very complex. Both Y121 mutants present a remarkable decrease of

the catalytic constant, and the kcat values for both mutants are increased by GlcNAc6P at a very low (nanomolar) concentration, although they never reach the typical kcat values for the wild-type enzyme. The present results are insufficient to propose a model to account for all these changes, produced by a point mutation. We can guess that the impaired catalytic function of the enzyme reveals that the residue at this position is also important for the structure of the active site. This role can be partially played by the W121 replacing for the original tyrosine, because Y121W deaminase is 14 times more active than Y121T enzyme in the absence of the activator, and twice more active, in the presence of GlcNAc6P. This structural role of the aromatic side chain in Y121W can be also seen in the comparison of the catalytical efficiencies (K_{cat}/K_m) of the wild-type and Y121W enzymes which are quite similar (Table I). On the other hand, the Y121T enzyme, is evidently a very less efficient enzyme, with a more disturbed structure at the catalytic site. The role of GlcNAc6P in stabilising the active structure of the enzyme distorted by the mutations, is also evident. The experiments of sulfhydryl protection by GlcNAc6P (Fig. 4) reveals that the occlusion of thiols from C118 and C239 accompanying the allosteric transition (Altamirano et al., 1992) correspond to the conversion of the intermediate conformer to the R-form, in the second phase of the biphasic activation curves observed in Fig. 3.

This set of observations, and the study of C118S and C239S mutant (Altamirano et. al, 1992) demonstrate that single substitutions affect simultaneously different functions of the enzyme as catalysis, GlcNAc6P activation of homotropic cooperativity, strongly suggesting that allosteric and catalytic sites and residues critical for allosteric conformerisation could be located in a restricted zone of deaminase molecule.

TABLE I

Kinetic properties of mutant forms of glucosamine-6-phosphate deaminase with replacements for Tyr 121

Enzyme	h_{MAX}	k_{CAT} s^{-1}	k_{CAT} s^{-1}	K^M Mm	k_{cat}/k_m $_{s-1}mM^{-1}$	c	L X 10^3
Wild-type	2.94	1800	1800	2	900	0.02	10
Y121W	3.42	245	584	.66	960	0.0005	7.7
Y121T	2.93	17	337	2.6	130	0.02	3.2

REFERENCES

Altamirano, M.M., Mulliert, G. and Calcagno, M. (1987) *Arch. Biochim. Biophys.* **258**, 95-100.

Altamirano, M.M., Lara-Lemus, R., Libreros-Minotta, C.A. and Calcagno, M. (1989) *Arch. Biochim. Biophys.* **269**, 555-561.

Altamirano, M.M., Plumbridge, J.A., Hernández-Arana, A., and Calcagno, M.L. (1991) *Biochim. Biophys. Acta* **1076**, 266-272.

Altamirano, M.M., Plumbridge, J.A. and Calcagno, M.L. (1992) *Biochemistry* **31**, 1153-1158.

Donovan, J.W. (1973) *Methods Enzymol.* **27**, 525-548.

Ellis, K.J. and Morrison, J.F., (1982) *Methods Enzymol.* **87**, 405-426.

Leloir, L.F. and Cardini, C. (1962) *Methods Enzymol.* **5**, 418-422.

Midelfort, C., and Rose, I.A. (1977) *Biochemistry* **16**, 1590-1596.

Monod, J., Wyman, J. and Changeux, J.P. (1965) *J. Mol. Biol.* **12**, 88-118.

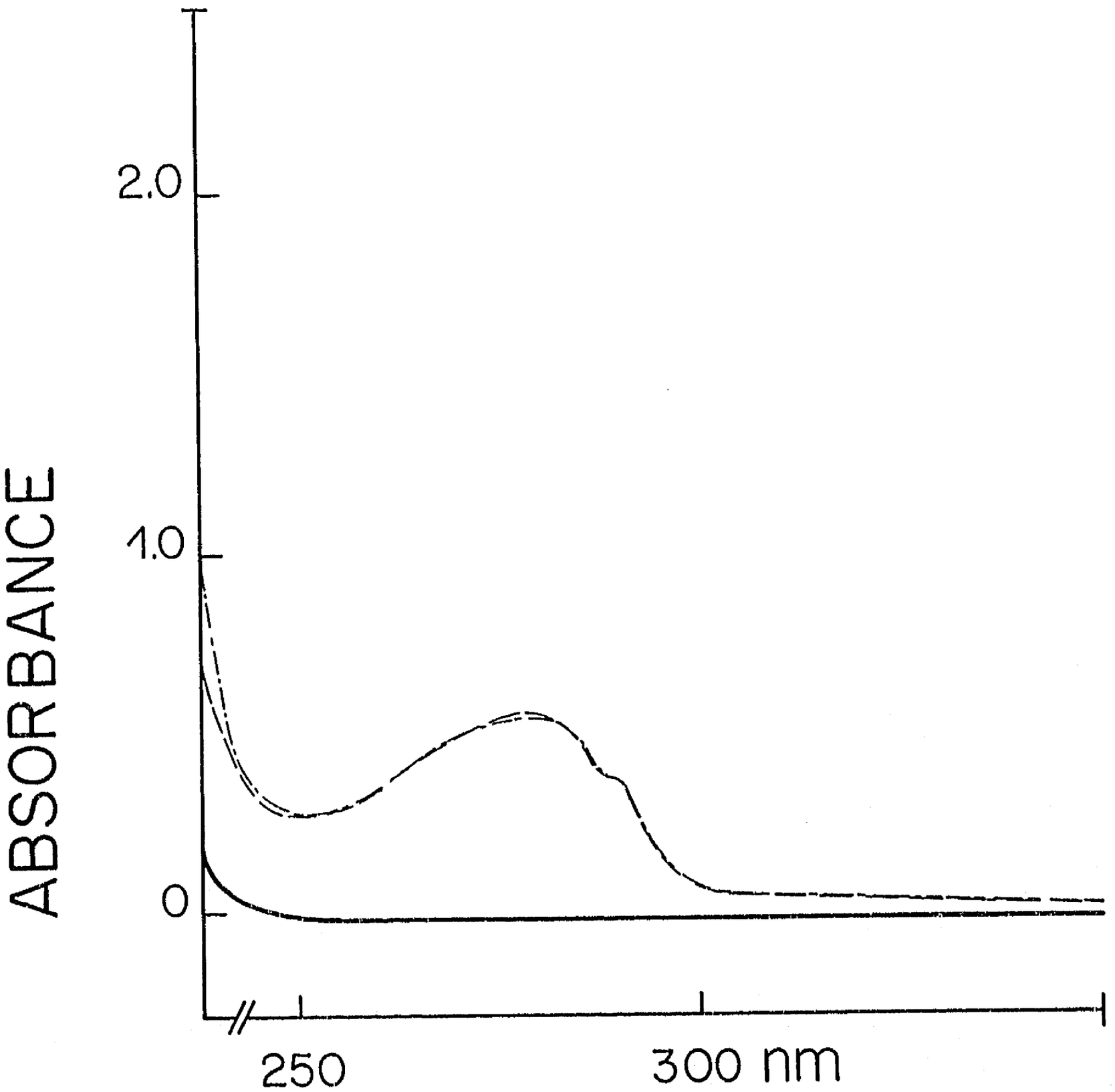
Plumbridge, J.A. (1991). *Mol. Microbiol.* **5**, 2053-2062.

Plumbridge, J.A., Cochet, O., Souza, J.M., Altamirano, M.M., C

Calcagno, M.L. and Badet, B. (1993) *J. Bacteriol.* **175**, *in press.*

Sambrook, J.; Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning, 2nd. Ed., Cold Spring Harbor
Laboratory Press, Cold Spring Harbor, N.Y.

Wyman, J. (1975) Proc. Nat. Acad. Sci. USA, 72: 3983-3987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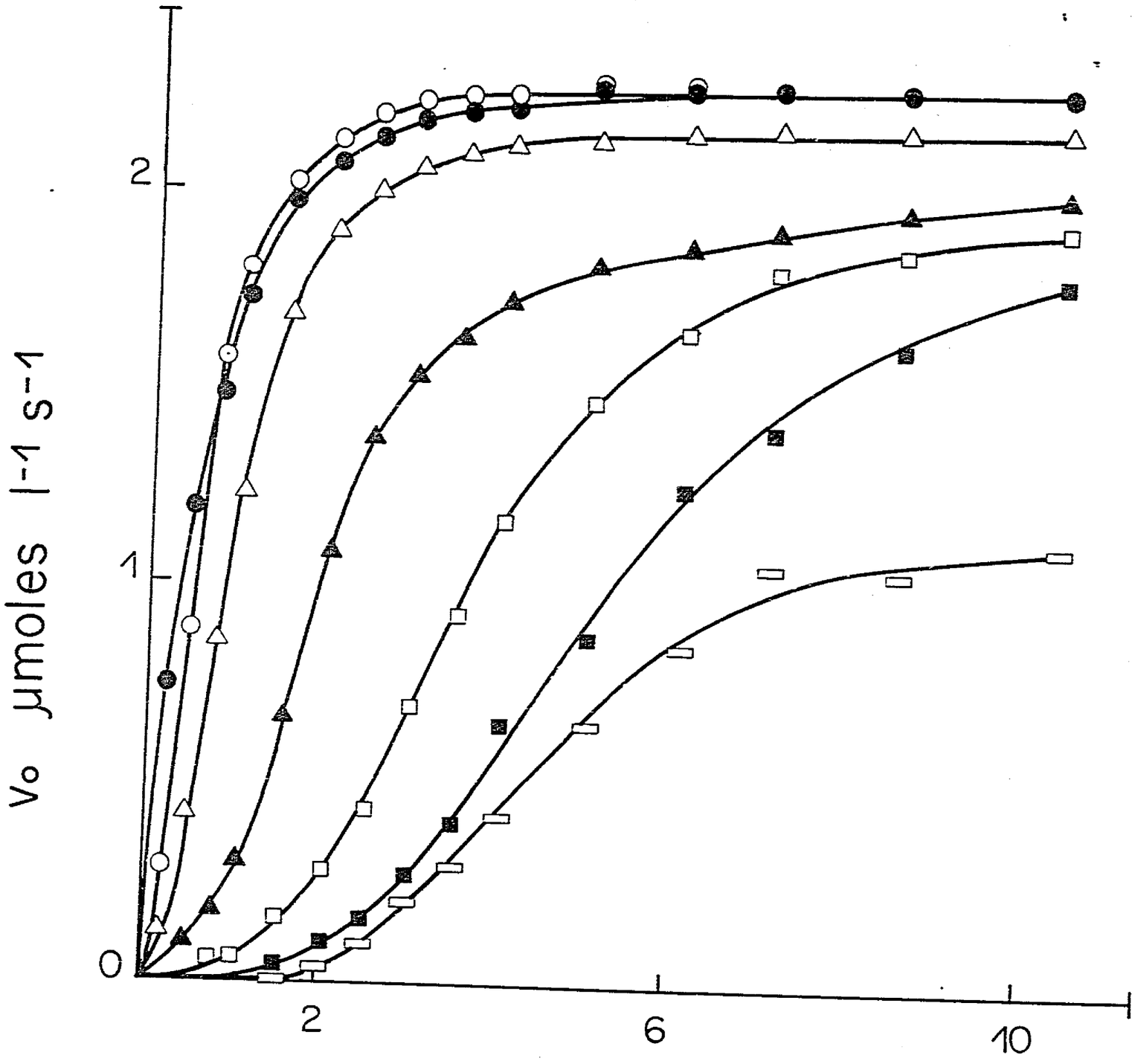
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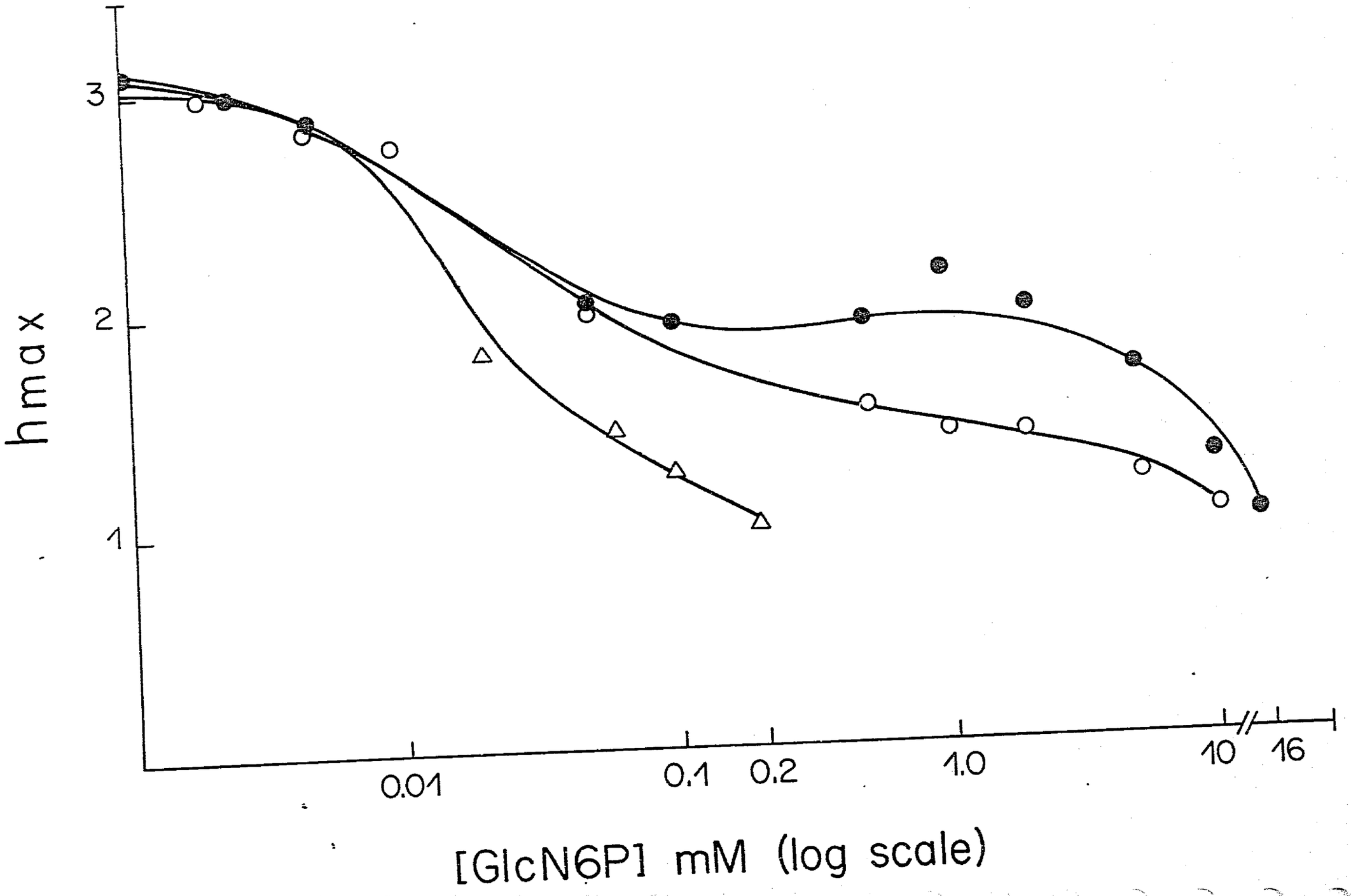
2
6
10
[GlcN6P] , mM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10
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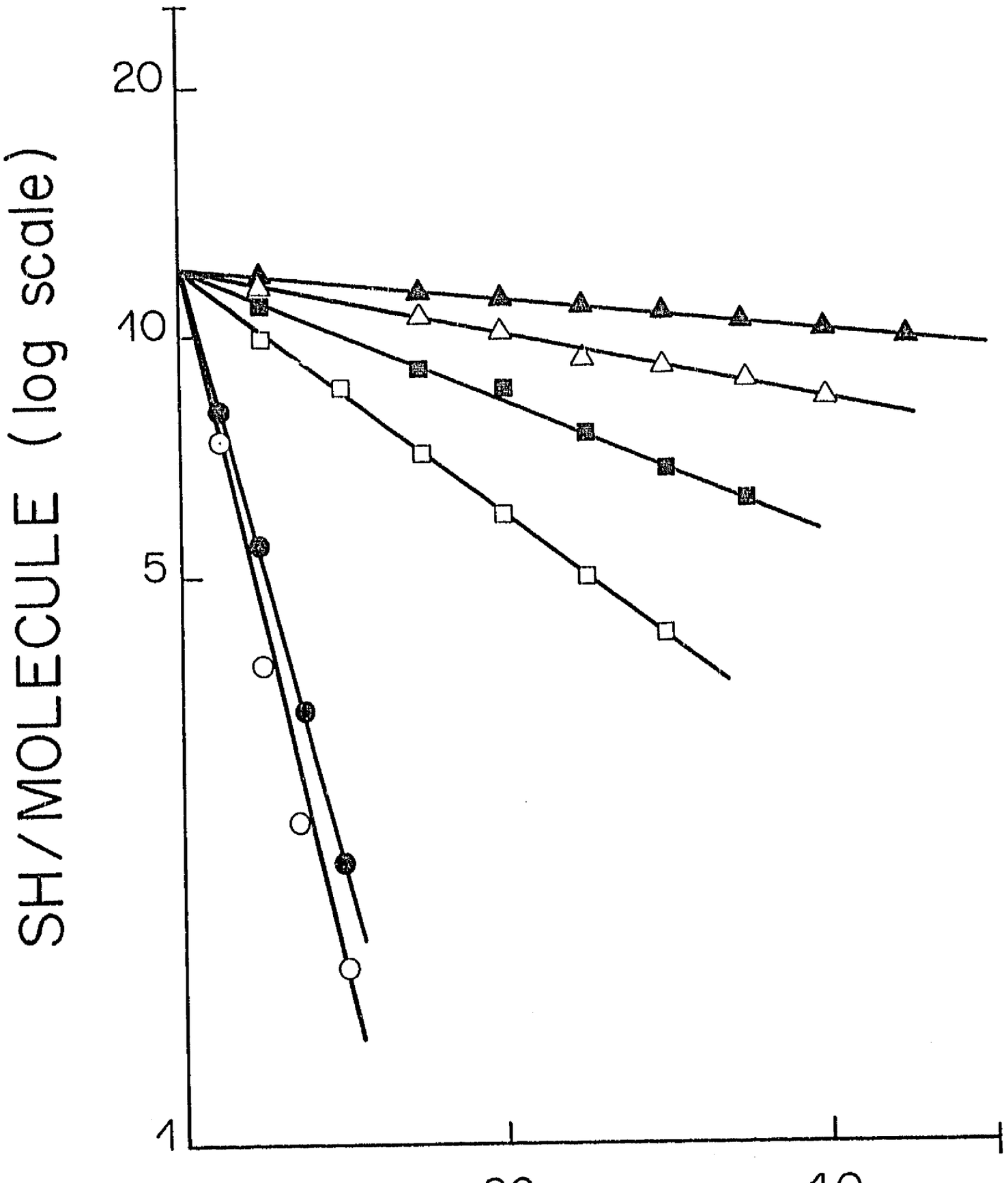
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20
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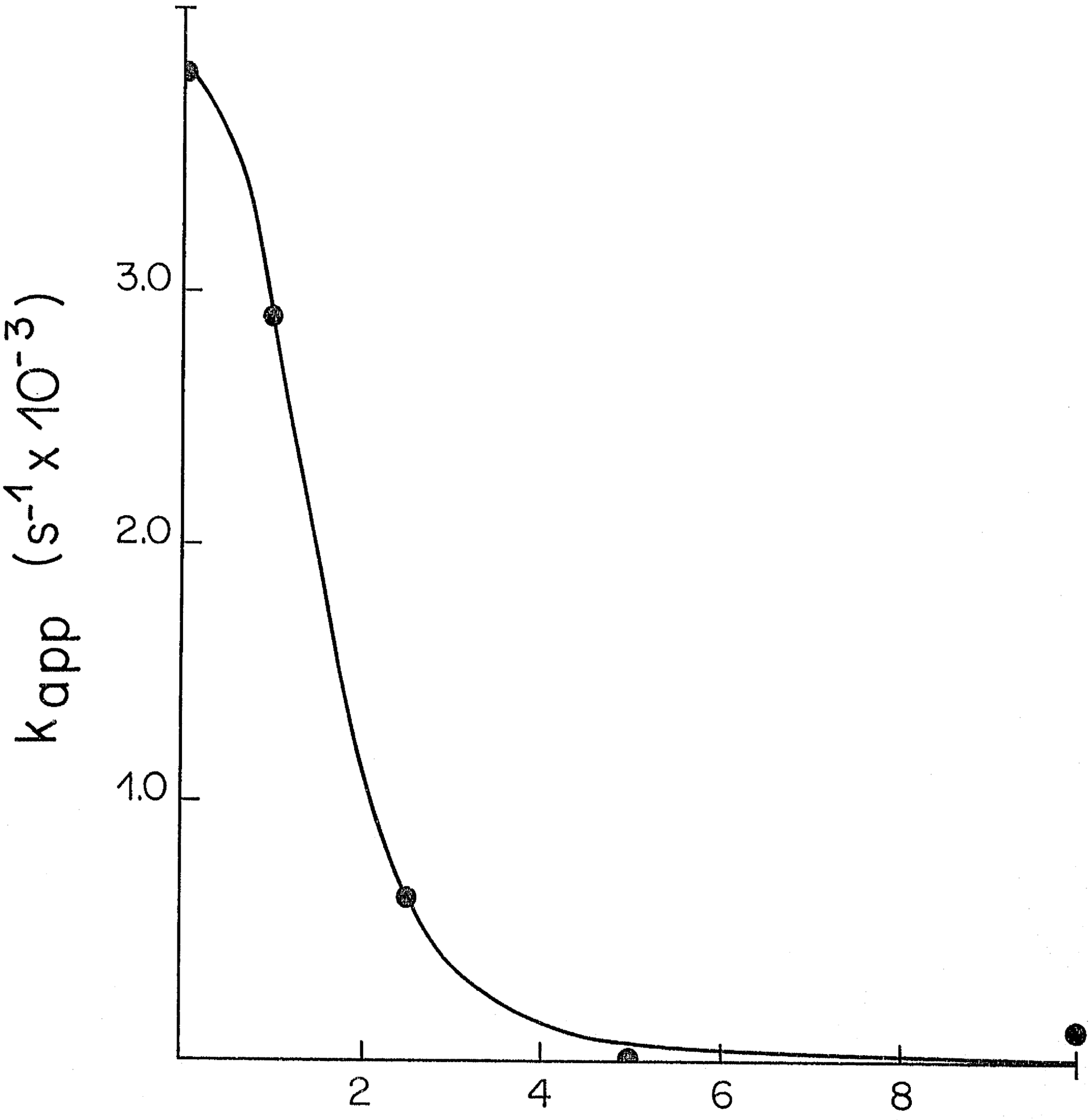
3.0
2.0
1.0
2
4
6
8
kapp (s-1 x 10-3)
[GlcNAc6P] , mM

LEGEND FOR THE FIGURES

Fig. 1.

Change in *near-UV spectrum of Y121W glucosamine-6-phosphate deaminase produced by the pH change 7.0 - 10.5.*

A 4.5 μM solution of the enzyme in 50 mM imizadole, 25 mM MES, 25 mM CAPS buffer, pH 10.5 (———) and pH 7.0 (----) were measured against a reference cell containing the buffer. The darker line is the differential spectrum.

Figure 2.

Activation of Y121W glucosamine-6-phosphate deaminase by GlcNAc6P.

A: direct plot of initial velocities versus substrate concentration at the following activator concentrations: (■), none; (○), 1 μM; (●), 10 μM; (▵), 25 μM; (▵), 50 μM; (□) 10 mM and (o) 16 mM. Assays were performed using 2 nM deaminase in a final volume of 200 μl, at pH 8.0 and 30 °C. as described elsewhere (Altamirano et al., 1989).

Figure 3

Extreme values of the Hill coefficient (hmax) plotted versus GlcNAc6P concentration. (○), Y121W mutant deaminase; (●), Y121T mutant deaminase; (□), Wild-type enzyme. See details in text.

III. La unidad alostérica mínima. Aspectos estructurales de la Glucosamina-6-fosfato desaminasa.

GLUCOSAMINE-6-PHOSPHATE DEAMINASE FROM *ESCHERICHIA COLI* HAS A TRIMER OF DIMERS STRUCTURE WITH THREE INTERSUBUNIT DISULFIDES

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SHORT TITLE (PAGE HEADING):

Disulfide bonds in glucosamine-6-phosphate deaminase

Proposed for the section on *Proteins*

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SYNOPSIS

Glucosamine-6-phosphate deaminase is an oligomeric protein composed of six identical 29.7 kDa subunits. Each subunit has four cysteinyl residues located at positions 118, 219, 228 and 239 on the sequence. We have previously shown that Cys118 and Cys239 form a pair of vicinal thiols whose reactivity changes with the allosteric transition. The site-directed mutations Cys→Ser corresponding to the other two cysteinyl residues have been constructed, as well as some selected multiple mutations involving the four cysteines. Sulfhydryl and disulfide measurements on the wild-type and mutant enzymes indicate that thiols from Cys219 are oxidised and form inter-chain disulfide bonds. The disulfide-linked dimer was demonstrated by SDS-PAGE. This result is consistent with preliminary crystallographic data and thermal denaturation studies, and strongly suggests that glucosamine-6-phosphate deaminase is a trimer of disulfide-linked dimers. The mutant form of the deaminase lacking the interchain disulfide bond or the thiol at Cys228 are both stable hexamers and they show the same sensitivity to urea denaturation as the wild-type protein. Furthermore, these Cys→Ser mutants display the same kinetics and allosteric properties as those already described for the wild-type enzyme.

INTRODUCTION

Glucosamine-6-phosphate deaminase (D-glucose-6-phosphate ketol isomerase, deaminating, EC 5.3.1.10), is an oligomeric protein composed of six identical polypeptide chains (Calcagno *et al.*, 1984) whose primary structure is known from the DNA sequence of its gene, *nagB* (Rogers *et al.*, 1988). The enzyme has been recently crystallised and preliminary crystallographic data are available from diffraction measurements at 2.1 Å resolution It has been found that this protein has a point group symmetry of the type 32, centred on a 3-fold axis; crystallographic evidence also suggests that the hexamer subunits are arranged as dimers with three local 2-fold axes passing through the centre of the three dyads forming the hexamer (Horjales *et al.*, 1992). This structure is also consistent with thermal denaturation data of the deaminase obtained by differential scanning calorimetry at neutral pH, which suggests that deaminase dissociates into dimers, in two successive steps (Hernández-Arana *et al.*, 1993).

The existence of disulfide bonds in the deaminase molecule can be presumed from the study of its four cysteinyl residues. Recently, we have identified Cys118 and Cys239 as the cysteines bearing two superficial and vicinal thiols whose reactivity changes with the allosteric transition. The other two cysteinyl residues are one we have described as "hidden", since it reacts with sulfhydryl reagents only under denaturing conditions, and a cysteinyl residue which does not react with thiol reagents at all, implying that it may be present in the disulfide form (Altamirano *et al.*, 1992). As the enzyme has only four cysteinyls, these residues must be Cys219 and Cys228. In this work we report the identification of these two cysteines by the analysis of proteins carrying the site-directed mutations Cys219→Ser and Cys228→Ser. Other proteins carrying multiple Cys→Ser mutations were also made by combination with the mutations at cysteinyls 118 and 239 constructed previously. These modified proteins were used to reexamine the question of the presence of a disulfide bond in the deaminase molecule and to assess its structural and functional role.

MATERIALS AND METHODS

Bacteria and enzyme

E. coli wild-type glucosamine-6-phosphate deaminase was prepared from an overproducing strain, as previously described (Altamirano *et al.*, 1991). Plasmids expressing the Cys219 to Ser and Cys228 to Ser mutant forms of the protein, were constructed as described previously (Altamirano *et al.*, 1992). The double mutation Cys219,228→Ser, the triple Cys219,228,239→Ser and the quadruple (all Cys changed to Ser) were also constructed. The host strain for the expression of these mutated proteins, was IBPC546R, which is *nagB::kan,recA1*, a recombination-deficient strain carrying a null mutation in the chromosomal copy of the *nagB* gene (Plumbridge, 1991). All mutations were checked by DNA sequencing.

Wild-type and genetically-modified forms of glucosamine-6-phosphate deaminase were obtained and assayed using previously reported procedures, using allosteric-site affinity chromatography (Altamirano *et al.*, 1991, 1992).

Reagents

2-nitro-5-thiosulfobenzoate (NSTB) was prepared according to Thannhauser *et al.* (1987) and conserved in solution. Biochemicals, protein standards and carbohydrate derivatives were purchased from Sigma Chemical Co., MO, U.S.A. Most chemicals were reagent-grade products.

Measurements of the reactive and total SH groups

The total thiol groups in the wild-type and the mutant forms of the deaminase were determined with 5,5'-dithiobis (2-nitrobenzoate), DTNB, at pH 7.7, at 25 °C, in the presence of 0.5 % SDS or 3.5 M guanidinium thiocyanate and 5 mM EDTA. The product of the thiol-disulfide exchange reaction, 2-nitro-5-thiobenzoate (TNB) was measured spectrophotometrically with a double-beam instrument. Calculations were made using the known molar absorption coefficient for this compound (Means and Feeney, 1971) and for glucosamine-6-phosphate deaminase (ε_{278} 20.0 x 10^4 M^{-1} cm^{-1}, Altamirano *et al.*, 1987).

Detection and measurement of disulfide bonds

Disulfide bonds were analysed by the method of Thannhauser *et al.* (1987). This procedure measures both thiols and disulfides. The reaction consists in a sulfitolysis of the S-S bonds by sulfite at pH 9.0, and the simultaneous reaction of preexisting thiols and those formed by sulfitolysis, with NSTB in the presence of 3.5 M guanidinium thiocyanate as protein denaturant.

Electrophoretic procedures

Electrophoreses of the different deaminase samples were made according to Laemmli (1970). When necessary, sample buffer was prepared without any thiol-reducing reagent. Electrophoreses under non-denaturing conditions were performed in pore gradient gels (T = 4-30 %), as previously described (Calcagno *et al.*, 1984). Electrophoreses in polyacrylamide gel slabs with transverse urea gradients, initially described by Creighton (1976) were prepared according to Goldenberg (1989).

High Performance Analytical Gel Filtration

Gel filtration was carried out on a TSK-G 3000 SW (LKB) column (7.5 x 600 mm) equilibrated with Tris-HCl 100 mM buffer pH 7.5, containing 0.075 mM potassium acetate. The following molecular weight standards were used: ferritin (440 Kda), catalase (240 Kda), wild-type glucosamine-6-phosphate deaminase (178 Kda), and hemoglobin (66 Kda).

RESULTS AND DISCUSSION

Measurements of reduced and total Cys residues

The reaction of wild-type glucosamine-6-phosphate deaminase with the NSTB-sulfite reagent (Thannhausser *et al.* 1987) produces 3.5 moles of TNB per mole of enzyme subunit. As the denatured enzyme has only three reactive thiols, and from the known stoichiometry of the Thannhausser *et al.* reaction, which releases one TNB anion per each disulfide cleaved as well as one TNB for each free sulfhydryl, this result indicates the existence of one disulfide bridge for two deaminase subunits. In Table I are shown the results of thiol and disulfide measurements

on wild-type and a set of Cys→Ser mutant enzymes. From these data it can be inferred that cysteinyl residues at position 219 are forming a disulfide bond between neighbouring polypeptide chains.

Electrophoretical isolation of the denatured dimer linked by a disulfide

In standard SDS PAGE, with samples prepared in a buffer containing mercaptoethanol or other reducing reagents, all enzymes from Table I, give a single electrophoretical band at 30 Kda(not shown). In the electrophoresis gels shown in Fig. 1, samples were prepared in a buffer without reducing agent. Wild-type enzyme gives two bands, corresponding to 30 and 60 Kda (Fig. 1, lanes 6 and 9). The mutant enzyme where only Cys219 has been replaced with serine, produces a single band corresponding to the monomer (lanes 1 and 7). The same result is obtained in other multiple Cys→Ser mutations containing the change of Cys219 to Ser (lanes 2 and 4) The triple Cys118,228,239→Ser mutation produces a band corresponding to the dimer, accompanied by a small amount of monomer (lane 5). The sample of this lane was prepared at room temperature. Another sample treated by heating for 1 min in a boiling water bath, gives a larger amount of monomer (nearly 25% by densitometry). These results and those obtained with the other Cys to Ser mutations studied (Fig. 1, lanes 4-6), confirm that Cys219 is the only cysteinyl residue involved in formation of the interchain disulfides. The cysteinyl residue at position 228, is identified as that having the "hidden" thiol, reacting only in the denatured protein (Table I). In wild-type enzyme the interchain disulfide is unstable and the lighter component predominates in non-reducing SDS gels, probably due to the reducing effect of the other three thiols present in the polypeptide chain. It would be expected that the triple mutation Cys118,228,239→Ser would give a pure 60 kDa band; the variable amounts of monomer always present, suggests that the denatured disulfide-linked dimers are partially unstable under the conditions of electrophoresis. The dimeric form after elution from the polyacrylamide gel and re-electrophoresis on a second gel, produces again a small and variable proportion of monomer. The amounts of dimer and monomer were determined by extraction of Coomassie blue from the stained protein bands excised from polyacrylamide gels, as described by Ball (1986). The ratio dimer/monomer obtained with the mutant Cys118,228,239→Ser was 5-6 for the first electrophoresis, and 12-14 for the re-electrophoresis.

Molecular weight and stability of Cys219→Ser deaminase

The mutant form of the deaminase lacking the interchain disulfide bond is an hexamer, as demonstrated by pore-gradient gel electrophoresis and gel filtration high performance liquid chromatography of the native proteins (not shown); in any case the presence of smaller oligomers was detected. The same results were obtained with Cys228→Ser mutation and the Cys219,228→Ser double mutant. To investigate the stability of the wild-type hexamer and our Cys→Ser enzyme mutants, we used the technique of transverse urea gradient gel electrophoresis (Creighton, 1976; Goldenberg, 1989). Wild-type enzyme (Fig. 2A), both Cys→Ser single mutants and the Cys219,228→Ser double mutant (Fig. 2B) behaved essentially identical; under these experimental conditions, discontinuities due to oligomer dissociation are not apparent.

Kinetics of the deaminases carrying the site-directed mutations Cys219→Ser and Cys228→Ser

The kinetics and allosteric properties of the mutant deaminases in which serine replaces cysteines 219 or 228, were the same as those already described for the wild-type enzyme (Altamirano *et al.*, 1992). The same result was obtained with the double mutation.

CONCLUDING REMARKS

We have demonstrated the presence of three interchain disulfide bonds in the deaminase molecule, formed between cysteinyls residues at position 219. According to the structural information available, the three S-S bonds in the deaminase molecule must be located on each of the three twofold symmetry axes (Horjales *et al.*, 1992). Surprisingly, this disulfide is not essential for the stability of the oligomer, under physiological conditions, and does not modify the profile of deaminase in electrophoreses in transverse urea gradients (Fig. 2). Nevertheless, quantitative denaturation studies, for instance, by differential scanning calorimetry, may be of value in the characterisation of the possible structural role of the thiol group from Cys219. Moreover the elimination of the "hidden" thiol from Cys228 or the interchain disulfide formed

by Cys219, does not produce any significant change in glucosamine-6-phosphate deaminase function.

This research was supported by grants from the Third World Academy of Sciences (grant BC 91-028), Dirección General Asuntos del Personal Académico (DGAPA), UNAM, Mexico), CNRS (France), and The European Economic Community, contract CI1-CT2-0038. MMA holds a doctoral fellowship, from DGAPA, UNAM, México.*

REFERENCES

Altamirano, M. M., Hernández-Arana, A., Plumbridge, J.A. and Calcagno M. (1991) Biochim. Biophys. Acta **1076,** 266-272

Altamirano, M.M., Mulliert, G. and Calcagno, M. (1987) Arch. Biochem. Biophys. **258**, 95-100

Altamirano, M. M., Plumbdridge. J.A. and Calcagno M. (1992) Biochemistry, **31**, 1153-1158

Ball, E.H. (1986) Anal. Biochem. **155**, 23-27

Calcagno, M., Campos, P.J., Mulliert, G., and Suástegui, J. (1984) Biochim. Biophys. Acta, **787,** 165-173

Creighton, T.E. (1978) J. Mol. Biol. **129**, 235-264

Goldenberg, David P. (1989) Analysis of protein conformation by gel electrophoresis. In Protein Structure: A practical approach. T.E. Creighton, Editor, IRL Press, Oxford.

Hernández-Arana, A., Rojo-Domínguez, A., Altamirano, M.M. and Calcagno, M.L. (1993) Biochemistry, Biochemistry (1993) **32**, 2644-3648

Horjales. E., Altamirano, M.M., Calcagno, M.L., Dauter, Z., Wilson, K, , Garratt. R.C., and Oliva, G. (1992) J. Mol. Biol **226**, 1283-1286

Laemmli, U.K. (1970) Nature (London) **227**, 680-685

Means, G.E. and Feeney, (1971) *Chemical Modification of Proteins,* Holden Day Inc., San Francisco, 1971.

Plumbridge, J.A. (1989) *Mol. Microbiol.* **3**, 505-515.

Rogers, N.J., Ohgi, T., Plumbridge, J. and Söll, D. (1988) *Gene,* **62**, 197-207

Thannhausser, R., Konishi Y., and H.A. Scheraga (1987) *Meth. Enzymol.* **143**, 115-119

TABLE I[a]

TITRATION OF SULFHYDRYLS AND DISULFIDES IN WILD-TYPE AND CYS→SER MUTATIONS OF *E. coli* GLUCOSAMINE-6-PHOSPHATE DEAMINASE

	SULFHYDRYLS[b]		SULFHYDRYLS + DISULFIDES[c]
	Native	Denatured[d]	
Wild-type	2.06	2.95	3.58
Cys219→Ser	2.10	3.07	3.11
Cys228→Ser	2.00	1.97	2.55
Cys219,228→Ser	1.94	2.12	2.10
Cys118,228,239→Ser	0	0	0.47
Cys118,219,228,239→Ser	0	0	0

[a] Average of three or four determinations. The number of thiol groups or disulfide links is expressed per chain of deaminase polypeptide.

[b] Measured with DTNB at pH 7.7, 30 °C.

[c] Measured with the reagent NSTB-sulfite (Thannhauser *et al.* 1987), at pH 9.0 in the presence of 3.5 M guanidinium thiocyanate.

[d] SH titrations in the presence of 3.5M guanidinium thiocyanate. When the denaturant was 0.5% SDS, similar results were obtained.

FIGURE 1

TITLE: Comparative analysis of wild-type and some Cys→Ser mutations of glucosamine-6-phosphate deaminase.

LEGEND:

SDS/PAGE of wild-type glucosamine-6-phosphate deaminase and some site-directed Cys→Ser mutations. The gel (T = 11.2%, C = 2.7%) was prepared and run according to Laemmli (1970), and run at 20 mA, 10 °C. Sample buffer was prepared without thiol-reducing agents. Lanes 1 and 7, Cys219→Ser; lane 2, Cys219,228→Ser; lane 3, Cys228→Ser; lane 4 Cys118,219,228,239→Ser (all four Cys residues changed to Ser); lane 5, Cys118,228,239→Ser (this sample was dissolved at room temperature); lanes 6 and 9, wild-type; lane 8, Cys118,228,239→Ser (this last sample was boiled for 1 min). The gel was stained with Coomassie brilliant blue R-250. Standards: bovine serum albumin (66 kDa); *E. coli* N-acetyl-D-glucosamine 6-phosphate deacetylase (41 kDa), carbonic anhydrase (30 kDa).

FIGURE 2.

TITLE

Transverse urea gradient PAGE of wild-type and Cys219,228→Ser double mutant of glucosamine-6-phosphate deaminase.

LEGEND. Deaminase samples were subjected to electrophoresis at pH 8.5, in a gel with a urea transversal concentration gradient from 0 to 8M prepared according to Goldenberg (1989). The gel (T12%, C2.4%) was loaded with 35 μg of protein, run at 4 °C at 20 mA for 2.5 h and stained with Coomassie brilliant blue R-250. Urea concentration gradient is from left to right **A:** wild-type glucosamine-6-phosphate deaminase. **B:** Cys219,228→Ser mutant enzyme

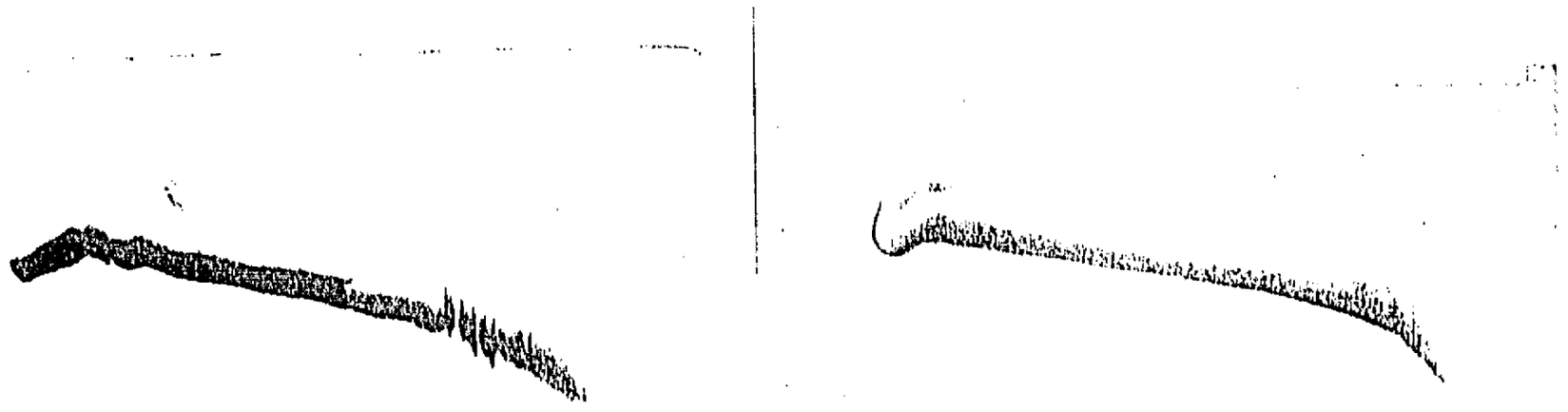
FOOTNOTES

‡ To whom correspondence should be addressed.

Abbreviations: DTNB, 5,5'-dithiobis (2-nitrobenzoate); TNB, 2-nitro-5-thiobenzoate; NSTB, 2-nitro-5-thiosulfobenzoate.

A B

Figure 1



A B

Figure 2

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06 May 93

REFERENCE: BJ 93/0545
TYPE: Full Paper
TITLE: Glucosamine-6-phosphate deaminase from Escherichia coli has a ...
AUTHOR(S): MM Altamirano et al

Dear Dr Calcagno

Thank you for submitting this paper to the Biochemical Journal. I am pleased to tell you that it will become acceptable for publication if satisfactory attention is given to the points in the enclosed editorial report, which is compiled from the comments of two reviewers, one of whom is a member of the editorial board. I am therefore returning your typescript to you so that you can consider these comments and make the appropriate amendments.

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Lyn Tomsett

for the Managing Editor

BIOCHEMICAL JOURNAL
This editorial report presents the combined opinions of the reviewers of your paper

REFERENCE: BJ 93/0545 VERSION: 1
TYPE: Full Paper
TITLE: Glucosamine-6-phosphate deaminase from Escherichia coli has a ...
AUTHOR(S): MM Altamirano et al

This interesting paper describes a number of experiments which clearly establish that the Cys 219 in glucosamine-6-phosphate deaminase is involved in inter-subunit disulphide bonds. Two small points require attention as indicated below.

Minor points

1. page 7, 2 lines of text up. More details should be provided about this re-electrophoresis experiment. How does the proportion of monomer compare with that on the first run?

2. page 8, line 2. The stability of the Cys 219 → Ser mutant compared with the wild-type enzyme is of considerable interest. Details of these experiments should be given. Have the authors tried other approaches, eg chemical or thermal denaturation, in order to assess the relative stabilities of mutant and wild-type enzyme?

Dr. Lyn Johnson
Biochemical Journal Editorial Office
59 Portland Place
London W1N 3AJ, U.K.

I am sending to The Biochemical journal, a revised version of the paper entitled: **Glucosamine-6-phosphate deaminase from *Escherichia coli* has a trimer of dimers structure with three inter-subunit disulfides** (BJ 93/0545) by M.M. Altamirano, J.A. Plumbridge and myself.

We introduced important changes in the text, according to the points raised by the referees.

Point 1. The quantitation of proteins in the re-electrophoresed bands was included in the text.

Point 2. We introduced additional information on the stability of Cys→Ser mutants compared with the wild type deaminase. New experiments were performed with a different approach, the use of Creighton's transversal urea gradients. Consequently, a new figure (Fig. 2, with part A and part B) was included, and the text modified accordingly in the sections Materials and Methods and Results and Discussion. To avoid un excess of gel pictures, we show the patterns for the wild type and for the double mutant. These experiments does not indicate any significant structural role for Cys228 or Cys219. The corresponding electrophoreses in transerval urea gradient were identical.
We consider that thermal denaturation studies of these mutants are out of the scope of this paper.

All changes are marked in a copy of the second version of the typescript.
I hope that in the present ammended version, the paper would be suitable for publication in your Journal.

Sincerey yours,

Mario L. Calcagno

J. Mol. Biol. (1992) 226, 1283–1286

Crystallization and Preliminary Crystallographic Studies of Glucosamine-6-phosphate Deaminase from *Escherichia coli* K12

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Hexameric glucosamine-6-phosphate deaminase from *Escherichia coli* has been crystallized isomorphously with both phosphate and ammonium sulphate as precipitants, over a wide pH range (6·0 to 9·0). The crystals belong to space group *R*32 and the cell parameters in the hexagonal setting are $a=b=125{\cdot}9$ Å and $c=223{\cdot}2$ Å. A complete native data set was collected to 2·1 Å resolution. Self-rotation function studies suggest that the hexamers sit on the 3-fold axis and have point group symmetry 32, with a non-crystallographic dyad relating two monomers linked by an interchain disulfide bridge. A possible packing for the unit cell is proposed.

Keywords: deaminase; glucosamine; hexamer; allostery; crystallization

The amino sugars D-glucosamine (GlcN) and *N*-acetyl-D-glucosamine (GlcNAc) are both potential carbon and nitrogen sources for the bacterium *Escherichia coli*, and its growth in the presence of amino sugars induces the expression of the enzymes necessary for their metabolism (White, 1968). The genes involved in the uptake and utilization of amino sugars were mapped at 15·5 minutes on the bacterial chromosome (White, 1968; Holmes & Russell, 1972; Jones-Mortimer & Kornberg, 1980). There are five genes arranged in two divergent operons, *nag*E-*nag*B,A,C,D, which have been cloned and sequenced and the control of their expression is well known (Rogers *et al.*, 1988; Plumbridge, 1989; Vogler & Lengeler, 1989). The gene *nag*B encodes the enzyme glucosamine-6-phosphate deaminase (E.C. 5.3.1.10), which catalyzes the conversion of D-glucosamine-6-phosphate (GlcN6P) into D-fructose 6-phosphate and ammonia. The reaction catalyzed is an aldo-keto isomerization coupled with an amination-deamination, termed an Amadori rearrangement (Hodge, 1955). This enzyme is allosterically activated by *N*-acetyl-D-glucosamine 6-phosphate (GlcNAc6P), the same metabolite identified as the co-inducer of the *nag* regulon (Plumbridge, 1991). Similar enzymes have been identified in several other microorganisms and animal tissues (Noltmann, 1972).

Glucosamine-6-phosphate deaminase from *E. coli* can be obtained easily from an overproducing strain and purified by allosteric site-affinity chromatography (Altamirano *et al.*, 1991). The enzyme is a hexameric homopolymer with subunits of 29·7 kDa, exhibiting an intense homotropic co-operativity towards GlcN6P, which is modulated by the allosteric activator GlcNAc6P (Calcagno *et al.*, 1984; Altamirano *et al.*, 1987). A search of the OWL

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sequence database version 12.1 (Bleasby & Wootton, 1990; 34,140 entries) has shown no significant homology with any other known protein family. Sequence-based secondary structure prediction, supported by circular dichroic spectroscopy, suggests that the enzyme has a dominant α/β structure (Altamirano *et al.*, 1991). Of the four cysteine residues in the sequence, two are titrable in the native protein, in the absence of ligands. The allosteric T to R transition produces complete loss of reactivity of this thiol pair (Cys118 and Cys239). These thiols are vicinal and participate in a high-affinity Zn-binding site. The two remaining cysteinyl residues are buried. One is titrable when the enzyme is denatured by urea or SDS and the fourth is exposed only after reduction, suggesting the presence of an interchain disulfide bridge (Altamirano *et al.*, 1989, 1992; Altamirano & Calcagno, 1990). This last observation indicates that the subunits are possibly arranged as dimers linked by a disulfide bridge, which then oligomerize as hexamers.

To increase our understanding of the structure and allosteric mechanism of deamination, we have initiated a crystallographic study of glucosamine-6-phosphate deaminase from *E. coli* K12. We report here the preparation of single crystals of the enzyme suitable for high-resolution X-ray structure determination and the results of the preliminary crystallographic study. We discuss the local symmetry of the hexamer and a possible packing for the molecules in the unit cell.

Glucosamine-6-phosphate deaminase from *E. coli* K12 was purified as described (Altamirano *et al.*, 1991), and stored precipitated in 2·5 M-ammonium sulfate with 5 mM-disodium EDTA. The enzyme suspension was dialyzed against a low ionic strength buffer (10 mM-Hepes/NaOH, pH 7·4) and concentrated to approximately 10 mg/ml. Isomorphous crystals were obtained using the vapor diffusion technique in hanging drops (McPherson, 1982) at 20°C under the following conditions: 1·4 M, 1·37 M and 1·33 M-Na/K phosphate buffer (pH 6·0, 7·0 and 8·0, respectively); 42% (w/v) ammonium sulfate in 50 mM-Tris·HCl (pH 8·0 and 9·0); and 42% ammonium sulfate in 50 mM-Hepes/NaOH (pH 7·0). The drops contained equal amounts (3 to 5 µl) of the dialyzed protein stock (10 mg/ml) and reservoir solutions. Crystal growth is visible after five days and usually complete within 21 days. The crystals are multifaceted and show variable morphology, growing up to 1·0 mm × 0·6 mm × 0·3 mm. SDS/polyacrylamide gel electrophoresis of the crystalline material in the presence of β-mercaptoethanol confirmed that the enzyme monomers were intact and migrated identically with those of glucosamine-6-phosphate deaminase before crystallization. The crystals belong to the rhombohedral system with space group $R32$. The unit cell dimensions of the crystals grown at 1·4 M-Na/K phosphate were measured from precession photographs obtained with a Rigaku RU200B rotating anode generator operating at 50 kV and 190 mA, and are $a=b=125{\cdot}9$ Å, $c=223{\cdot}2$ Å (1 Å = 0·1 nm) for the hexagonal setting. The unit cell volume of $3{\cdot}064\times10^6$ Å^3 is compatible with two monomers in the asymmetric unit, yielding a calculated V_m value (Matthews, 1968) for these crystals of 2·87 Å^2/dalton. Assuming a protein partial specific volume of 0·737 cm^3/g, estimated from the amino acid composition (Calcagno *et al.*, 1984), the solvent content of the crystal is approximately 60%. Rotation photographs taken on a high-brilliance synchrotron radiation source show that the crystals diffract to at least 2 Å resolution.

Complete native data to a resolution of 2·1 Å were collected from one crystal on the EMBL Protein Crystallography Station X31 in HASYLAB at DESY, Hamburg, using an image-plate detector. The crystals are very sensitive to CuKα radiation ($\lambda=1{\cdot}54$ Å) but proved to be stable for over 24 hours during the synchrotron data collection performed using radiation of wavelength 1·0 Å. Processing of the data was performed with the

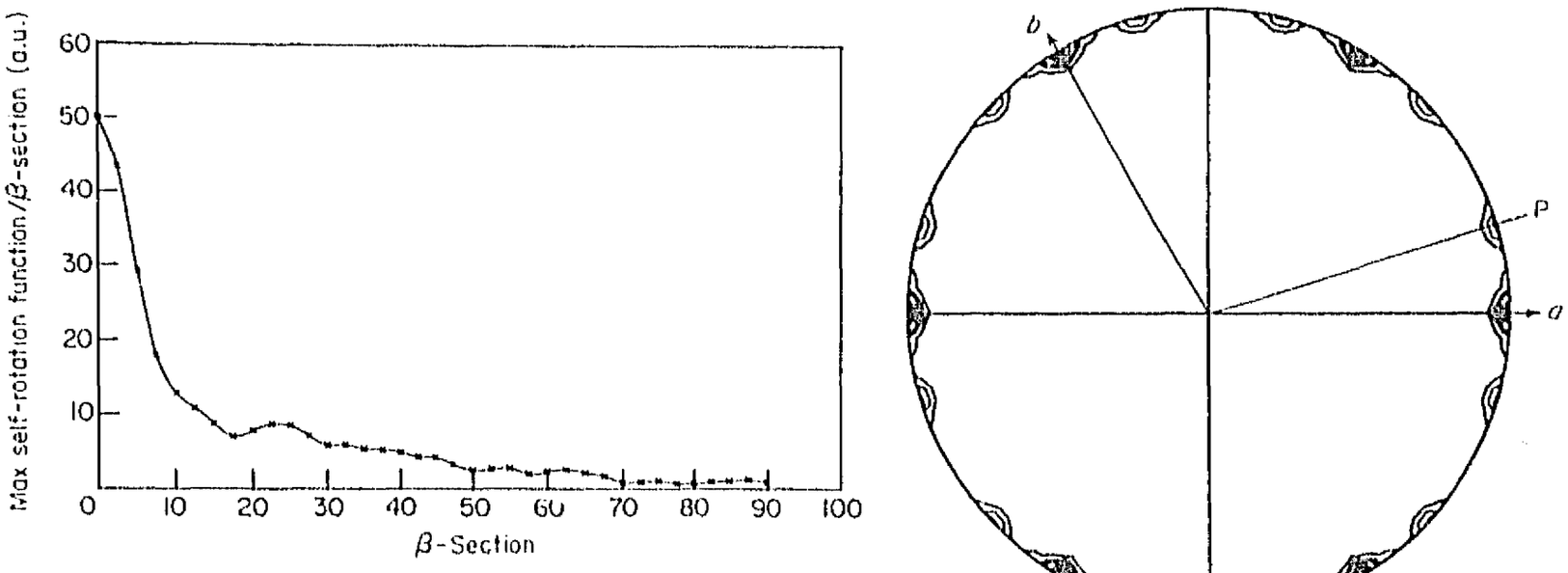
Table 1

Data collection statistics for the 2·1 Å native dataset of glucosamine-6-phosphate deaminase

Resolution shell lower limit (Å)	R_{sym}†	R_{cum}	Reflections measured	Independent reflections	Percentage of completeness	Cumulative % of reflections with $I>3\sigma(I)$
5·01	0·060	0·060	14,864	2586	87·1	99·2
3·60	0·089	0·080	35,515	4912	99·3	97·4
2·95	0·116	0·091	41,184	6231	99·3	93·2
2·56	0·110	0·093	32,555	7270	99·2	85·6
2·30	0·153	0·097	36,491	8232	99·4	78·2
2·10	0·201	0·101	40,083	9137	99·6	68·5
Totals	0·101	0·101	200,692	38,368	97·4	81·6

† $R_{sym}=\dfrac{\sum_h\sum_i|I_{hi}-\langle I_h\rangle|}{\sum_h\langle I_h\rangle}$,

where I_{hi} is the scaled intensity of the ith measurement of reflection h or its equivalent and $\langle I_h\rangle$ is the average intensity of reflection h.



(a) (b)

Figure 1. Self-rotation function of glucosamine-6-phosphate deaminase. The resolution range is 1·5 to 2·5 Å, with a radius of integration of 2 to 20 Å, using normalized structure factors. The rotations are assumed to be γ about the crystal c-axis, β about the reciprocal a^*-axis and α again about the c-axis. (a) Maximum value of the self-rotation function for each β-section, in arbitrary units (a.u.) normalized to a value of 50 for the origin peak, with a map root-mean-square value of 6 a.u.; (b) stereographic projection of the diagonal cross-section ($\alpha+\gamma=180^\circ=\chi$) of the self-rotation map down the crystal c-axis, contoured at 2·5, 3·5, 4·5, 5·5, 6·5 and 7·5 times the map root-mean-square value, showing the only significant peak apart from the origin, which is at section $\beta=0^\circ$ with a height of 26 a.u, and corresponds to a non-crystallographic 2-fold axis (P) parallel to the crystal ab-plane, 15° away from the a-axis.

MOSFLM (Wonacott *et al.*, 1985) package and the merging of the 200,692 intensity measurements, resulted in 38,368 independent reflections with an overall R_{sym} of 10% to 2·1 Å. Data collection statistics are given in Table 1.

To determine the non-crystallographic symmetry relation between the two independent monomers in the asymmetric unit, a self-rotation function was calculated using the Fast Rotation Function of Crowther (1972) as implemented in the program ALMN (CCP4, 1985). The data included were in the resolution range of 20 to 2·5 Å and the outer radius of integration was varied between 15 Å and 25 Å, and all of them produced similar results. Figure 1(b) is the stereographic projection of the diagonal cross-section ($\alpha+\gamma=180^\circ=\chi$) of the self-rotation function map, showing a single peak (P) corresponding to a non-crystallographic dyad perpendicular to the crystallographic 3-fold, and displaced from the nearest crystallographic 2-fold by 15°. This result indicates that the hexamer has 32 point group symmetry, centered on the 3-fold axis and necessarily displaced from the origin. This is compatible with the titration experiments that suggest the presence of an interchain disulfide bridge (Altamirano *et al.*, 1992), which can be achieved only if the hexamer subunits are arranged as dimers with the local 2-fold passing through the center of the interchain disulfide bridge and further associated by the crystallographic 3-fold axis to produce the hexamer. The offset of 15° between the non-crystallographic and crystallographic 2-folds could allow for two hexamers related by the crystallographic 2-fold to pack face-to-face in an optimal staggered configuration. In such a way, each monomer from a given hexamer would lie between two monomers of the 2-fold related hexamer. On the basis of preliminary electron microscopy experiments that suggest a disk-like configuration for the hexamer, and assuming a spherical shape for each monomer with approximate radius of 20 Å to give the correct partial specific volume for the protein molecule, an optimal model for the packing of the hexamers in the unit cell can be achieved, with no superposition of the spheres. A search for suitable heavy-atom derivatives in in progress.

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References

Altamirano, M. M. & Calcagno, M. (1990). Zinc binding and its trapping by allosteric transition in glucosamine-6-phosphate deaminase from *Escherichia coli*. *Biochim. Biophys. Acta*, **1038**, 291–294.

Altamirano, M. M., Mulliert, G. & Calcagno, M. (1987). Sulfhydryl groups of glucosamine-6-phosphate isomerase deaminase from *Escherichia coli*. *Arch. Biochem. Biophys.* **258**, 95–100.

Altamirano, M. M., Libreros-Minotta, C. A., Lara-Lemus, R. & Calcagno, M. (1989). Evidence for vicinal thiols and their functional role in glucosamine-6-phosphate deaminase from *Escherichia coli*. *Arch. Biochem. Biophys.* **269**, 555–561.

Altamirano, M. M., Plumbridge, J. A., Hérnandez-Arana, A. & Calcagno, M. (1991). Secondary structure of *Escherichia coli* glucosamine-6-phosphate deaminase

from amino acid sequence and circular dichroism spectroscopy. *Biochim. Biophys. Acta*, **1076**, 266–272.

Altamirano, M. M., Plumbridge J. A. & Calcagno, M. (1992). Identification of two cysteine residues forming a pair of vicinal thiols in glucosamine-6-phosphate deaminase from *Escherichia coli* K12, and study of their functional role by site-directed mutagenesis. *Biochemistry*, **31**, 1153–1158.

Bleasby, A. & Wootton, J. (1990). Construction of a validated, non-redundent composite sequence database. *Protein Eng.* **3**, 153–159.

Calcagno, M., Campos, P. J., Mulliert, G. & Suastegui, J. (1984). Purification, molecular and kinetic properties of glucosamine-6-phosphate isomerase (deaminase) from *Escherichia coli*. *Biochim. Biophys. Acta*, **787**, 165–173.

CCP4 (1985). *Collaborative Computing Project in Protein Crystallography*. SERC, Daresbury Laboratory, Warrington, UK.

Crowther, T. (1972). The fast rotation function. In *Molecular Replacement Method* (Rossmann, M. G., ed.), pp. 173–178, Gordon and Breach, New York.

Hodge, J. E. (1955). Amadori arrangement. *Advan. Carbohyde. Chem.* **10**, 169–205.

Holmes, R. P. & Russell, R. R. B. (1972). Mutations affecting amino-sugar metabolism in *Escherichia coli* K12. *J. Bacteriol.* **111**, 290–291.

Jones-Mortimer, M. C. & Kornberg, H. L. (1980). Amino-sugar transport systems in *Escherichia coli* K12. *J. Gen. Microbiol.* **117**, 369–376.

Matthews, B. W. (1968). Solvent content of protein crystals. *J. Mol. Biol.* **33**, 491–497.

Noltmann, E. A. (1972). In *The Enzymes* (Boyer, P. D., ed.), 3rd edit., vol. 6, pp. 314–318, Academic Press, New York.

McPherson, A. (1982). *Preparation and Analysis of Protein Crystals*, 1st edit., pp. 96–97. Wiley and Sons, New York.

Plumbridge, J. A. (1989). Sequence of the *nag*BACD operon in *Escherichia coli* K12 and pattern of transcription within the *nag* regulon. *Mol. Microbiol.* **3**, 506–515.

Plumbridge, J. A. (1991). Repression and induction of the *nag* regulon of *Escherichia coli* K12: The roles of *nag*C and *nag*A in maintenance of the induced state. *Mol. Microbiol.* **5**, 2053–2062.

Rogers, M. J. Ohgi, T., Plumbridge, J. A. & Söll, D. (1988). Nucleotide sequences of the *Escherichia coli nag*E and *nag*B genes: The structural genes for *N*-acetylglucosamine transport protein of the bacterial phosphoenopyruvate : sugar phosphotransferase system and for glucosamine-6-phosphate deaminase. *Gene*, **62**, 197–207.

Vogler, A. P. & Lengeler, J. W. (1989). Analysis of the *nag* regulon from *Escherichia coli* K12 and *Klebsiella pneumoniae* and of its regulation. *Mol. Gen. Genet.* **219**, 97–105.

Womacott, A. J., Dockerill, S. & Brick, P. *MOSFLM, a Program for the Measurement of Integrated Intensities on Oscillation Photographs*, Imperial College, University of London.

White, R. J. (1968). Control of amino-sugar metabolism in *Escherichia coli* and isolation of mutants unable to degrade amino-sugars. *Biochem. J.*, **106**, 847–858.

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RECAPITULACION

ALLOSTERIC REGULATION OF GLUCOSAMINE-6-PHOSPHATE DEAMINASE FROM *ESCHERICHIA COLI*

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Synopsis

The pathway for utilisation of amino sugars in *Escherichia coli* is controlled both by regulating the level of the expression of the genes coding for the catabolic enzymes and the level of enzyme activity. The key regulated enzyme in the pathway is D-glucosamine-6-phosphate deaminase, an allosteric enzyme activated by N-acetyl-D-glucosamine-6-phosphate. The enzyme is a hexamer composed of identical subunits arranged as a trimer of disulfide-linked dimers. However, kinetic studies indicate that the unit of the allosteric conformational change is the whole hexamer. The allosteric transition is concerted and can be accurately described by the MWC model. The concomitant conformational change occludes the side chains of two cysteinyl residues, identified as Cys118 and Cys239. A site-directed mutagenesis study demonstrated that the thiol of Cys239 contributes to the structure of the T allosteric conformer with nearly 4 kJ mole^{-1}. Tyr121 is also a critical residue in the allosteric regulation of the enzyme, since it is involved in GlcNAc6P binding at the allosteric site.

Introduction

Glucosamine-6-phosphate deaminase (D-glucose-6-phosphate ketol isomerase, deaminating, EC 5.3.1.10) catalyses the reversible conversion of D-glucosamine-6-phosphate (GlcN6P) into D-fructose-6-phosphate (Fru6P) and ammonia (1-3). This is a key step in the pathway of amino sugar utilisation in *Escherichia coli*, and is regulated at the transcriptional as well as the enzymatic levels. The gene *nagB* encoding the deaminase is part of the *nagE-nagBACD* regulon, whose expression is induced when the bacteria are grown with D-glucosamine (GlcN) or N-Acetyl-D-glucosamine (GlcNAc) as the carbon source (4-6). While the deaminase and the amidotransferase which synthesise GlcN6P (glutamine fructose-6-phosphate amidotransferase, E.C. 2.6.1.16), are two enzymes catalysing physiologically opposing reactions, the expression of their genes is coordinated so as to avoid the operation of a futile cycle (5,7). The molecule that induces the regulon is N-acetyl-D-glucosamine-6-phosphate (GlcNAc6P), the intracellular product of GlcNAc transport. This metabolite is also the allosteric activator of the deaminase, and the substrate of the other *nag* enzyme, GlcNAc6P deacetylase. GlcNAc6P controls both the expression and the activity of deaminase; the regulation of its activity derives from the allosteric nature of the enzyme. The long-range goal of our work on *E. coli* GlcN6P deaminase is aimed at characterising this regulation. The cloning of the *nagB* gene in the last few years, together with the other genes of the regulon (8,9), has facilitated considerably the study of the deaminase by making available large amounts of protein for structural studies and allowing for the construction of site-directed mutants. The present review summarizes our main results from kinetic and structural studies on this enzyme, with emphasis on its allosteric properties.

Structure

GlcN6P deaminase is a hexameric homopolymer with subunits of 29.7 kDa (11) which appear in electron micrographs in an hexagonal array (Fig. 1). The deaminase was recently crystallised and preliminary crystallographic data have been reported. Although the complete structure is not yet available, X-ray diffraction data obtained at 2.1 Å resolution, have confirmed the hexagonal structure of the oligomer, and strongly suggests a "trimer of dimers" structure with a single 3-fold axis and three 2-fold axes of symmetry (12). We have recently demonstrated the presence of three interchain disulfide bonds between cysteinyl residues 219 in the deaminase molecule (13). According to the structural information that is available, the three S-S bonds in the deaminase molecule must be located on each of the three 2-fold symmetry axes. This particular disulfide is not essential for the stability or function of the oligomer, as revealed by the study of the site-directed mutant Cys219→Ser (13). The proposed structure is also consistent with thermal denaturation curves of the deaminase obtained by differential scanning calorimetry, which indicate that at high temperatures, deaminase dissociates into dimers in two sequential steps (14). Figure 2 is a schematic representation of the quaternary structure of this enzyme.

The secondary structure of the deaminase was investigated both by circular dichroism spectrometry in the far UV, and empirical prediction methods that employ a joint prediction scheme which combines six predictive algorithms with defined rules based on different physico-chemical indices (10). The predicted structure has a considerable degree of alternancy of α-helices and β-sheets. Two-thirds of the helices are amphipathic while 90% of the β-sheets are hydrophobic, strongly suggesting that deaminase is an α/β protein having probably a C-terminal cluster of helices.

Kinetics

GlcN6P deaminase catalyses a reversible reaction, and assays can be performed in both directions, although the equilibrium favours deamination (K_{EQ} = 0.22 M at pH 7.7, 30 °C) (11). Under normal physiological conditions, deaminase functions only as a catabolic enzyme (*c.f.* ref. 6). The enzyme displays intense homotropic cooperative kinetics towards its substrate,

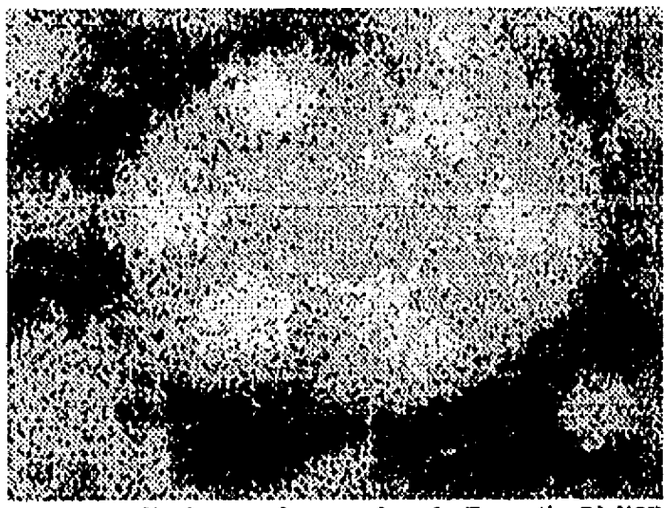

Figure 2. Electron micrograph of E. coli GlcN6P Deaminase (negative stain).
= 75 Å

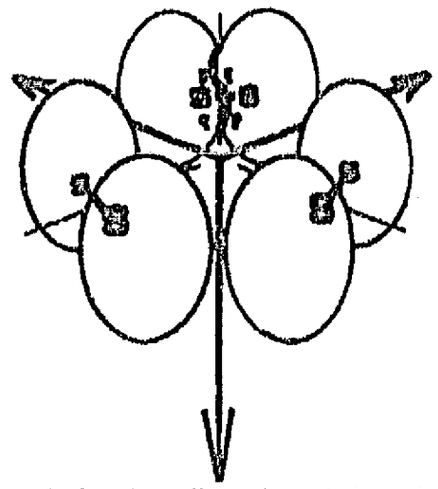

Figure 1. A schematic representation of the quaternary GlcN6P deaminase

GlcN6P (maximum Hill coefficient, 2.9 at pH 7.7). This value declines at pH values below 7.4 and above 8.2, approaching unity at both extremes of pH. The reverse reaction, that is, the formation of GlcN6P, also exhibits homotropic cooperativity towards Fru6P In contrast, however, ammonia behaves as a Michaelian substrate. The maximum Hill coefficient for Fru6P is 1.4, and is independent of the ammonia concentration (11).

The allosteric kinetics of GlcN6P deaminase can be described by the non-exclusive substrate binding case of the model of Monod *et al.* (MWC model, (15)). Non-linear regression analyses give good fits, with **n** values (the number of interacting sites) close to six (Fig. 3). The allosteric activator, GlcNAc6P, behaves as an exclusive-binding activator, producing at saturating concentrations, Michaelis-Henri kinetics. The number of allosteric sites was confirmed as being six by equilibrium dialysis experiments (16). Dissociation constants obtained by model-fitting kinetic data and by direct measurement of GlcNAc6P binding were in close agreement (30-35 μM). Our kinetic data prove that the entire deaminase oligomer is the allosteric unit, excluding the existence of a putative smaller cooperative unit, such as the disulfide-linked dimer. Evidence for the concerted nature of the allosteric transition, comes from two additional experiments: a) the enzyme immobilised on N-ε-amino-hexanoyl-glucosamine-6-phosphate agarose, which binds the enzyme by the allosteric site, displays hyperbolic kinetics over a wide range of protein/gel ratios, and b) glucitolamine-6-phosphate (GlcN-ol-6P), a dead-end inhibitor of the deaminase and an alternative homotropic ligand, produces paradoxical activation at low substrate concentrations, as predicted by the concerted allosteric model (16).

Kinetics studies of the forward and backward reactions, under conditions of hyperbolic kinetics, give initial velocity and product inhibition patterns consistent with a rapid equilibrium random mechanism (16), therby validating the use of equilibrium models for the analysis of the allosteric kinetics.

Structural events associated with the allosteric transition.

The deaminase is in the T (low affinity) conformation has two reactive thiols; the T→R transition induced by saturation with either homotropic or heterotropic ligands produces a complete protection of these groups. These thiols are vicinal in the native protein (17, 18). They were located in the primary structure by peptide mapping after specific cyanylation followed by cleavage at the S-cyanocysteines and hence identified as Cys118 and Cys239 (19). The two other cysteines in the deaminase polypeptide chain were also identified. They are the residues that form the interchain disulfide (Cys219) and the cysteine bearing a "hidden" thiol that can be exposed only after denaturation (Cys228) (13). The sulfhydryl groups of Cys118 and Cys239 can bind Zn^{2+} with very high affinity (K_{DIS} = 1.5 x 10 $^{-7}$ M). This affinity for Zn^{2+} is manifested only in the T conformer; when the T→R isomerisation is induced by a high concentration of allosteric activator or competitive inhibitor, the enzyme can no longer bind zinc. On the other hand, if the Zn-bound enzyme changes to the R-form, the metal is sequestered inside the protein and no longer equilibrates with Zn^{2+} in solution. Furthermore, the bound zinc can be released only after the protein is retumed to the T conformation (21). While the possible physiological role of this Zn binding site remains unclear, these observations demonstrate that Cys118 and Cys239 are situated in a region of the molecule that undergoes a drastic conformational change during the allosteric transition.

We investigated the role of these thiols in deaminase function using a combined approach

of chemical modification, and site-directed mutagenesis. If these thiols are blocked by methylation, sulfomethylation or by reaction with trivalent arsenicals (*e.g.* arsenite, phenylarsine oxide), the modified enzyme has a decreased homotropic cooperativity (the Hill coefficient changes from 3.0 to 1.6), and a k_{CAI} that is only half of the value for the native enzyme (18). The same result is obtained when these thiols are oxidised to form a disulfide or when the enzyme is assayed in the presence of Zn^{2+} (18, 19). The observed change in cooperativity in the SH-blocked deaminase is due to a 4 kJ mol^{-1} lower apparent free energy of interaction between oligomers. This change is due mainly to a decrease in K_T; the modified enzyme behaves as if it were positioned in an intermediate conformation between the limit conformations R and T of the native protein. This strongly suggests that one or both of these sulfhydryls contribute significantly to the stabilisation of the T conformer.

The role of Cys239 in the allosteric transition.

Because Cys118 and Cys239 have the same reactivity, the chemical approach does not permit the separate evaluation of the role of these two cysteines in the allosteric transition. This issue was investigated by the construction of the corresponding site-directed Cys→Ser mutations. A kinetic study of the mutant enzymes demonstrated that only Cys239 is necessary for full cooperative behaviour (19). The enzyme with the mutation Cys118→Ser

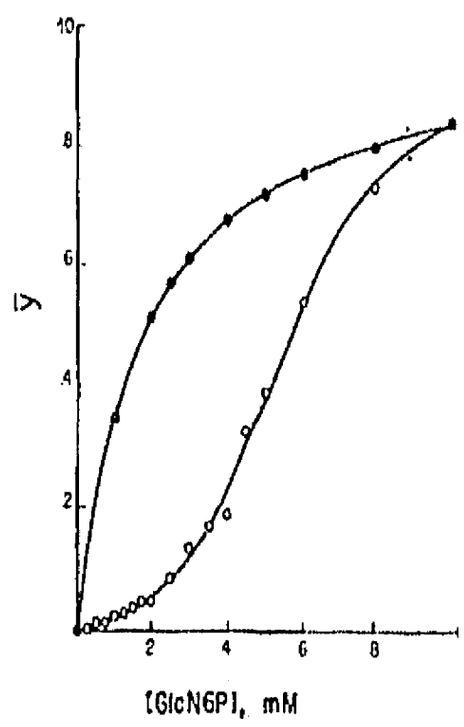

Figure 3 . ,Fit of the kinetic data (v/V against substrate) to MWC model. L: 1300; n: 5.7 c : 0.02. ⓘAllosteric activation by 2mM GlcNAc6P.

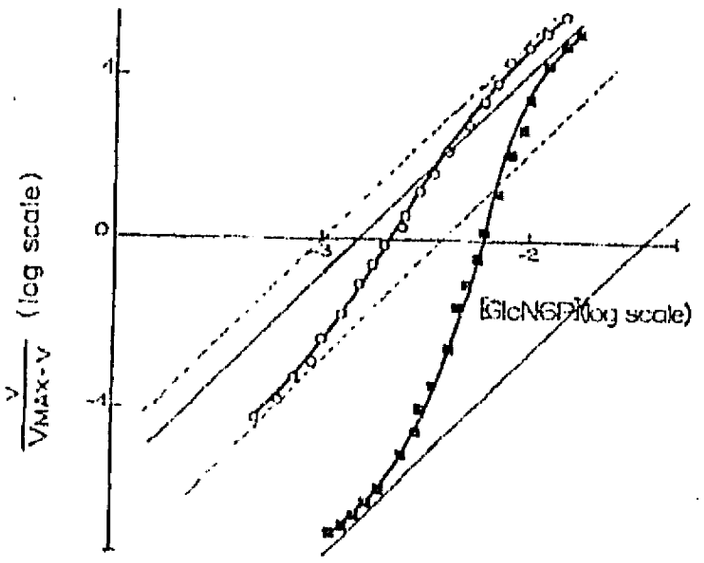

Figure 4 . Kinetic data presented as Hill plots. ■ : Wild type K_R 1 .5 mM: K_T 35 mM. O Cys239-Ser mutant Kr 1 mM, Kt 3.8 mM.

is just as cooperative as the wild-type protein, but the Cys239→Ser replacement produces a change in the K_R/K_r ratio and consequently in the value of Wyman's apparent interaction energy (20), similar to the thiol-blocked enzymes (Fig. 4). This finding proves that only Cys239 participates in the interactions that stabilise the allosteric T-conformer of GlcN6P deaminase. However, both vicinal thiols are necessary for the full catalytic activity of the enzyme, since each single mutation produces the change caused by chemical modification (*i.e.*, the reduction in the apparent k_{CAT} to nearly half of the value for the wild-type enzyme (19)). Although the precise nature of this effect remains to be established, this result demonstrates that the changes in cooperativity and k_{CAT} found by chemical blockage of both cysteines cannot be explained by assuming that the modified enzyme displays half-of-the-sites kinetics.

The cooperativity of deaminase decreases above pH 8.2, and displays hyperbolic kinetic at pH 9.0. The effect of pH on the Hill coefficient is complex, but the comparison of the curves obtained with wild-type and Cys239→Ser mutant enzymes strongly indicates that one of the protonic equilibria involved in this effect is the dissociation of Cys239.

The role of tyrosine 121.

Native GlcNAc6P deaminase has a single tyrosyl residue whose phenolic hydroxyl can be spectrophotometrically titrated with alkali (pK = 8.75). This residue is no longer titrable in the presence of saturating concentrations of the allosteric activator. The dead-end inhibitor, GlcN-ol-6P does not have this protective effect. The allosteric transition is accompanied by spectral changes (absorbance, circular dichroism and tryptophan fluorescence), which indicate involvement of aromatic residues. The differential circular dichroism spectrum of deaminase produced by saturation with GlcNAc6P exhibits a maximum at 274-275 nm, which probably corresponds to a tyrosyl residue. This peak is not present in the difference spectrum obtained by saturating the enzyme with the competitive inhibitor, GlcN-ol-6P. Together, these results, suggest that an acidic tyrosyl residue is located at or near the allosteric site, but is not involved in the associated T→R conformational change. Recently we have identified this tyrosyl residue as Tyr121 and have constructed the site directed mutants Tyr121→Thr and Tyr121→Trp; their ability to bind GlcNAc6P is impaired, but not abolished, and their homotropic cooperativity remains unaffected (22).

Concluding remarks

The work described herein shows that GlcN6P deaminase provides an interesting model for the study of allosteric interactions. It is a comparatively simple protein, since it is composed by six identical subunits, each with one allosteric site. Furthermore, it can be easily produced, purified and crystallysed. The reversibility of the reaction it catalyses and its special quaternary structure are also interesting features. On the other hand, a better knowledge of the regulation of deaminase may lend to a better understand the role of its modulation by GlcNAc6P and H^+ ions, and to the regulation of amino sugar catabolism under different physiological conditions. The construction of site-directed mutants with altered allosteric properties may be an useful tool in this research.

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References

1.Leloir, L.F. and Cardini, C.E. (1956) *Biochim. Biophys. Acta* 54, 273-282.
2. Comb, D.G. and Roseman, S. (1958) *J. Biol. Chem.* 232, 807-827.
3. Midelfort, C. and Rose, I.A. (1977) *Biochemistry* 16, 1590-1596.
4. White, R.J., (1968) *Biochem J.* 106, 847-858.
5. Vogler, A.P.S., Trentman, S., and Lengeler, J.W. (1989) *J.Bacteriol.* 171, 6582-6592.
6. Plumbridge, J.A. (1990) *J. Bacteriol.* 172, 2728-2735.
7. Plumbridge,J.A., Cochet, O., Souza, J.M., Altamirano, M.M., Calcagno, M.L. and Badet, B. (1993) *J. Bacteriol.* In press.
8. Rogers, M.J., Ohgi, T., Plumbridge, J., and Söll, D. (1988) *Gene* 62, 197-207.
9. Plumbridge, J.A. (1989) *Mol. Microbiol.* 3, 506-515.
10.Altamirano, M. M., Plumbridge, J. A., Hernández-Arana, A. and Calcagno, M.L. (1991) *Biochim.Biophys.Acta* 1076, 266-272
11. Calcagno, M., Campos, P.J., Mulliert, G. and Suástegui, J. (1984) *Biochim. Biophys. Acta* 787, 165-173.
12. Horjales, E., Altamirano, M.M., Calcagno, M.L., Dauter, Z., Wilson, K., Garratt, R.C. and Oliva, G. (1992) *J. Mol. Biol.* 226, 1283-1286.
13. Altamirano, M.M., Plumbridge, J.A., Barba, H.A. and Calcagno, M.L. (1993) *Biochem. J.* In press.
14. Hernández-Arana, A., Rojo-Domínguez, A., Altamirano, M.M. and Calcagno, M.L. (1993) *Biochemistry*, in press.
15. Monod, J., Wyman, J. and Changeux, J-P. (1965) *J. Mol. Biol.* 12, 88-118.
16. Altamirano, M.M. and Calcagno, M.L. Unpublished results.
17. Altamirano, M.M., Mulliert, G. and Calcagno, M. (1987) *Arch. Biochim. Biophys.* 258, 95-100.
18. Altamirano, M.M., Lara-Lemus, R., Libreros-Minotta, C.A., and Calcagno, M. (1989) *Arch. Biochim. Biophys.* 269, 555-561.
19. Altamirano, M.M., Plumbridge, J.A. and Calcagno, M.L. (1992). *Biochemistry* 31, 1153-1158.
20. Wyman, J. (1964) *Adv. Prot. Chem.* 19, 223-286.
21. Altamirano, M.M. and Calcagno, M. (1990) *Biochim. Biophys. Acta* 1038, 291-294.
22. Altamirano, M.M. (1993) Ph. D. Thesis, Universidad Nacional Autónoma de México, UACPyP.