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COLEGIO DE CIENCIAS Y HUMANIDADES

GRUPOS SULFHIDRILO

DE LA GLUCOSAMINA 6 FOSFATO DESAMINASA DE ESCHERICHIA COLI:

CORRELACION ESTRUCTURA-FUNCION

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INTRODUCCION

I. METABOLISMO DE LOS AMINOAZUCARES.

En *Escherichia coli* y en otras bacterias gram negativas, los aminoazúcares N-acetil-D-glucosamina (GlcNAc) y D-glucosamina (GlcN) son precursores esenciales de dos macromoléculas fundamentales de la envoltura celular, el peptidoglicano[1] y los lipopolisacáridos[2] (Fig.1). Los aminoazúcares son indispensables para el crecimiento de la bacteria y pueden provenir tanto de la biosíntesis intracelular como de fuentes exógenas.

El primer paso en la biosíntesis de los aminoazúcares es la formación de la D-glucosamina 6 fosfato (GlcN6P) a partir de la D-fructosa 6 fosfato (Fru6P) y la L-glutamina; esta reacción es catalizada por la Glucosamina 6 fosfato isomerasa dependiente de glutamina, (EC 5.3.1.19.). Las mutantes defectuosas para el gen de esta enzima son estrictamente dependientes de los aminoazúcares exógenos para su crecimiento. El agotamiento de estos compuestos produce una rápida pérdida de la viabilidad de las bacterias y la lisis de las células[3,4].

Además de su papel esencial como precursores de los componentes de la pared celular, los aminoazúcares pueden ser utilizados como fuentes

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

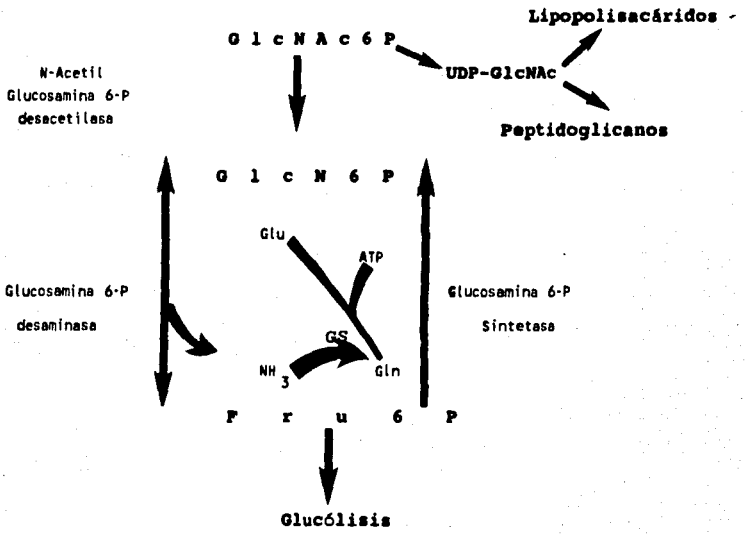
[2] Raetz, C. R. (1987). In "*Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology". American Society for Microbiology, Washington, D. C.

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

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

de carbono y de nitrógeno. Los aminoazúcares se catabolizan principalmente por la actividad de dos enzimas: La N-acetil glucosamina 6 fosfato desacetilasa (EC. 3.5.1.25) y la Glucosamina 6 fosfato isomerasa desaminasa (EC. 5.3.1.10).

FIG.1. METABOLISMO DE LOS AMINOAZUCARES EN LAS ENTEROBACTERIAS



Glu: L-glutámico, Gln: L-Glutamina, GS: Glutamina sintetasa.

Los estudios con mutantes permitieron identificar los genes de estas enzimas a 15.5 min en el mapa cromosómico de *Escherichia coli*[5,6]

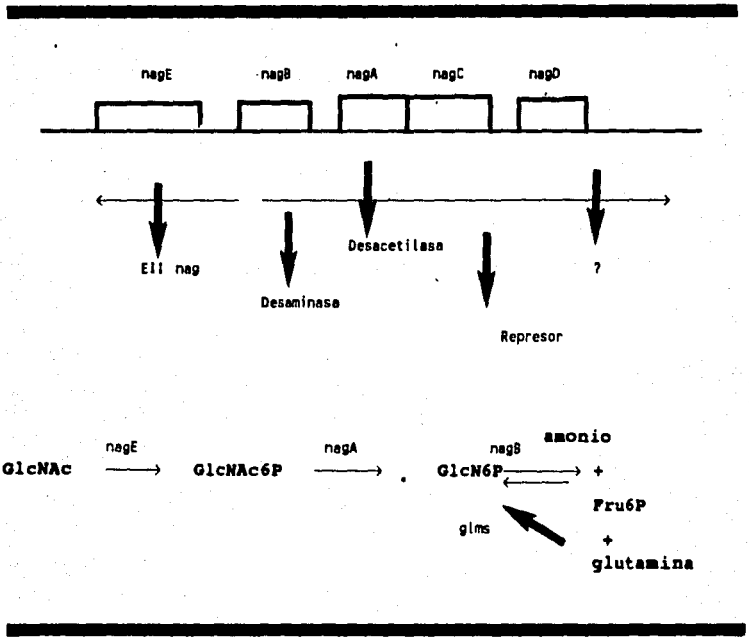
La síntesis de la GlcN6P a partir de la Fru6P y la glutamina por la sintetasa y su catabolismo a Fru6P y NH3 por la desaminasa, constituyen un clásico ciclo improductivo cuyo resultado es la

[5] Holmes, R.P. y Russel, R. (1972). J. Bacteriol 11:290-291.

[6] Plumbridge, J.(1987). Mol. Gen. Genet. 209:618-620.

aumento de la concentración intracelular de la GlcNAc6P desencadena su propio catabolismo a través de la regulación de la expresión de los genes y por la activación directa de la desaminasa.

FIG.2. REGULON DE LOS AMINOAZUCARES



Los progresos en el conocimiento del metabolismo de los aminoazúcares son producto del avance en la identificación y el estudio de los genes estructurales y reguladores correspondientes y además del progreso en la caracterización funcional y estructural de las enzimas que participan en la biosíntesis y degradación de estas moléculas.

II. ENZIMA GLUCOSAMINA 6 FOSFATO ISOMERASA.

La enzima glucosamina 6 fosfato isomerasa o glucosamina 6 fosfato

hidrólisis de la glutamina en glutamato y NH3, lo que implica una pérdida indirecta de ATP (Fig. 1). La operación de éste ciclo está impedida gracias a una estricta coordinación de la expresión y la actividad de todas las enzimas participantes en la síntesis y degradación de los aminoazúcares. Los primeros estudios revelaron una regulación antagónica de las enzimas catabólicas y biosintéticas: la GlcNAc induce la desaminasa y reprime la sintetasa [7,8]. Recientemente Rogers[9] y Plumbridge[10] mediante análisis de secuencias de DNA y estudios de complementación de mutantes comprobaron que los genes del catabolismo de los aminoazúcares están organizados en un regulón divergente formado por dos operones (Fig. 2). Los genes identificados, comprenden el *nagE* que codifica para un componente EII específico para N-acetil glucosamina del sistema de transferencia de fosfato (PTS), que se transcribe en dirección horaria y 4 genes que se transcriben en la dirección opuesta. Estos son: *nag*B que codifica la enzima desaminante, *nag*A que codifica la enzima desacetilasa, *nag*C que produce una proteína represora con afinidad por la región intergénica del regulón y el gen *nag*D de función desconocida. Plumbridge (1990)[11] ha demostrado que la GlcNAc6P es el coinductor que desencadena la inducción del regulón. Es interesante destacar que esta misma molécula es el activador alostérico de la proteína codificada por el gen nagB (la glucosamina 6 fosfato isomerasa desaminasa) y el sustrato de la desacetilasa, producto del gen nagA. De esta manera un

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

[8] Imada,A., Nozaki,F., Kawashima,F., y Yonida M. (1977). J.Gen, Microbiol. 100:329-337.

[9] Rogers, J., Ohgi, T., Plumbridge,J., Söll, D. (1988). Gene 62:197-207.

[10] Plumbridge, J. (1989). Molec. Microbiol. 3:505-515.

[11] Plumbridge, J. (1990). Mol. Microbiol. 3:506-515.

desaminasa, [2-amino-2-desoxi-D-glucosa 6 fosfato cetol isomerasa (desaminante), EC 5.3.1.10], producto del gen *nagB* (Fig. 1,2); es hasta ahora la proteína mejor conocida del regulón *nagE-BACD*. La enzima fue descrita inicialmente en *Neurospora crassa*, por Leloir y Cardini[12], quienes describieron por primera vez su activación por la GlcNAc6P. Esta enzima fue posteriormente detectada y purificada parcialmente a partir de diversas fuentes microbianas y animales[13]. El primer estudio en *Escherichia coli* fue realizado por Comb y Roseman[14], quienes trabajando con una enzima parcialmente purificada demostraron que para su actividad óptima era necesaria la GlcNAc6P. La reacción que cataliza la desaminasa (Fig. 1 y 2) ha sido considerada fisiológicamente irreversible[4], sin embargo, *in vitro* es reversible, aun cuando el equilibrio favorece la reacción catabólica[14,15]. Recientemente Vogler y col[16]., han demostrado que en bacterias con una mutación del gen que codifica para la enzima GlcN6P sintetasa (*glsm*), la enzima GlcN6P desaminasa es funcional en la biosíntesis de los aminoazúcares cuando se impide la expresión del gen represor (*nagC*) con una inserción que bloquea su transcripción completa.

Como ya se ha visto, la desaminasa cataliza una reacción reversible que es una isomerización cetoaldólica acoplada a una aminación-desaminación. Esto constituye un ejemplo biológico de la

[12] Leloir, L y Cardini, C. (1956) Biochim. Biophys. Acta 20: 33-42.

[13] Nolman, E (1972) in "The enzymes" 3rd Edn. (Boyer, P. ed). Academic Press, New York.

[14] Comb, D. y Roseman, S. (1958) J. B. Chem. 232, 807-827.

[15] Midelfort, C. y Rose, I. (1977). Biochem. 16:1590-1596.

[16] Vogler, A., Trentmann, S. y Lengeler, J. (1989). J. Bacteriol. 171:6586-6592.

reacción de Amadori[17]. Irwing Rose[18,19,20] quien estudió el mecanismo químico de las isomerasas cetoaldólicas, publicó con Midelfort[15], un análisis mecanístico de la reacción de la desaminasa de *E. coli* parcialmente purificada mediante el empleo de técnicas de intercambio de Tritio a partir de posiciones específicas en los sustratos. Estos autores proponen el mecanismo de reacción, ejemplificado en la Fig.3, en el cual el NH_4^+ pierde un protón al iniciarse el ciclo catalítico y el NH_3 ataca al C carbonílico de la Fru6P. Se forma entonces un aminoalcohol intermediario que pierde agua. Esto da lugar a la enolización de la Fru6P que forma una enolamina intermediaria, probablemente con configuración *cis*[15]. (FIG.3).

La estereoquímica de la reacción en el C1 de la Fru6P, en relación con la estereoespecificidad en R para la posición C2, es la típica de estas isomerasas cetoaldólicas[18]. Una característica única de la glucosamina 6 fosfato desaminasa es que durante el curso de la reacción, diferentes grupos (agua, NH_3, o el oxígeno del OH en C5 del sustrato), pueden atacar al C2 de la Fru6P, lo que revela una notable versatilidad de los grupos funcionales del sitio activo que rodean al carbono carbonílico de la Fru6P.

La desaminasa es una proteína que se induce al cultivar las bacterias utilizando en el medio glucosamina como única fuente de carbono y de nitrógeno. Calcagno y col.,[21] publicaron un procedimiento de purificación a homogeneidad de la proteína de *Escherichia coli B*,

[17] Hodge, J. E. (1955). The amadori rearrangement. Adv. Carbohydr. Chem. 10: 169-205.

[18] Rose, I.(1975). Adv. Enzymol. 43:491-517.

[19] Rose, I., O'connell, E. y Mehler, A. (1970). J. Biol. Chem. 245: 2219-2228.

[20] Rose, I. (1970). in "The enzyme". 3a ed. Academic Press, New York

[21] Calcagno, M., Campos, P., Mulliert, G. y Suastegui, J. (1984), Biochem. Biophys. Acta. 787:165-173.

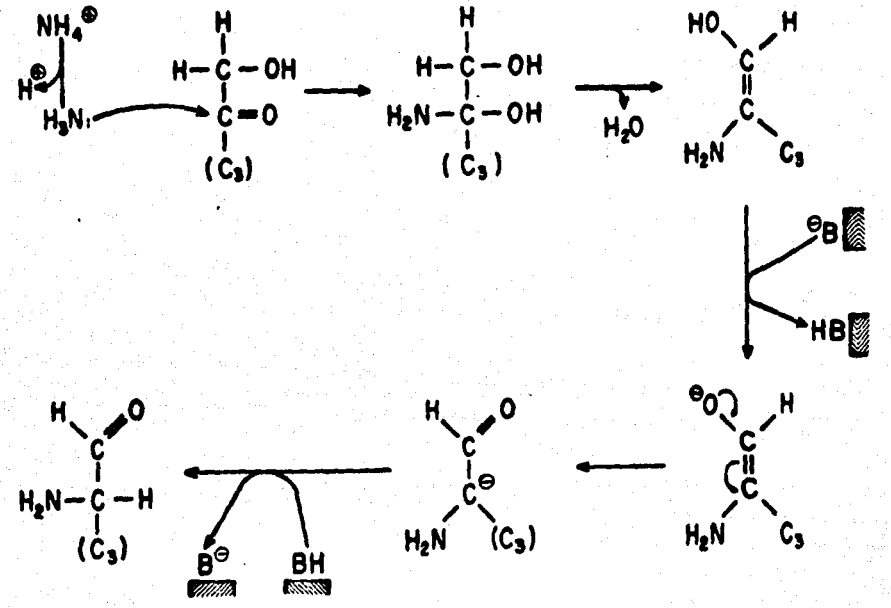


Figura 3. Mecanismo propuesto por Mildelfort y Rose (ref. 15) para la reacción de la glucosamina 6-P desaminasa de Escherichia coli

por cromatografía de afinidad alostérica, utilizando como ligando un análogo del activador alostérico: la ε-aminocaproil-GlcN6P, fijada a agarosa . La enzima es un oligómero hexamérico de 178 kDa. (cadenas polipéptidicas de 29.7 kDa.); su carácter homopolimérico se había deducido inicialmente por experimentos de electroenfoque en medios desnaturalizantes[21] y por el dato que en 31 ciclos de degradación de Edman realizados para establecer la secuencia N-terminal, se encontró solamente un residuo por ciclo[22]. Esto fue confirmado al conocerse la secuencia completa a partir de la secuencia del gen *nagB*[9]. Estudios de microscopía electrónica permiten proponer una distribución de las seis subunidades de la desaminasa con una simetría hexagonal que podría ser C6 o D3[18] .

Desde el punto de vista cinético[21], la enzima presenta una intensa cooperatividad homotrópica positiva con respecto a la GlcN6P (h_{max} = 3), a pH de 7.6-8.0, lo que coincide con el pH óptimo de la desaminasa. El activador (GlcNAc6P) a concentraciones saturantes hace desaparecer la cooperatividad y la enzima adquiere una cinética hiperbólica. Los valores de S0.5 disminuyen a medida que aumenta la concentración del activador (efecto K), pero la velocidad máxima no se modifica, por lo tanto, es un sistema alostérico de tipo K puro. La obtención de cinética hiperbólica por saturación con GlcNAc6P indica un comportamiento de fijación exclusiva del activador.

III. GRUPOS SULFHIDRILO Y PROTEINAS.

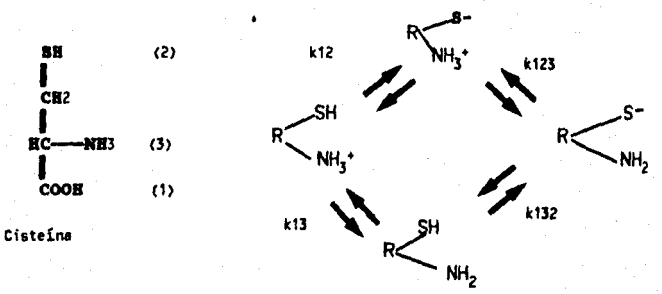
A. ESTRUCTURA ELECTRONICA Y REACTIVIDAD QUIMICA.

El azufre se localiza en el grupo VI de la tabla periódica de los elementos; su peso atómico es de 32, su configuración electró-nica es: $[Ne]3s^2 3p^4$, puede existir en diferentes estados de oxidación desde -2 a +6. En las proteínas, existe solamente en los estados de oxidación de S^{-2} (Cys) y S^{-1} (CySSCy). Los compuestos orgánicos de azufre

[22] Calcagno, M. y colaboradores. Datos no publicados.

presentan algunas características muy diferentes a sus correspondientes análogos de oxígeno. En primer lugar, el azufre es mucho menos electronegativo o electrofílico que el oxígeno. En segundo término, la tendencia a formar uniones π es menor entre el C y el S que entre el C y el O. La formación de enlaces π entre el S y el O (como en el anión sulfato) es importante en la química de los compuestos de oxidación superior del azufre. El azufre forma puentes de hidrógeno más débiles, en comparación con el nitrógeno o el oxígeno, ya que una electronegatividad alta es un requisito para la formación de puentes de hidrógeno estables.

Edsall y Wyman[23] calcularon la constante de ionización del grupo tiol y amino de la cisteína (Cys), utilizando diferentes condiciones y métodos experimentales; estos autores formulan sus resultados en términos de un conjunto de constantes microscópicas, de acuerdo con el siguiente esquema:



La cisteína tiene tres grupos ácidos: el carboxilo, el sulfhidrilo y el amonio, que denominaremos 1, 2 y 3 respectivamente. El pK1

[23] Edsall,J. y Wyman, J. (1958).in "Biophysical Chemistry" vol.1 . Academic, Press. New York.

representa esencialmente la ionización del grupo carboxilo. La adjudicación del pK2 y el pK3 a los grupos SH y amonio no es inmediata, puesto que el carácter ácido del amonio es del mismo orden de magnitud que el del grupo SH. Sin embargo el esquema de ionización puede escribirse en forma análoga al de la glicina.

Las constantes macroscópicas pueden relacionarse con los valores de pK utilizando la ecuación:

$$K2 = k12 + k13 \text{ y } K2\ K3 = k12k123 = k12k132$$

TABLA I* VALORES DE pK APARENTE DE LA CISTEINA

pK	Benesch y Benesch	Elson y Edsall	Grafius y Nielands
pK_{12}	8.53	8.50	8.65
pK_{13}	8.86	8.85	8.75
pK_{123}	10.36	10.35	10.05
pK_{132}	10.03	10.00	9.95

*Liu,T. (1977) in "The proteins" (Neurath, H. y Hill, R. ed.), Academic Press. New York.

B. REACCIONES DE LOS GRUPOS SULFHIDRILO

I. Intercambio tiol-disulfuro [24,25]

El intercambio tiol-disulfuro se produce por una transferencia de equivalentes reductores entre dos pares tiol-disulfuro. Los grupos sulfhidrilo de las proteínas o de moléculas no proteícas (PSH) pueden

[24] Liu, T. (1977) in "The proteins". (Neurath, H. y Hill, R. ed.) vol. III. Academic Press, New York.

[25] Gilbert, H. (1990). Adv. Enzymol. 69-171.

reaccionar con un disulfuro (RSSR) en un desplazamiento bimolecular, para formar una proteína que tiene un disulfuro (PSSR).

$$2PSH + RSSR \leftrightarrow PSSP + 2\ RSH \qquad K1$$

este proceso ocurre en dos etapas:

$$PSH + RSSR \leftrightarrow PSSR + RSH \qquad K2$$

$$PSH + PSSR \leftrightarrow PSSP + RSH \qquad K3$$

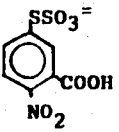
donde K2K3 = K1 (constante de equilibrio).

Los estudios cinéticos revelan que la velocidad de la reacción es proporcional a la concentración de RS^- y no a la de su ácido conjugado RSH. El mecanismo de reacción consiste en un ataque nucleofílico de un anión tiolato a un disulfuro con la formación de un nuevo disulfuro y un nuevo tiolato. Siendo el tiolato la especie reactiva, el ión hidrógeno se comporta como un ligando protector; el efecto de la variación del pH sobre la velocidad de la reacción permite conocer la constante de disociación del grupo sulfhidrilo. En las proteínas, la proximidad de una carga positiva o de un sustituyente electrofílico, disminuye el pK del tiol. Cuando el sulfhidrilo se encuentra en una zona muy hidrofóbica de la proteína, los valores de pK pueden aumentar considerablemente con respecto a los SH expuestos. La velocidad de la reacción del intercambio tiol disulfuro aumenta cuando disminuye el pK del grupo saliente.

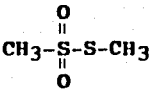
Un reactivo ideal para titulación de SH por intercambio tiol disulfuro debe tener un grupo saliente cuyo tiolato sea más ácido que el sulfhidrilo de la cisteína, y que presente una intensa absortividad en una región del espectro en la que no interfiera la absorción de la proteína o del reactivo. El DTNB (ácido 5',5'-ditio-bis(2-nitrobenzoico), o reactivo de Ellman cumple estos requisitos: es muy soluble en medio acuoso y es estable a pH 7.0. Sin embargo introduce en la proteína un grupo voluminoso y polar que puede distorsionar a la molécula en estudio. Existen otros reactivos que funcionan por el mismo principio de intercambio tiol-disulfuro pero que incorporan a la proteína sustituyentes pequeños como el: -CN, en el caso del NTCB (2-nitro,5-tiocianatobenzoato), el-SCH_3 del metil metano
tiosulfonato (MMTS), o el $-SO_3^-$ del 2-nitro 5-tiosulfonatobenzoato

(NSTB).[26,27]

NTCB



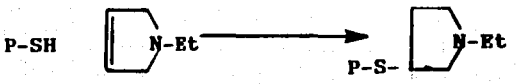
NSTB



MMTS

II.Reacciones de adición

El anión tiolato es çapaz de adicionar a dobles ligaduras, deacuerdo al mecanismo general de la adición de Michel. Un reactivo muy utilizado, que forma un aducto estable, es la N-etil maleimida, NEM[28,29]. En este trabajo la hemos utilizado como un reactivo de bloqueo irreversible de tioles.



III.Sustituciones nucleofilicas.

El anión tiolato es un nucleófilo que puede participar en reacciónes de sustitución, SN1 o SN2. En este mecanismo ocurre un

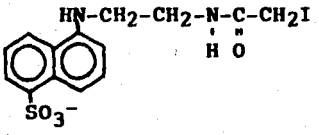
[26] Eyzaguirre, J. (1987) in "Chemical modification of enzymes", (Eyzguirre, J. ed.), Halsted Press. New York.

[27] Brockehurst, K. (1979). Int. J. Biochem. 10:259-274.

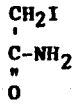
[28] Cohen, L. A. (1968). Annu. Rev. Biochem. 37:695-715.

[29] Cohen, L. A. (1970). in "The enzyme" (P. Boyer, ed). Vol.I. Academic Press.

ataque nucleofílico a un carbono tetraédrico, que tiene unido un grupo saliente adecuado, por lo general más electronegativo que el grupo de ataque, para obtener condiciones termodinámicas favorables. El grupo saliente preferido para estos compuestos es el I^-, por lo que muchos reactivos para tioles contienen la función yoduro de alquilo[30]. Es el caso del yoduro de metilo[31], la yodoacetamida o el ácido monoyodoacético. El bromo es también un buen anión de salida, y se ha utilizado en algunos reactivos para modificación de proteínas. Un inconveniente de los haluros de alquilo es su menor especificidad para grupos SH, ya que pueden reaccionar con otros nucleófilos como el grupo amino. Esto se puede controlar con el pH de la reacción, ya que el ácido conjugado correspondiente, el grupo amonio, no es un reactivo nucleofílico. En muchas ocasiones, debido a la proximidad entre los valores de pKa de los grupos -SH y $-NH_4^+$, estos reactivos resultan menos específicos. Existen algunos derivados interesantes del yodoacetato, como el IAEDANS (N-Iodoacetil-N'- (sulfo- 1- naftil) etilendiamina), que es una compuesto que contiene un *p*-dimetilamino naftaleno sulfonilo (dansilo) que le confiere fluorescencia[32].



IAEDANS



YODOACETAMIDA

YODURO DE METILO

[30] Jocelyn, P., (1974) in "Biochemistry of the SH group"., Academic Press,New York.

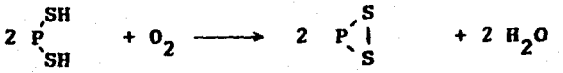
[31] Stark, G. R. y Stein, W.,(1964). J. Biol. Chem. 239:3755-3768.

[32] Jocelyn, P. (1987) in "Methods in enzymology", vol. 143. Academic Press, New. York.

IV.Oxidación

Dos grupos SH pueden oxidarse con la pérdida de un protón y un electrón por tiol, formando una unión disulfuro, -S-S-. Esta reacción se logra con oxidantes suaves, como el dioxígeno, en medio acuoso. Un excelente catalizador para esta oxidación es el complejo *o*-fenantrolina-Cu^{2+} [33]. Bajo condiciones más rigurosas, se logran estados superiores de oxidación del azufre, que a diferencia de la oxidación a disulfuro, resultan irreversibles. Por ejemplo, el ácido perfórmico, es muy utilizado para oxidar los residuos de cisteinilo a sulfonilalanina, como paso previo a la hidrólisis ácida para el análisis de aminoácidos.

$$4\ PSH + O_2 \longrightarrow 2\ P\text{-}S\text{-}S\text{-}P + 2\ H_2O$$



V. Reacción con metales[34,35]

[33] Kobashi, M.. (1968). Biochim. Biophys. Acta 158:239-245.

[34] Kidani, Y. y Hirose (1986). in "Zinc enzymes" (Bertinei, I., Luchinat C, Maret, W. ed.), Vol. 1. Birkhauser Boston Inc., Boston.

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Al igual que los protones, los iones metálicos son electrófilos o ácidos de Lewis, capaces de aceptar un par de electrones y formar una unión . A diferencia de los protones, los metales por su caracter polivalente, pueden formar quelatos de diferentes estructuras tridimensionales. Si bien el residuo más importante como donador de electrones para la formación de quelatos metálicos es la histidina, en muchas proteínas son los tioles de la cisteína los principales grupos ligantes de metales: por ejemplo el zinc, en la anhidrasa carbónica de eritrocito o en la alcohol deshidrogenasa de levadura, etc..

El Pb(II), Hg(II), Cu(II), Cd(II), Sn(II), Ni(II), Hg(I), Cu(I) y Ag, forman complejos estables con los sulfhidrilos, cuyas constantes de disociación son del orden nanomolar o micromolar. La sustitución de Zn^{2+} por $^{113}Cd^{2+}$ ha permitido generar señales de NMR que facilitan el estudio de la interacción y la identificación de los donadores de electrones que forman el campo ligante.[36]

VI.Reacción de los tioles con los arsenicales.

Los derivados arsenicales actúan en los sistemas biológicos únicamente a través de su capacidad para reaccionar con los grupos SH de las proteínas. Los arsenicales monosustituidos con la estructura general R-As→O, forman compuestos muy estables con los ditioles en los cuales los SH estan integrando un ciclo de cinco átomos, con la estructura de un ditioarsinito.

Estos ditioarsinitos cíclicos son mucho mas estables que los que se formarían con dos moléculas de un monotiol, lo que permite

[36] Nowak, T. (1987) in "Chemical Modification of enzymes", (Eyzaguirre, J. ed), Halsted Press, New York.

diferenciarlos.

VII. Papel de los sulfhidrilos en las proteínas.

Las propiedades físicas y cinéticas de algunas enzimas o proteínas dependen del estado redox de los tioles y disulfuros presentes en su estructura. Algunas proteínas como la ribonucleasa A, el inhibidor de la tripsina pancréatica, o la lisozima, son activas cuando sus disulfuros nativos estan reducidos. En contraparte, se encuentran proteínas como la glucosa 6 fosfato deshidrogenasa, la fosfofructocinasa, la 3-hidroxi-3-metilglutaril CoA reductasa, o la fructosa 1,6 bifosfatasa que son activas sólo cuando sus grupos SH se encuentran en estado reducido.

Los grupos SH juegan un importante papel en los sitios activos de diversas enzimas que utilizan sustratos fosforilados y se ha demostrado la existencia de residuos vecinales próximos al sitio de unión del éster fosfato en siete enzimas que actúan sobre azúcares-fosfato [37,38].

Las técnicas de modificación química han sido utilizadas para identificar la naturaleza de los residuos localizados en los sitios activos u otras regiones de la proteína. De esta forma se han localizado aminoácidos importantes para la función y la interacción entre subunidades, y se han obtenido proteínas estructural y funcionalmente modificadas. Dentro de los aspectos funcionales susceptibles de modificación química, podemos mencionar: la especificidad de la reacción, la cooperatividad tanto homotrópica como heterotrópica y el comportamiento iónico de los grupos funcionales. La modificación química permite alterar la especificidad de las enzimas,

[37] Rippa, M., Bellini, T. Signorini, M. y Dalocchio, F. (1981). J. Biol. Chem. 256:451-455.

[38] Kaiser, T., Lawrence, S. y Rokita, S. (1985). Ann, Rev. Biochem. 54:565-595.

su curva de pH, cambiar patrones de inhibición o activación, etc. Estos cambios sobre la especificidad enzimática, pueden expresarse a través de su constante catalítica (k_{cat}), y su constantes relacionadas con los ligandos (constante de Michaelis, constantes de inhibición, constantes de disociación, etc.), y en los sistemas alostéricos, a través del efecto sobre la cooperatividad, valorado por el coeficiente de Hill máximo (h_{max}) o la desensibilización a los moduladores alostéricos.

La transformación de las cadenas laterales de los aminoácidos, aporta datos cinéticos y estructurales esenciales para conocer el funcionamiento y regulación de las proteína, y constituye una base fundamental para iniciar estudios de mutagénesis dirigida.

OBJETIVO

ESTUDIAR EL PAPEL DE LOS GRUPOS SULFHIDRILO DE LA GLUCOSAMINA 6 FOSFATO DESAMINASA EN LA CATALISIS Y EN LA TRANSICION ALOSTERICA.

ASPECTOS METODOLOGICOS

En cada uno de los cinco artículos que integran esta tesis, sedescribe la metodología usada. En los anexos se detallan algunas preparaciones, como la síntesis y valoración de la GlcN-ol-6P, que salvo algunas menciones en las publicaciones, está esencialmente inédita.

En el curso de estas investigaciones, se desarrolló un método para dializar volúmenes muy pequeños, que se describe muy brevemente en el cuarto artículo, y que proyectamos publicar por separado en una revista metodológica.

En general, hemos utilizado una metodología multidisciplinaria, combinando técnicas bacteriológicas, de purificación de proteínas, estudios cinéticos y ajuste de modelos, modificación química de proteínas, métodos fisicoquímicos de estudio de proteínas en solución y algoritmos empíricos de predicción de estructuras.

ARTICULO I

Sulfhydryl Groups of Glucosamine-6-phosphate Isomerase Deaminase from *Escherichia coli*.

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Sulfhydryl Groups of Glucosamine-6-phosphate Isomerase Deaminase from *Escherichia coli*

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Glucosamine-6-phosphate isomerase deaminase (2-amino-2-deoxy-D-glucose-6-phosphate ketol isomerase (deaminating), EC 5.3.1.10) from *Escherichia coli* is an hexameric homopolymer that contains five half-cystines per chain. The reaction of the native enzyme with 5',5'-dithiobis-(2-nitrobenzoate) or methyl iodide revealed two reactive SH groups per subunit, whereas a third one reacted only in the presence of denaturants. Two more sulfhydryls appeared when denatured enzyme was treated with dithiothreitol, suggesting the presence of one disulfide bridge per chain. The enzyme having the exposed and reactive SH groups blocked with 5'-thio-2-nitrobenzoate groups was inactive, but the corresponding alkylated derivative was active and retained its homotropic cooperativity toward the substrate, D-glucosamine 6-phosphate, and the allosteric activation by *N*-acetyl-D-glucosamine 6-phosphate. Studies of SH reactivity in the presence of enzyme ligands showed that a change in the availability of these groups accompanies the allosteric conformational transition. The results obtained show that sulfhydryls are not essential for catalysis or allosteric behavior of glucosamine-6-phosphate deaminase.

Sulfhydryl groups play an important role either in ligand binding or in catalysis of several enzymes acting on phosphorylated substrates, and Rippa *et al.* (1) have found evidence for the involvement of vicinal thiols near the phosphate ester binding sites of seven enzymes studied.

Recently we purified and characterized the glucosamine-6-phosphate isomerase deaminase (glucosamine-6-phosphate deaminase, 2-amino-2-deoxy-D-glucose-6-phosphate ketol isomerase (deaminating), EC 5.3.1.10) from *Escherichia coli* B. This enzyme catalyzes the conversion of GlcN6P[2] in Fru6P and ammonia, and is allosterically activated by GlcNAc6P. It is composed of six apparently identical subunits, and amino acid analysis reveals five cysteine residues per polypeptide chain (2). The enzyme from *E. coli* has been reported to be inhibited by *p*-hydroxymercuribenzoate (3). The deaminase purified by Das and Datta (4) from *Candida albicans*, which seems to be a different protein, is also inhibited by sulfhydryl reagents.

The present study was undertaken to elucidate the status of cysteine residues in the glucosamine-6-phosphate isomerase deaminase obtained from *E. coli* and to evaluate the role of these sulfhydryl groups in the catalysis and the allosteric control of this enzyme.

[1] To whom correspondence should be addressed.

[2] Abbreviations used: GlcN6P, D-glucosamine 6-phosphate; Fru6P, D-fructose 6-phosphate; GlcNAc6P, *N*-acetyl-D-glucosamine 6-phosphate; GlcN-ol-6P, 2-amino-2-deoxy-D-glucitol 6-phosphate or sorbitol-amine 6-phosphate; DTNB, 5',5'-dithiobis-(2-nitrobenzoic acid); TNB, 5'-thio-2-nitrobenzoic acid; SDS, sodium dodecyl sulfate.

0003-9861/87 $3.00

MATERIALS AND METHODS

Enzyme. Glucosamine-6-phosphate isomerase deaminase from *E. coli* B (strain ATCC 11303) was purified and stored essentially as previously described (2). The protamine sulfate step was omitted in the purification scheme, without appreciable change in the characteristics of the enzyme obtained. The purity of each enzyme batch was always verified by denaturing polyacrylamide gel electrophoresis. The specific activity of the enzyme at pH 7.7 and 30°C was in the range 650–680 μmol min^{-1} mg^{-1}. The same value was obtained in the presence of 5 mM dithiothreitol and after its removal by dialysis. Enzyme concentrations were calculated from the molar absorptivity $\epsilon_{278} = 20.02 \times 10^4$ M^{-1} cm^{-1}, determined by the gravimetric method of Kupke and Dorrier (5) by weighing three enzyme samples of 0.5 mg each. The value obtained from the complete amino acid composition, using the absorptivity data published by Edelhoch (6), was somewhat lower (16.0×10^{-4} M^{-1} cm^{-1}). Thus, the value previously reported (2) was inaccurate.

Enzyme assays were run as previously described (2) in the direction of GlcN6P deamination. For each assay, substrate concentration was varied from 0.5 to 10 mM in the presence or absence of 2.5 mM GlcNAc6P. Data were fitted to the Michaelis-Henri equation according to Wilkinson (7). Homotropic cooperative kinetics was fitted to the Hill equation by the Atkins procedure (8) to evaluate the maximal velocity and the Hill coefficient.

Materials. GlcNAc6P was prepared from GlcN6P following the procedure of Leloir and Cardini (9); GlcN-ol-6P was obtained by reduction of GlcN6P with sodium borohydride and purified by ion-exchange chromatography essentially as described by Midelfort and Rose (10). Purity of both products was verified by thin-layer chromatography on cellulose plates with two different solvent systems (ethyl acetate:acetic acid:water:ammonia, 6:2:2:1, and isopropanol:ammonia, 4:1). Other biochemicals and DTNB were from Sigma Chemical Co. (St. Louis, MO). Methyl iodide was from J. T. Baker (U.S.A.) and was distilled before use. All other chemicals were reagent-grade products.

Sulfhydryl group analysis. Sulfhydryls were determined spectrophotometrically with varying amounts of DTNB at 25°C in 0.1 M Tris-HCl buffer with 5 mM EDTA in a final volume of 500 μl. The reactions were carried out at room temperature under pseudo-first-order conditions, i.e., with a high molar excess (600- to 1200-fold) of DTNB. Reactions were started by addition of the enzyme, while the absorbance was recorded at 412 nm with a Pye Unicam SP-1800 double beam spectrophotometer. The number of sulfhydryl groups that reacted per mole of enzyme was determined from the absorbance at the end of the reaction from the molar absorptivity $\epsilon_{412} = 14{,}140$ M^{-1} cm^{-1} (11). Kinetic measurements of sulfhydryl reactivity were carried out under the same conditions, both in the absence and in the presence of ligands. Pseudo-first-order or second-order rate constants were determined by fitting the corresponding equations in their linear form by means of the least-squares method.

Alkylation of sulfhydryl groups with methyl iodide was achieved in a 1.0 μM enzyme solution in 50 mM Tris-HCl buffer (pH 8.0) overnight, at room temperature in a closed tube. The reaction was started by the addition of the alkylating agent (2 μl) per milliliter of reaction mixture) to a final concentration of 32 mM.

Treatment of the enzyme with *m*-periodate was performed in 0.1 M Tris-HCl buffer (pH 8.0) at 20°C with a 5 μM enzyme solution and a 120 or 1200 times molar excess of sodium *m*-periodate. Samples of the reaction mixture were taken at different times and diluted into 100 vol of a 0.1 M glycerol solution to destroy the excess of *m*-periodate anions and to prepare the enzyme for assay.

RESULTS

Determination of Sulfhydryl Groups of Glucosamine-6-phosphate Deaminase with DTNB

When the release of TNB anions in the presence of a high molar excess of DTNB was followed spectrophotometrically, simple pseudo-first-order kinetics was obtained (Fig. 1). In the presence of 8 M urea or 0.5% SDS the reaction was almost instantaneous. From these experiments, 12 sulfhydryl groups per mole of native enzyme and 18 per mole of enzyme denatured by urea or SDS (2 and 3 residues per subunit, respectively) can be calculated (Table I). In another experiment, the enzyme was treated simultaneously with denaturing and reducing reagents. An enzyme sample (0.4 nmol) was dialyzed against 500 vol of a solution containing 8 M urea, 50 mM Tris-HCl buffer (pH 8.0, 20°C), and 10 mM dithiothreitol. The content of the dialysis sack was freed of the latter reagent by further dialysis against a deaerated urea-Tris solution of similar composition, and the sulfhydryls were determined with DTNB under the conditions described. The results (Table I) indicate that the number of cysteines in denatured and reduced enzyme is 28 per mole, i.e., 5 residues per chain (nearest integer). The table also shows that in

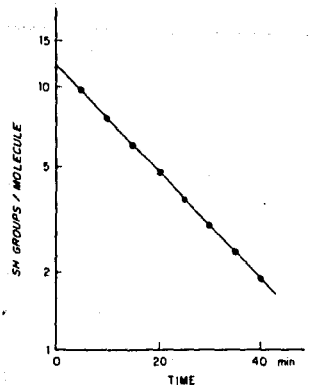

FIG. 1. DTNB titration of SH groups of native glucosamine-6-phosphate isomerase deaminase. In a 500-μl microcell, 0.65 μM enzyme and 0.5 mM DTNB were mixed under the conditions described under Materials and Methods. Absorbance data at 412 nm were read against a blank without enzyme. The total number of SH groups per mole of enzyme (12) was calculated from absorbance at the end of the reaction and used to determine the unreacted groups for each time.

absence of the denaturant, dithiothreitol does not produce any change in the number of sulfhydryls reacting.

In another experiment, a sample of native enzyme was treated with methyl iodide for 30 min under the conditions described under Materials and Methods and dialyzed exhaustively against 50 mM Tris-HCl (pH 8.0); afterward, sulfhydryls were determined by DTNB reaction. As shown in Table I, methylation blocks the two reactive sulfhydryls per subunit, whereas a third one remains unchanged and can be determined with the DTNB reaction in the presence of SDS.

Effect of Modification of Reactive SH Groups on Enzyme Activity

Native enzyme, fully reacted with DTNB and containing two TNB groups per polypeptide chain, was totally inactive. In other series of experiments, samples of the reaction mixture containing native enzyme and DTNB were taken at different time intervals and the fraction of V_{max} with respect to zero time was determined for each one. The plot of the fraction of activity, as a function of the number of sulfhydryl groups reacting per mole of enzyme, is shown in Fig. 2.

To reactivate the enzyme, modified by the addition of TNB groups, a fully reacted sample (0.9 nmol) was dialyzed against a volume excess of 10 mM dithiothreitol in 50 mM Tris-HCl buffer, pH 7.7, for 6 h at 4°C. Under these conditions, 65% of the original activity was recovered (average of two determinations).

The enzyme having the 12 reactive thiols modified by methylation remains practically unchanged in its kinetic behavior. Homotropic cooperativity toward GlcN6P (Hill coefficient = 2.3) and activation by GlcNAc6P, giving hyperbolic kinetics in its presence at saturating concentrations (K_m for GlcN6P = 0.5 ± 0.04 mM), were essentially the same as in the untreated enzyme. Nevertheless, the specific activity of the modified enzyme was 463 μmol min^{-1} mg^{-1}, 76% of the expected value (average of two

TABLE I

SULFHYDRYL GROUPS REACTING WITH DTNB PER MOLE OF ENZYME[a]

Reaction conditions	Thiol groups per mole enzyme
Unmodified	12.2 ± 0.3
8 M urea[b]	17.3 ± 0.9
0.5% SDS[b]	18.4 ± 1.5
10 mM dithiothreitol[c,d]	11.9
10 mM dithiothreitol + 8 M urea[c,d]	28.0
Methylated enzyme[c,d]	0
Methylated enzyme, 0.5% SDS[c,d]	6.1

[a] All reactions were performed at 20°C with 0.7 μM enzyme, 0.5 mM DTNB, 5 mM EDTA, and 0.1 M Tris-HCl buffer (pH 8.0) in a final volume of 500 μl (data from 6 determinations).

[b] Reaction carried out in the presence of the denaturing agent.

[c] See details in the text.

[d] Average of two independent experiments.

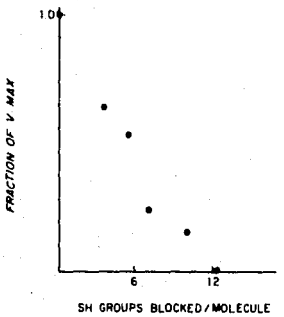

FIG. 2. Effect of modification of reactive SH groups with DTNB on the activity of glucosamine-6-phosphate isomerase deaminase. Enzyme (0.65 μM) was reacted with DTNB (2.0 mM) under the conditions described under Materials and Methods. At appropriate times during the reaction, 15-μl samples of the reaction mixture were taken and the reaction stopped by a 20× dilution with water. The enzyme was assayed immediately as described, and fraction of V_{max} was plotted against the number of SH groups reacting with DTNB per mole of enzyme at the time when the reaction was brought to a halt.

experiments). This methylated enzyme is insensitive to inhibition by DTNB.

m-Periodate Treatment of the Enzyme

The enzyme, treated with *m*-periodate under the conditions described under Materials and Methods, retained its specific activity for 30 min; after this time, no modifications in thiol content were detected with DTNB. The enzyme kinetics and the activation by GlcNAc6P were the same as in the untreated enzyme. Similar results were obtained when a 120 or 1200 times molar excess of *m*-periodate was used.

DTNB Reaction and Effect of Ligands on the Reactivity of Thiol Groups

The 12 exposed groups present in the native enzyme displayed pseudo-first-order kinetics when reacting with DTNB under the large molar excess of the reagent used (Fig. 1). The reaction order with respect to DTNB was also one (Fig. 3).

The second-order rate constant at pH 8.0 and 20°C was about 2.0–2.5 $M^{-1} s^{-1}$. In comparison, the second-order rate constant for the reaction between reduced glutathione and DTNB, at the same pH and temperature, was four orders of magnitude larger ($4 \times 10^4 M^{-1} s^{-1}$).

The reactivities of exposed SH groups toward DTNB at pH 8.0 in the presence of several enzyme ligands are compared in Table II. None of the ligands tested influenced the kinetic pattern of thiol reactivity or the number of groups that reacted. When pseudo-first-order rate constants are compared, some inhibition of reactivity was observed in the presence of 20 mM GlcN6P or Fru6P; no effect was produced by ammonia or its dead-end analog methylammonium. The allosteric activator, GlcNAc6P, markedly diminished the availability of thiols to DTNB. The same effect was obtained with the competitive inhibitor, GlcN-ol-6P (Table II).

DISCUSSION

Sulfhydryl group analyses of glucosamine-6-phosphate isomerase deaminase reveal three of these groups per polypeptide chain, two exposed and reactive and a third unreactive one, which is either buried in the enzyme structure or involved in some kind of side-chain interactions that make it unavailable to the reagent. The simple

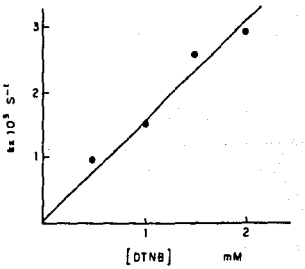

FIG. 3. Reaction order with respect to DTNB. Pseudo-first-order rate constants, represented on the vertical axis, were obtained under the same experimental conditions described for Fig. 2, except that the reagent concentration was varied.

TABLE II

EFFECT OF LIGANDS ON THE REACTIVITY OF THIOL GROUPS OF GLUCOSAMINE-6-PHOSPHATE ISOMERASE DEAMINASE[a]

Ligand concentration (mM)	2nd-order rate constant ($M^{-1} s^{-1}$)	Reactivity[b]
None	2.43	1
GlcNAc6P		
0.75	1.08	0.44
1.50	0.64	0.26
2.5	0.18	0.07
GlcN-ol-6P		
0.012	1.60	0.66
0.030	0.21	0.09
20	0	0
GlcN6P 15	2.07	0.85
Fru6P 15	1.80	0.74

[a] Conditions were the same as in Table I, except that DTNB was 2 mM. From the spectrophotometric determination of the reaction, the pseudo-first-order rate constant was fitted by linear regression of the semilogarithmic form of the first-order equation; the corresponding second-order constant was obtained dividing this result by the DTNB concentration.

[b] Calculated from each second-order rate constant relative to its value in absence of ligands.

pseudo-first-order kinetics of the reaction implies also that the exposed groups react at the same rate, indicating a similar local environment. A true first-order reaction (zero order for DTNB) was not found, as would be expected if there were a rate-limiting first-order step, such as the unfolding of protein structure. On the other hand, second-order kinetics is expected for groups exposed on the protein surface. Nevertheless, reactivity of these exposed groups toward DTNB is considerably lower than that of glutathione sulfhydryl, taken as a model of an interaction-free group. This finding shows that significant interactions also exist in which these thiols participate.

The reduction experiment of the urea-denatured enzyme with dithiothreitol reveals two additional sulfhydryl groups per enzyme subunit. This observation could be explained as the reduction of a preexisting disulfide linkage in each subunit. This result accounts for the five cysteic acid residues per oxidized polypeptide chain reported to be present (2), although disulfide-containing proteins are not generally produced by *E. coli* (12) because of its high intracellular reducing potential. We cannot exclude the oxidation of the enzyme during preparation or storage, or even the presence of the two thiols being blocked in some other way. Further experiments are necessary to explain this finding.

The exposed sulfhydryl groups were not modified by treatment of the enzyme with *m*-periodate under the same conditions that according to Rippa *et al.* (1) produce oxidation of vicinal thiols in different proteins. This result reveals a noticeable structural difference from enzymes studied by these authors.

Although the reaction of the 12 exposed sulfhydryls with DTNB completely inactivates the enzyme (Fig. 2), its alkylation with methyl iodide, a smaller and charge-free reagent, does not produce significant kinetic changes, except for a decrease in the turnover number. This could be due to side reactions of methyl iodide with other amino acid side chains. Alkylation experiments prove directly that SH groups are not involved in the catalysis or the allosteric control of the enzyme. Inhibition by DTNB or mercurials (3) can be a consequence of conformational distortion of protein structure by the introduction of bulky charged groups. This change can be only partially reversed by dithiothreitol.

Enzyme ligands decrease the reactivity toward DTNB of the 12 exposed sulfhydryl groups. The allosteric activator, GlcNAc6P, and the competitive inhibitor, GlcN-ol-6P, markedly reduce the reactivity of these SH groups and protect them entirely at high concentrations. The change of the rate constant produced by the allosteric effector parallels the effect of this ligand to reduce positive cooperativity for the reaction of GlcN6P deamination, [(2), Fig. 1]. GlcN-ol-6P, an analog of the open-chain form of the substrate (10) and able to occupy the entire active site, produces the same effect. The inhibited enzyme displays hyperbolic kinetics, as expected for an allosteric K-system (Hill coefficient is near 1 between 20 and 30 μM GlcN-ol-6P).

Fru6P and GlcN6P, both allosteric substrates (2), also produce some inhibition of SH reactivity at the concentrations tested. It should be taken into account that, under these conditions, saturation kinetics is not attained, because of the low apparent affinity of the deaminase for the sugar phosphates in the absence of the activator [(2), Fig. 2]. Ammonium, a substrate, and the inhibitor methylammonium do not modify the reactivity of the exposed thiols. Since the kinetic mechanism of the enzyme is still to be determined, this result may be explained in terms of an ordered sequential addition of substrates, but it is also interesting to note that ammonium is not an allosteric substrate for this enzyme (2).

The results presented, taken as a whole, rule out the possible participation of sulfhydryl groups in the active or allosteric site of the enzyme or their participation in interactions essential for the allosteric conformational change. A similar finding, the inhibition by DTNB and iodoacetamide, and the protection against these inhibitors provided by methylating the reactive surface sulfhydryls, has been described also for pig muscle 3-phosphoglycerate kinase by Dékány and Vas (13).

Reactivity of exposed sulfhydryls is notably modified by ligands that induce the allosteric conformational transition. This correlation suggests that the reactivity changes found are a simple consequence of a conformational change in which the thiols do not play an essential role.

ACKNOWLEDGMENTS

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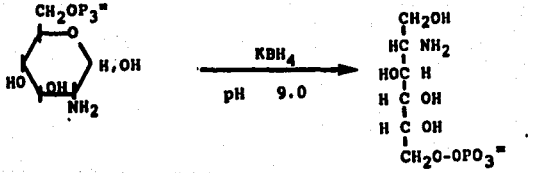
ANEXO I

En el artículo precedente, se ha utilizado el inhibidor sin salida, 2-desoxi-2-amino glucitol 6-fosfato, (Sorbitolamina 6 fosfato), como ligando homotrópico en los experimentos de protección. Este compuesto fue descrito por Midelfort y Rose,[39] cuando trataron con borohidruro de sodio una mezcla del complejo enzima-sustrato (desaminasa-GlcN6P). Estos autores observaron una inhibición total de la enzima, producida por un compuesto de bajo peso molecular, al que identificaron como la sorbitolamina 6 fosfato.

En nuestro trabajo requeríamos de un ligando de gran afinidad por el sitio activo, que fuese estable; un inhibidor sin salida (competitivo), parecía el ligando ideal. En consecuencia, sintetizamos la sorbitolamina 6-P, ya que no es un producto comercial. Hicimos a continuación la valoración de su pureza y su capacidad como inhibidor de la glucosamina 6 fosfato.

SINTESIS DEL GlcN-ol-6P

El procedimiento se basó en la reducción de la GlcN6P por el ión H^-, del borohidruro de sodio de acuerdo con la siguiente reacción:



GlcN6P GlcN-ol-6P

Procedimiento:

Se disuelve 1 g de GlcN6P, forma ácida, en 10 ml de agua. Se

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

agrega $NaHCO_3$ en polvo, hasta ajustar el a pH 9.0; posteriormente se agregan 5 mmoles de borohidruro de Na, previamente disuelto en KOH 0.02 M. La mezcla se ajusta a pH 9.0 y se deja reaccionar, tomando muestras para cromatografía en capa fina. Se deja la mezcla a temperatura ambiente de 4 a 6 h, y se destruye el borohidruro ajustando el pH momentáneamente a 1. La mezcla se pasa por una columna de Dowex-50, forma H^+, y posteriormente se eluye con acetato de amonio 0.02M. La sorbitolamina 6 fosfato sale de la columna en un único pico, las fracciones se liofilisan y el producto se conserva desecado a -20 C.

Valoración del Producto

Pureza:

Se verificó la pureza del compuesto obtenido por medio de cromatografía en capa fina sobre celulosa microcristalina en los siguientes sistemas: acetato de etilo:acido acético:agua:NH_3, 6:2:2:1 y en isopropanol:NH_3 6:1. Las placas se revelaron con ninhidrina, y con un procedimiento para ésteres fosfóricos con el reactivo de Hanes-Isherwood[40]. Se obtuvo un producto cromatográficamente puro, de acuerdo con estas condiciones de análisis. El compuesto en solución se detectó y se cuantificó determinando el fosfato orgánico.

Ensayo de la GlcN-ol-6-P como inhibidor de la desaminasa.

Se ensayó el compuesto en el sentido desaminante de la reacción. En la Fig. I-1 se muestra el patrón de inhibición de la glucosamina 6 fosfato desaminasa por esta poliolamina fosfato. El experimento se hizo en condiciones de cinética hiperbólica, en presencia del activador alostérico (GlcNAc6P). Se observa inhibición competitiva lineal , con una *Ki* del orden de 2.8 μM. En el sentido biosintético de

[40] Hanes, C., Isherwood, A (1949). Nature 164, 1107.

la reacción (no se muestra), también se obtiene inhibición competitiva, tanto con respecto a la Fru6P como al ión amonio. En ausencia del activador alostérico, en condiciones en las que se manifiesta la cinética intensamente cooperativa con respecto a la GlcN6P, se observa el comportamiento característico de un inhibidor competitivo en un sistema alostérico de tipo K: a bajas concentraciones de inhibidor y de sustrato se produce activación. (Fig. I-2). En la figura I-3 se muestran los resultados de una simulación con parámetros tomados de datos experimentales de nuestra ensima, en la que se observa esta activación paradójica. (Datos de Calcagno y col.1989).

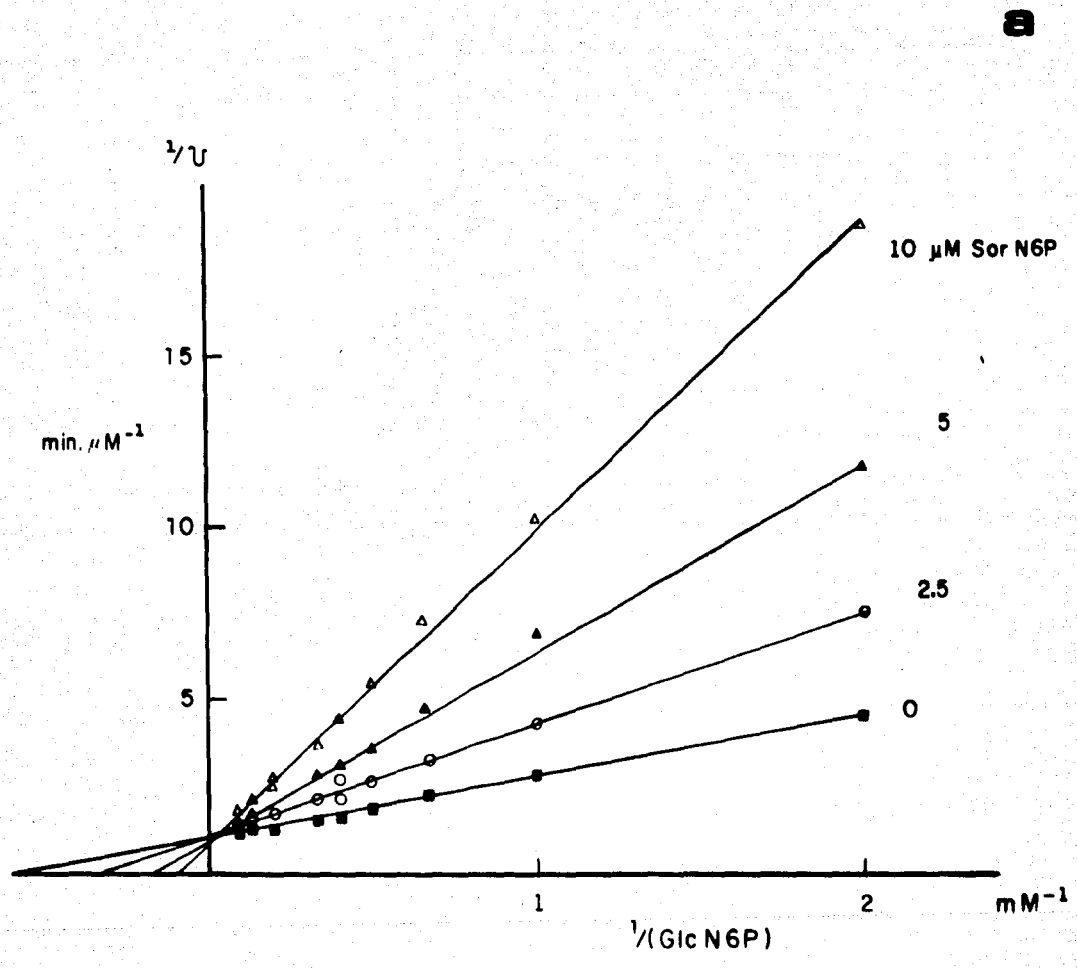


FIGURA I-1

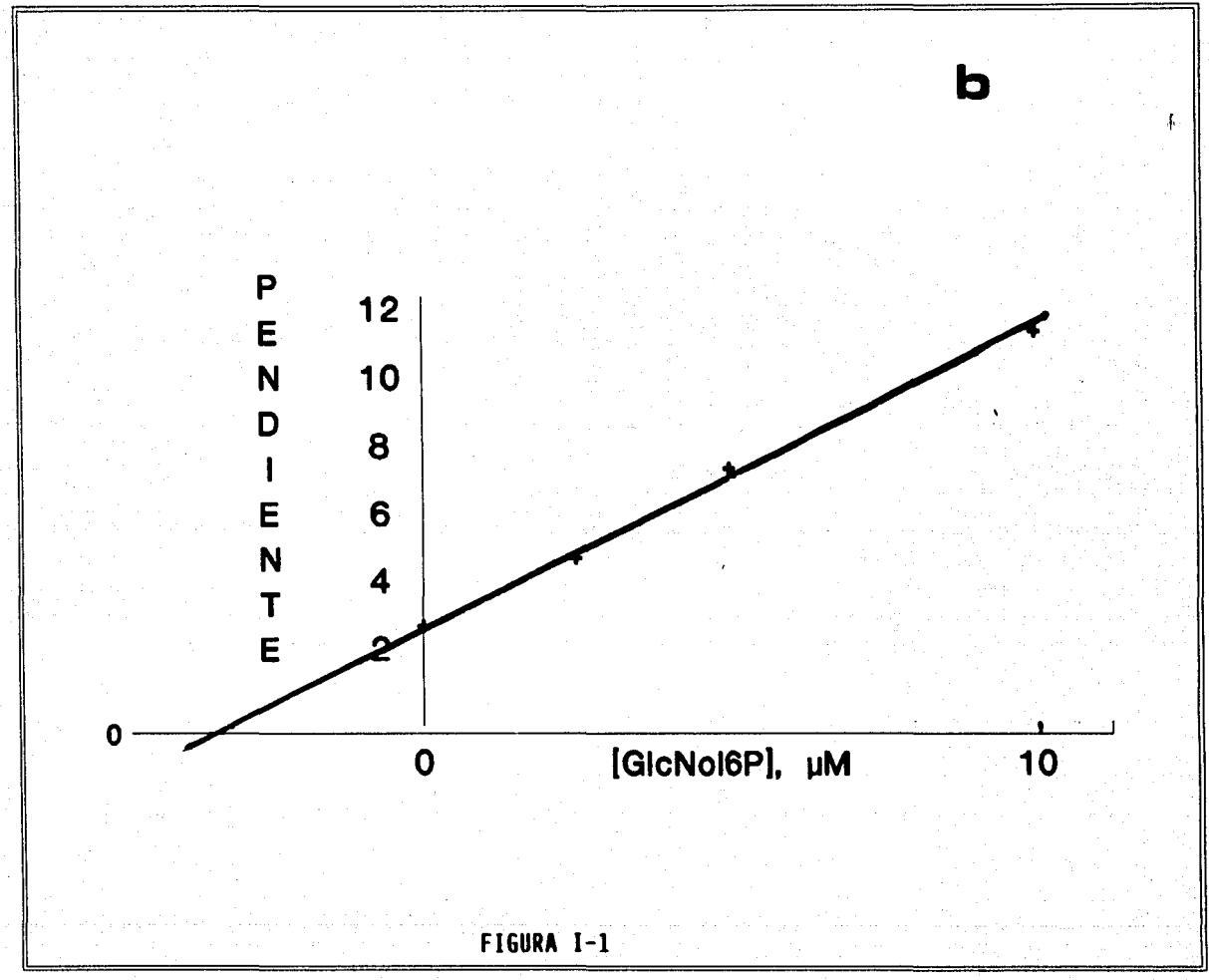
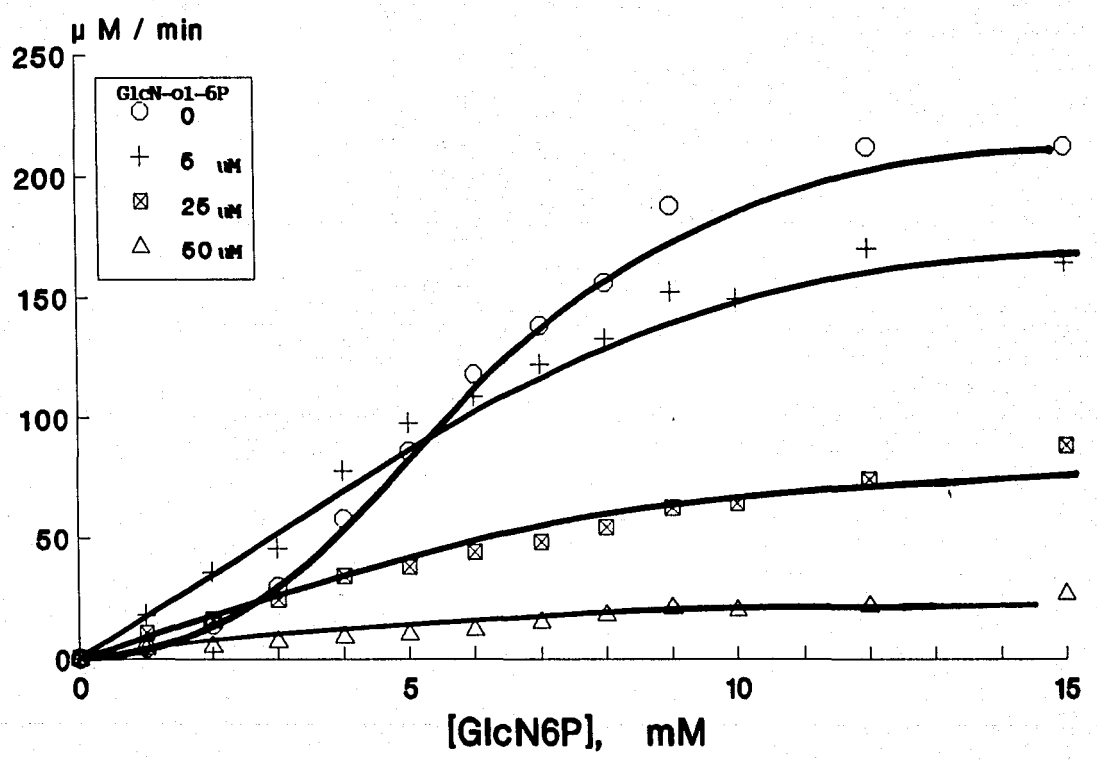


FIGURA I-1

Figura I-1.
Patrón de inhibición de la Glucosamina 6-P desaminasa por 2-desoxi 2-amino glucitol 6-P (SorN6P o GlcN-ol-6-P). La mezcla de reacción contenía una concentración saturante del activador alostérico, GlcNAc6P, en este caso, 1 mM. La concentración de la enzima fue 2 nM final en el ensayo.

Parte: **a**: presentación de los patrones de velocidad inicial en forma doble recíproca. Las rectas fueron ajustadas por separado, mediante el ajuste a la hipérbole, según Wilkinson (Véase la parte metodológica del artículo 1).
Parte **b**: regráfico de pendientes (Km/Vmax) en función de la concentración del inhibidor, con los datos del mismo experimento. Ajuste por mínimos cuadrados Se calcula una Ki de 2.8 uM.

FIGURA I-2


µ M / min
250
200
150
100
50
0
GlcN-o1-6P
0
6 uM
25 uM
50 uM
0
5
10
15
[GlcN6P], mM

$$v/V_{MAX.} = \frac{L\,c\alpha\,(1+c\alpha+d\theta)^{n-1} + \alpha\,(1+\alpha+\beta)^{n-1}}{L'\,(1+c\alpha+d\theta)^{n} + (1+\alpha+\beta)^{n}}$$

FIGURA I-3

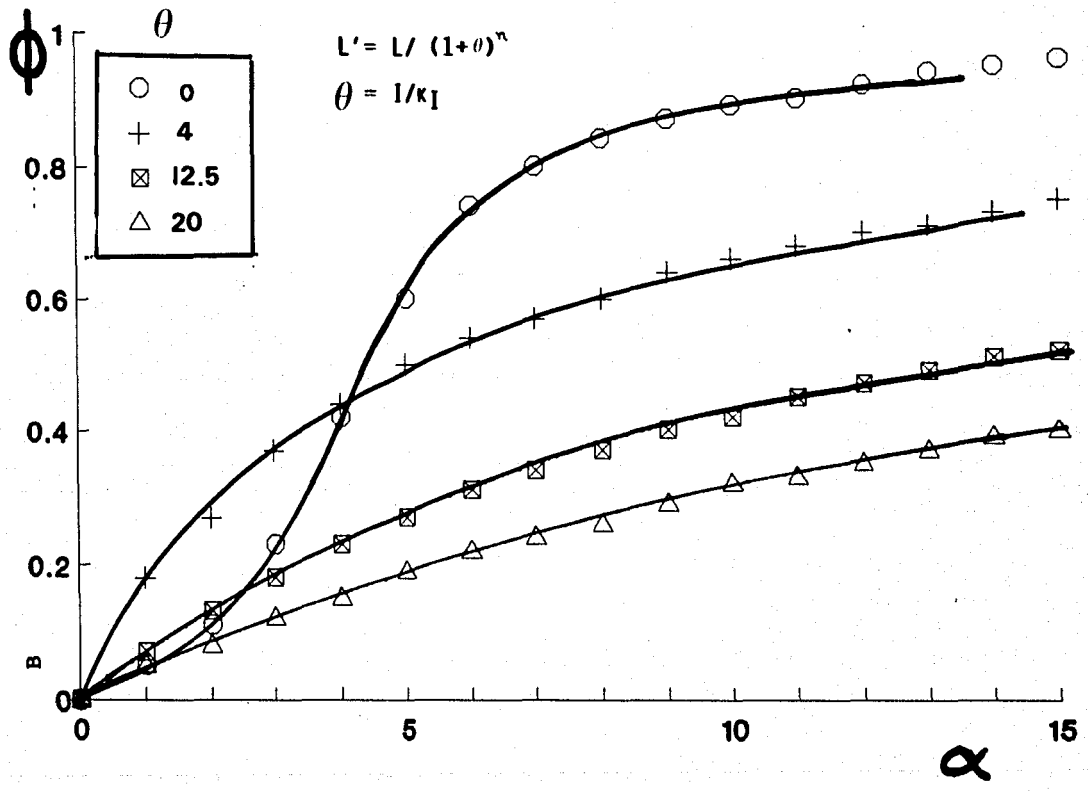


Figura I-2
Inhibición de la glucosamina-6-P desaminasa por GlcN-ol-6P, en ausencia del activador. Gráficas directas de velocidad inicial en función de la concentración de sustrato. A baja concentración del inhibidor, (teta = (I)/Ki = 4), y baja concentración de sustrato, se observa la activación paradójica producida por el efecto homotrópico.

Figura I-3
Simulación de la curva de velocidad/sustrato, según el modelo de Monod, Wyman y Changeux, para el caso general, (fijación no exclusiva). Se utilizaron los siguientes parámetros: n (sitios) , 6; L (constante alostérica) , 400, c = 0.03 (tomada de los valores de Kr = 2 mM y Kt = 65 mM).
Se incluye también una serie de curvas simuladas con la ecuación para inhibición competitiva dentro del modelo de fijación no exclusiva del inhibidor, suponiendo que la constante de fijación no exclusiva para el GlcN-ol-6P es igual a la constante c para el sustrato.
Compárese con las curvas experimentales de la figura I-2

ARTICULO II

Evidence for Vicinal Thiols and Their Functional Role in Glucosamine-6-phosphate Deaminase from *Escherichia coli*.

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Evidence for Vicinal Thiols and Their Functional Role in Glucosamine-6-phosphate Deaminase from *Escherichia coli*

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Methylation of glucosamine-6-phosphate isomerase deaminase (2-amino-2-deoxy-D-glucose-6-phosphate ketol-isomerase, deaminating, or glucosamine-6-phosphate deaminase, EC 5.3.1.10), from *Escherichia coli* produces a modified protein having two alkylated sulfhydryls per each polypeptide chain. The enzyme is still active and allosteric, but exhibits a lower homotropic cooperativity and its V_{max}/E_{total} is almost exactly half that of the native enzyme. Arsenite produces comparable kinetic changes that can be reversed with ethanedithiol but not with 2-thioethanol or dialysis. Thiols can be oxidized by molecular oxygen using the $(1,10\text{-phenanthroline})_3$-Cu(II) complex as catalyst; the enzyme obtained no longer has titrable SH groups with 5,5'-dithiobis(2-nitrobenzoic acid) and displays kinetic behavior similar to that of the other chemically modified forms of the deaminase using monofunctional or bifunctional reagents. The results reported indicate that the involved sulfhydryls are vicinal groups, and are located in a region of the molecule that moves as a whole in the allosteric transition.

Glucosamine-6-phosphate isomerase (deaminase) (2-amino-2-deoxy-D-glucose-6-phosphate ketol-isomerase, deaminating, or glucosamine-6-phosphate deaminase, EC 5.3.1.10) from *Escherichia coli*, which catalyzes the reversible conversion of GlcN6P[2] into Fru6P and ammonia, is a hexameric homopolymer that contains five cysteinyl residues per chain (1). In a previous publication (2) we have shown that the native enzyme has two titrable sulfhydryl groups in the absence of ligands, and three in the presence of denaturants. When the allosteric conformerization is induced by the activator, GlcNAc6P, or by homotropic ligands (the substrates Fru6P or GlcN6P and the dead-end inhibitor 2-amino-2-deoxy-D-glucitol-6-phosphate), the reactivity of these groups toward DTNB is practically abolished. Reduction in the presence of denaturants causes the appearance of five thiols per polypeptide chain, as expected according to amino acid analysis. The possible existence of disulfide bridges in this protein has already been discussed (2).

The enzyme fully modified in the reactive sulfhydryls by the introduction of the 5-thio-2-nitrobenzoate group or other bulky substituents becomes inactive, but the methylation of the same cysteinyl residues with methyl iodide produces an active modified enzyme that has a lower apparent catalytic constant (V_{max}/E_{total}) and is still susceptible to activation by GlcNAc6P (2). Thus, it is apparent that these two cysteinyl residues in each polypeptide chain change their environment remarkably as a

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[2] Abbreviations used: GlcN6P, D-glucosamine 6-phosphate; Fru6P, D-fructose 6-phosphate; GlcNAc6P, *N*-acetyl-D-glucosamine 6-phosphate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); SDS, sodium dodecyl sulfate.

0003-9861/89 $3.00

consequence of the allosteric transition, but they would not seem to have an essential role in this conformational change or in the catalytic cycle of the enzyme.

The present study describes the kinetics of the methylated deaminase and the changes produced by arsenite and oxidation, in an attempt to further characterize the functional role of these two reactive sulfhydryls present in each subunit and their relation to the catalytic and allosteric properties of the enzyme.

MATERIALS AND METHODS

Materials. GlcNAc6P was synthesized from GlcN6P by the procedure of Leloir and Cardini (3); other biochemicals were products from Sigma Chemical Company (St. Louis, MO). Chemicals were reagent-grade products.

Enzyme. Glucosamine-6-phosphate isomerase deaminase from *E. coli* B (strain ATCC 11303) was purified, stored, and assayed as described by Calcagno *et al.* (1), except that 5 mM disodium EDTA was added to the assay mixture. The enzyme used was electrophoretically homogeneous and its molecular activity for the forward direction of the reaction was in the range 1740–1770 s^{-1} at 30°C and pH 7.7; its concentration was calculated from the molar absorptivity (ϵ_{278} = 20.0×10^4 M^{-1} cm^{-1} (2).

Preparation of the S-methyl derivative of glucosamine-6-phosphate deaminase. Reaction with methyl iodide was performed at 20°C in a closed vial containing 400 μl of a reaction mixture composed of 50 mM Tris-HCl buffer, pH 7.0, 5 mM EDTA, 50 mM methyl iodide, and 1.4 μM glucosamine-6-phosphate deaminase. Aliquots were taken periodically for sulfhydryl assay with the DTNB-SDS reagent, as previously described (2). SDS was used because the reaction is practically instantaneous with denatured enzyme, making the time course of the methylation reaction easier to follow. Due to the presence of a third cysteinyl residue that reacts only in the presence of the denaturant, 18 thiols per molecule were titrated at zero time and six at the end of the reaction. The time course of this reaction is shown in Fig. 1. According to these data, the fully methylated enzyme was prepared by incubating the reaction mixture for 240 min, i.e., five half-lives of the reaction; afterward, it was dialyzed against 500 vol of 50 mM Tris-HCl buffer (pH 8.0) containing 2.5 mM EDTA and 5 mM dithiothreitol for 8 h and, finally, dialyzed in 50% glycerol containing the same Tris-HCl buffer and stored at −20°C. This modified enzyme, when assayed without denaturants, does not have thiols titrable with DTNB.

Reaction rate measurements. Enzyme assays were made in the forward (deaminating) direction of the reaction, by means of a stop-time colorimetric determination of Fru6P with the resorcinol-hydrochloric acid reaction (4).

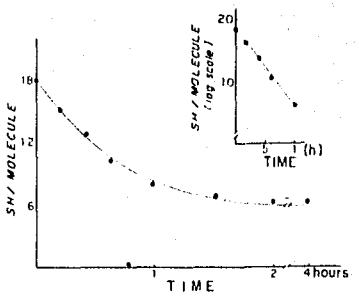

FIG. 1. Kinetics of methylation of native glucosamine-6-phosphate deaminase. Reaction conditions are described under Materials and Methods. Aliquots (100 μl) were taken at different times to determine total SH groups with 1 mM DTNB in the presence of 0.5% SDS, to make the reaction instantaneous. Observe that one additional thiol group per chain (six per molecule) is titrated, because SDS was used in the reaction mixture (see the text). Inset: semilogarithmic plot of the same data that fits to pseudo-first-order kinetics. The apparent first-order rate constant was 2.4×10^{-4} (half-life, 48 min).

Data analysis. Kinetic data obtained in the presence of an excess of the allosteric activator, GlcNAc6P, were adjusted to the Michaelis-Henri equation according to Wilkinson (5). Under homotropic cooperative kinetics the values for V_{max} were estimated using the iterative procedure of Atkins (6) or by hyperbolic fit of a simultaneous series run in the presence of saturating concentrations of GlcNAc6P. Both sets of data are usually similar, as expected for an allosteric K-system (7).

RESULTS

Under the conditions used, it was possible to methylate stoichiometrically the two available sulfhydryls in each polypeptide chain. Completeness of the reaction was verified by the absence of titrable sulfhydryls with DTNB in the undenatured enzyme.

Kinetics of the S-modified enzymes. Kinetic studies of the methylated enzyme in

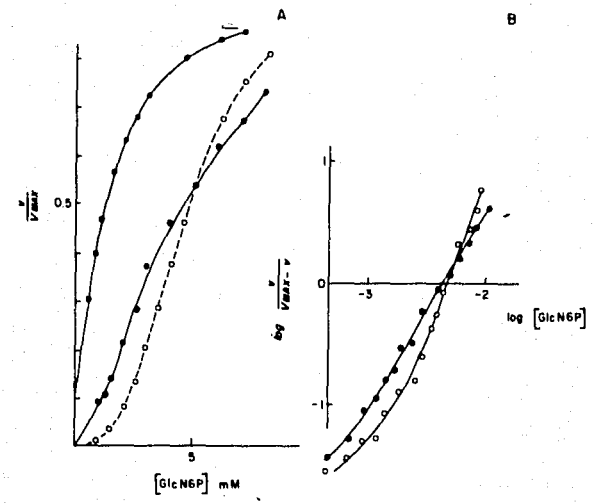

FIG. 2. Kinetics of glucosamine-6-phosphate deaminase modified by methylation. Experimental conditions are described under Materials and Methods. Data plotted are averages from two separate experimental series. (A) Squares: in the presence of 2.0 mM GlcNAc6P (K_m 1.63 mM); full circles: in the absence of the allosteric activator; open circles: data from a similar series using native enzyme are included for comparison. (B) Hill plots of data from A; the same symbols are used. Hill coefficients are 3.2 and 1.67 for the native and the methylated enzyme, respectively.

the presence of saturating concentrations of the allosteric activator (Fig. 2A) gave good hyperbolic fits. The corresponding K_m values for GlcN6P were similar for the native and the methylated enzyme; the same approximate values for $S_{0.5}$ (GlcN6P concentration that gives $v_0/V_{max} = 0.5$) were also found in absence of the activator. The ratio V_{max}/E_{total} appeared multiplied by a factor that is almost equal to 0.50 in all experiments (Table I). Homotropic cooperativity was remarkably lower for methylated enzyme, as can be seen from the corresponding maximal Hill coefficient, h_{max}, obtained from the slope of Hill plots in its central linear portion (Fig. 2B, Table I). As the methylated enzyme is still homotropic, it is able to be activated by GlcNAc6P (Fig. 2A).

Effect of arsenite. The enzyme was modified with sodium arsenite, taking into account that it may react as a bifunctional reagent with high affinity toward close pairs of sulfhydryls (8-12). Sodium arsenite was added to a diluted enzyme solution (0.1 μM) in 50 mM Tris-HCl (pH 7.8) to yield a final concentration of 1 mM. Kinetics of the arsenite reaction was followed by determining periodically the V_{max} in the presence of 2.0 mM GlcNAc6P (Fig. 3A). The activator serves also to stop the reaction, because the active form of the enzyme does not have reactive sulfhydryls and the allosteric activation occurs instantaneously for the time scale of the experiment (2). The reaction gave pseudo-first-order kinetics and its half-life under the conditions described was 195 min. The arsenite complex of the enzyme was generally prepared by incubating a similar reaction mixture for 18 h at 20°C.

TABLE I

	V_{max}/E_{total} (s^{-1})	f^a	K_m (mM)	h_{max}	$s_{0.5}$
Native enzyme[b]	1739 ± 87	1.00	2.05 ± 0.14	3.00 ± 0.02	4.83 ± 0.40
Methyl-enzyme[b]	887 ± 32	0.51	1.63 ± 0.19	1.73 ± 0.10	4.59 ± 0.35
Arsenite-enzyme[b]	904 ± 39	0.52	1.63 ± 0.21	1.67 ± 0.12	5.60 ± 0.25
Arsenite-enzyme + ethanedithiol[c]	1739	1.00	1.73	3.07	4.80
Native enzyme + $(Ph)_3Cu(II)$[d]	956	0.55	1.60	1.50	8.50

[a] Ratio between V_{max}/E_{total} values of native and modified enzyme. Data from experiments in the presence of 2 mM GlcNAc6P.

[b] Data from four series of experiments. Details are explained in the text.

[c] Enzyme was treated with 1 mM sodium arsenite as described in Fig. 3A for 24 h and then incubated with 2 mM 1,2-ethanedithiol for 24 h before assay. Average of two experiments.

[d] Enzyme treated with (1,10-phenanthroline)$_3$-Cu(II) complex as catalyst of the sulfhydryl oxidation by molecular oxygen.

Arsenite-treated deaminase displayed noticeable kinetic similarities with the methylated enzyme. The same change in V_{max}/K_m was found, whereas K_m for GlcN6P were almost the same as those obtained with native or methylated enzymes (Table I). Cooperativity with respect to GlcN6P concentration also diminished in a similar fashion, and the enzyme was capable of being allosterically activated; $S_{0.5}$ values are not significantly different in sulfhydryl-modified enzymes (Fig. 4, Table I). Methylated enzyme was not further modified by preincubation with 1 mM sodium arsenite, and the arsenite-treated enzyme no longer had available SH groups when assayed with DTNB (not shown). The kinetic changes produced by arsenite remained unchanged after exhaustive dialysis against a 50 mM Tris-HCl buffer, pH 8.0.

Attempts to remove arsenite were made using a monothiol (2-thioethanol) and a dithiol (1,2-ethanedithiol). Only the dithiol was able to reverse the effects of arsenite, as shown in Fig. 3B. The enzyme treated with 5 mM ethanedithiol for 8 h displayed the usual homotropic kinetics of the native enzyme.

Effect of 1,10-phenanthroline-copper(II) complex. Attempts were made to oxidize the reactive sulfhydryl groups to disulfide bridges, using the (1,10-phenanthroline)$_3$-copper(II) complex. This reagent is an efficient catalyst for thiol oxidation by molecular oxygen (11). An enzyme solution, diluted sufficiently to avoid polymerization (0.2 μM enzyme in 50 mM Tris-HCl buffer, pH 8.0, at 20°C), was incubated for 12 h at room temperature with a 100 μM cupric complex. After exhaustive dialysis against 50 mM Tris-HCl buffer (pH 8.0) containing 5 mM EDTA, an aliquot was taken for kinetic experiments, and the remnant was assayed for titrable sulfhydryls with DTNB. As shown in Table I, the oxidized deaminase behaves as the other two sulfhydryl-modified forms. Furthermore, practically no reactive thiols were detected in the oxidized enzyme (less than 0.2 thiol per polypeptide chain).

DISCUSSION

Methyl iodide was used to methylate the two reactive cysteinyl residues present in each enzyme subunit. Conditions improved compared to those previously described (2) were used to minimize undesirable reactions. Alkylation was performed for a controlled time and at pH 7.0, and the product was treated with 5 mM dithiothreitol to reconstitute possible alkylated methionines. Side reactions, on the other hand, are im-

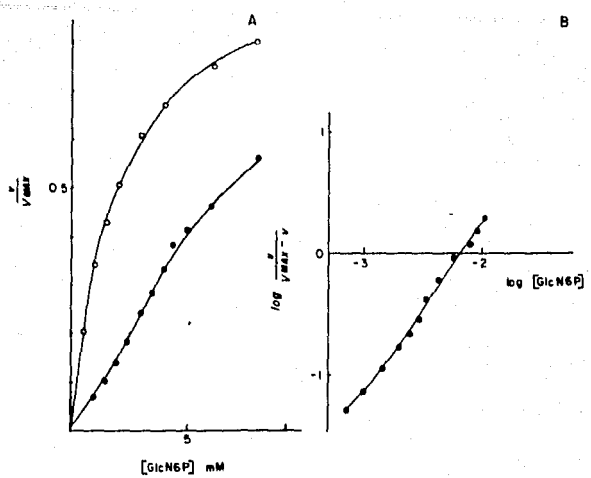

FIG. 3. Kinetics of arsenite-treated glucosamine-6-phosphate deaminase. Experimental conditions are described under Materials and Methods; plotted data are averages from three separate experimental series. (A) Open circles: assays in the presence of 2.0 mM GlcNAc6P (K_a 1.63 mM); full circles: in the absence of the allosteric activator. (B) Hill plot of data from A; Hill coefficient is 1.66.

probable in arsenite experiments as trivalent arsenicals are highly specific sulfhydryl reagents, and there are no other protein groups able to react with them (12). Sodium arsenite can react as a bifunctional reagent, giving stable cyclic dithioarsinite derivatives with vicinal sulfhydryls (8-12).

Kinetics of methylated, arsenite-bound or oxidized enzyme are quite similar (Table I); the ratio V_{max}/E_{total}, that is, the k_{cat} multiplied by the number of active sites, changes to almost half of its value for the native enzyme, and homotropic cooperativity is noticeably reduced. Cooperativity of all modified enzymes was in the h_{max} range 1.6-1.7, and an appreciable degree of activation by GlcNAc6P was still observed (Figs. 2 and 4).

The observed change in V_{max}/E_{total} for the modified enzymes may be a consequence of the formation of a catalytically less efficient central complex because of a conformational distortion produced by sulfhydryl modification. Change in cooperativity may be explained by a modification of the free energy of interaction between oligomers. Data in Fig. 2 and 4 allow the estimate of the intrinsic constant for the first binding site (K_1) from the abscissa intercept of the initial asymptote of the Hill plots. This value is near 22 mM for the native enzyme and 12 mM for the methylated enzyme. The constants for the nth site (K_n) can be taken as the K_m values determined at saturating concentration of the allosteric activator (Table I). The use in our case of constants obtained kinetically as equilibrium constants is an approximation, because we do not have evidence in favor of a rapid equilibrium model for this enzyme.

According to Wyman (13), the free energy of intersubunit interaction can be calculated from the equation

$$\Delta G_i = -RT \ln(K_n/K_1).$$

At 30°C, these values are −5.8 and −5.1 kJ mol^{-1} for native and modified enzymes, re-

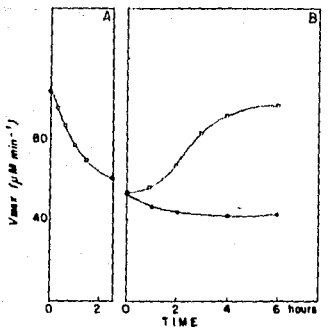

FIG. 4. (A) Time course of arsenite modification of glucosamine-6-phosphate deaminase, evaluated by means of the V_{max}: 1500 μl of a solution containing 0.1 μM glucosamine-6-phosphate deaminase, 1 mM sodium arsenite, 20 mM Tris-HCl buffer (pH 8.0, 20°C), and 5 mM EDTA was incubated at room temperature; 100-μl aliquots were taken at different times. Enzyme assays (colorimetric) were run in the presence of 2.0 mM GlcNAc6P; each point was obtained from a series of five different GlcN6P concentrations. The reaction follows pseudo-first-order kinetics, with a rate constant equal to 5.8×10^{-5} (half-life, 195 min). (B) Treatment of the arsenite-enzyme with 2 mM 2-thioethanol (full circles) and 2 mM ethanedithiol (open circles). The enzyme was incubated with arsenite under the same conditions described in A for 18 h, in two samples of 1.0 ml. Then, the monothiol was added to one and the dithiol to the other, to a final concentration of 2 mM. Aliquots for enzyme assay were taken at different times.

spectively. This change accounts for the different cooperativity of the modified enzymes and suggests that the involved cysteinyl residues may do some contribution in the stabilization of the allosteric conformers.

Another possible explanation that takes into account the kinetic changes found and the observation that the V_{max}/E_{total} is exactly halved by sulfhydryl modification is that half of the active sites of the enzyme were blocked. If this were the case, it would be necessary to assume that the chemical change does not modify the intersubunit interactions, and that two sets of structurally different but functionally equivalent sites exist, one of them containing the involved thiols; the lower homotropic cooperativity would then be a consequence of the change in the number of active sites. The phenomenon of half-site reactivity in chemical modifications has been described for several enzymes (14, 15). Further experiments will be needed to clarify these aspects, particularly the direct determination of the number of active sites using ligand-binding techniques.

Glucosamine-6-phosphate deaminase forms an arsenite complex that remains stable after exhaustive dialysis or treatment with a monothiol reagent, but its effects are reversed by ethanedithiol (Fig. 3B), as expected if a dithioarsinite cycle were formed with a pair of vicinal sulfhydryls (8-12); furthermore, the vicinal condition of these groups was confirmed by oxidation. Allosteric transition is not hindered when two cysteinyl residues per chain become crosslinked by combination with trivalent arsenic or by the formation of a disulfide bond. This behavior suggests that the protein region containing the vicinal thiols moves in the allosteric conformerization, keeping the same mutual geometrical relationships.

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ANEXO II

En el artículo precedente se analizó la cooperatividad homotrópica con respecto a la GlcN6P mediante el gráfico de Hill. Este método ha sido discutido teóricamente por Jeffries Wyman [41], quien demostró el cálculo de las constantes extremas, y del cambio de energía libre asociado a la transición alostérica. En el léxico del modelo concertado de Monod, Wyman y Changeux [42], las constantes extremas son Kr y Kt, y en un sistema K con un activador de fijación exclusiva, Kr es igual a la Km en presencia de concentraciones saturantes de activador, (suponiendo un mecanismo cinético de equilibrio rápido al azar). A su vez, el G de la transición, está relacionado con la constante alostérica L, en este modelo.

Hemos recurrido muchas veces a la nomenclatura del modelo de Monod y col., pero no mencionamos en ningún artículo si nuestros datos se ajustan a este modelo, o a modelos más generales, como es el caso de los diferentes modelos secuenciales. De los datos que se muestran en la Fig. II-1, se puede obtener un Kt = 65 mM; el Kr (igual al Km) es de 2.0 mM. Con estos datos se puede ajustar la ecuación de Monod para el caso más general (fijación no exclusiva del sustrato). Se utilizó la siguiente linearización de la ecuación, y se llevó a cabo un ajuste por mínimos cuadrados:

$$\text{Log}\frac{Y\,(1+\alpha)-\alpha}{c\alpha - Y(1+c\alpha)} = \text{Log } L + (n-1)\ \text{Log}\ \frac{1+c\alpha}{1+\alpha}$$

Se obtuvieron los valores de L y n, la constante alostérica de Monod, y el número de sitios. Es interesante destacar que los valores de *n* fueron, en todos los casos aproximables a 6, que es lo esperado en el caso de un homopolímero hexamérico, ya que correspondería a un

[41] Wyman J. (1964), Adv. Prot. Chem. 19:223-286.

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sitio activo por cadena polipéptidica. Sin embargo cuando se simula un experimento real y se superponen las curvas de velocidad específica contra concentración específica de sustrato, se observa que a pesar de tener un coeficiente de correlación de 0.964, la superposición no es completa, sugiriendo que el ajuste a este modelo no es más que una aproximación.

Por otra parte, el ajuste de los datos de las curvas de velocidad inicial contra concentración de GlcN6P, para las enzimas modificadas (tanto metilada, como arsenilada), da valores de 3 para el número de sitios. Si bien esto podría interpretarse en términos de una cinética de medios sitios, esto no es más que una posibilidad, que debe verificarse por titulación directa de los sitios activos.

FIGURA II-1

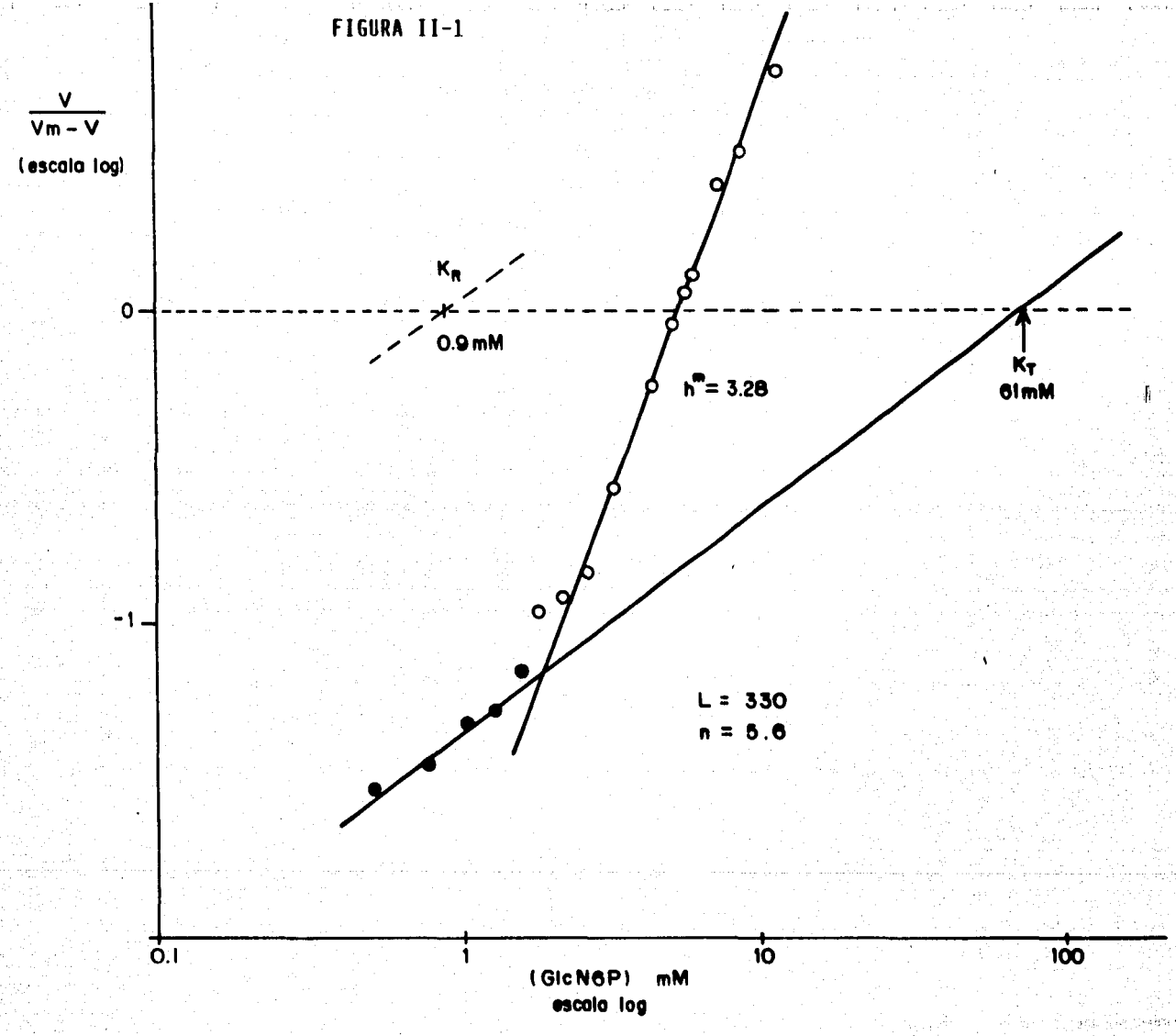
V / Vm - V
(escala log)
0
-1
K_R
0.9 mM
h^m = 3.28
K_T
61 mM
L = 330
n = 5.6
0.1
1
10
100
(GlcN6P) mM
escala log

Figura II-1

Gráfica de Hill correspondiente a los valores de velocidad inicial en función de la concentración de GlcN6P, en ausencia de activador. Se observa la intensa cooperatividad homotrópica, que para este conjunto de datos, da un coeficiente de Hill de 3.28. El ajuste a la ecuación de Monod, Wyman, y Changeux para el caso de la fijación no exclusiva, con los valores de Kt y Vmax tomados de este experimento, y haciendo Km = Kr, puesto que se atrata de un sistema K puro, obtenemos un ajuste con una correlación de 0.97, y los valores de n = 5.6 (próximo entero, 6), y L 330.

ARTICULO III

Zinc binding and its trapping by allosteric transition in glucosamine-6-phosphate deaminase from *Escherichia coli*.

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Zinc binding and its trapping by allosteric transition in glucosamine-6-phosphate deaminase from *Escherichia coli*

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Key words: Glucosamine-6-phosphate deaminase; Vicinal sulfhydryl; Sulfhydryl modification; Zinc binding

Glucosamine-6-phosphate isomerase deaminase from *Escherichia coli*, a typical allosteric enzyme, becomes less cooperative and 50% inhibited when treated with zinc. This metal cation behaving as a tight-bound and slow partial inhibitor. Modification of a pair of vicinal reactive thiols with some sulfhydryl reagents mimics this effect. On the other hand, sulfhydryl reactivity disappears in the presence of saturating concentrations of Zn^{2+}, which does not modify the kinetics of *S*-methylated enzyme, a finding that indicates that vicinal thiols are an essential part of the zinc-binding site. Allosteric activation of the deaminase causes trapping of the metal, which cannot be released by dialysis against a buffer containing EDTA. Cadmium and nickel(II) cations also produce a similar effect.

Introduction

Glucosamine-6-phosphate isomerase deaminase (D-glucose-6-phosphate ketol-isomerase (deaminating), EC 5.3.1.10) from *Escherichia coli* is an allosteric enzyme of the K-type that is activated by *N*-acetyl-D-glucosamine 6-phosphate (GlcNAc6P) [1,2]. The enzyme, an hexameric homopolymer, has two vicinal sulfhydryl groups per polypeptide chain that are titrable in the absence of ligands, but become completely hindered in the presence of active- or allosteric-site ligands [2]. These reactive thiols are not essential for full activity or allosteric activation of the enzyme. Indeed, when these thiols are blocked with small and uncharged substituents the enzyme presents a reduced cooperativity and its V_{max}/E_t becomes 50% of the value for the native enzyme [3].

To study the possible role of these reactive sulfhydryls in the regulation of the deaminase activity, we investigated its ability to bind divalent cations, mainly Zn^{2+}, which could be a natural ligand of the enzyme.

Materials and Methods

Reagents

GlcNAc6P was prepared by acetylation of D-glucosamine 6-phosphate (GlcN6P) and purified by ion-exchange chromatography, according to Leloir and Cardini [4]. Other biochemicals were products from Sigma Chemical Co. (St. Louis, MO). Chemicals were reagent-grade products.

Enzyme

Glucosamine-6-phosphate isomerase deaminase from *E. coli* (strain ATCC 11303) was purified, stored and assayed as previously described [1]. The enzyme was electrophoretically homogeneous and its molecular activity for the forward (deaminating) reaction was in the range of 1700–1770 s^{-1} at 30°C (pH 7.7); its concentration was calculated from the molar absorptivity $\epsilon_{278} = 20.0 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ [2].

Enzyme methylated in its reactive vicinal sulfhydryls was prepared as described, following the reaction course by 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) titration [3].

Kinetic data analysis

Data corresponding to hyperbolic kinetics were fitted by the procedure of Wilkinson [5]. Under homotropic cooperative kinetics, V_{max} and $S_{0.5}$ were obtained according to Atkins [6], and the Hill coefficient was calculated by least-squares fit, from the slope of the central linear portion of the Hill plot.

Abbreviations: E_t, total molar concentration of the enzyme; GlcN6P, D-glucosamine 6-phosphate; GlcNAc6P, *N*-acetyl-D-glucosamine 6-phosphate; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid).

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Results and Discussion

Kinetic changes produced by Zn^{2+}

Treatment of *E. coli* glucosamine-6-phosphate deaminase with 1 μM zinc acetate for 2 h produced the kinetic modifications shown in Table I. The V_{max}/E_t ratio was decreased to one half of the untreated enzyme and its cooperativity towards GlcN6P was reduced, as can be seen from the Hill coefficient. This zinc-enzyme is also 50% inhibited when assayed in the presence of saturating concentrations of the allosteric activator, GlcNAc6P, and displays hyperbolic kinetics, as expected. The same kinetic changes have been described for the deaminase having its vicinal sulfhydryls blocked by methylation, arsenite-binding or oxidation to disulfide [3]. On the other hand, zinc did not affect the *S*-methylated enzyme (Table I), whereas the zinc-saturated enzyme had no titrable sulfhydryls with DTNB (data not shown). Zinc treatment had no effect upon the enzyme in the presence of 1 mM GlcNAc6P (Table I). These findings, taken as a whole, indicate that sulfur atoms from vicinal cysteine residues are essential for this effect of zinc, although nitrogen or oxygen atoms from other amino acid side-chains may also be part of the zinc-binding site. Data in Table I also show that unspecific zinc binding, if significant at the concentration used in these experiments, had no kinetic consequences and did not affect the reported results.

TABLE I

Kinetic changes of ligand-free or modified E. coli glucosamine-6-phosphate deaminase produced by Zn^{2+} [a]

	V_{max}/E_t (s^{-1})	Partial inhibition [b] coefficient	h_{max} [c]
Untreated enzyme	1740 ± 80	–	3.00 ± 0.09
Zinc-enzyme [d]	890 ± 65	0.50 ± 0.01	1.60 ± 0.08
Untreated enzyme [e] assayed + GlcNAc6P	1700 ± 90	–	1.05 ± 0.06
Zinc-enzyme [f] assayed + GlcNAc6P	895 ± 60	0.51 ± 0.01	1.00 ± 0.07
GlcNAc6P-enzyme [g] + zinc	1730 ± 75	–	1.00 ± 0.09
Methylated enzyme [h]	880 ± 35	0.51 ± 0.02	1.73 ± 0.12
Methylated enzyme3 [i] + zinc	885 ± 40	0.51 ± 0.01	1.70 ± 0.15
Zinc-enzyme, dialyzed [j] against EDTA	1690 ± 95	–	2.91 ± 0.17

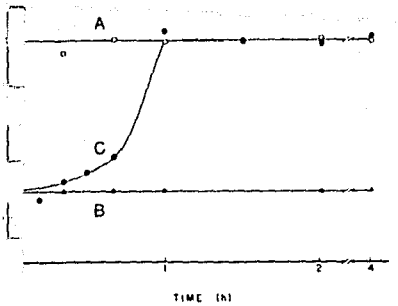
[a] Data are from at least four experiments.
[b] Calculated as the ratio of V_{max}/E_t for fully inhibited and native enzyme.
[c] Obtained from the slope of the central linear portion of the Hill plot.
[d] A 20 nM enzyme solution in 50 mM Tris-HCl buffer (pH 8.0) was incubated for 2 h with a 1 μM zinc acetate. According to data in Fig. 1, the incubation time corresponds to 10-times the half-life of the zinc-binding reaction. The enzyme was then assayed in the absence of the activator.
[e] Assayed in the presence of 1 mM GlcNAc6P.
[f] A zinc-enzyme sample prepared as in d, was assayed in the presence of 1 mM GlcNAc6P. Addition of 2.5 mM EDTA in an assay mixture, already containing the allosteric activator, does not modify this result.
[g] To a 20 nM enzyme solution in the same buffer as in d, GlcNAc6P was added to obtain a final concentration of 1 mM. Then, zinc acetate was added to a final concentration of 1 μM and left to stand for 2 h before assaying it.
[h] Prepared as described in a previous paper [4].
[i] The same methylated enzyme used in h was treated with Zn^{2+} as explained in d.
[j] The Zn-enzyme prepared as explained in d, was dialyzed twice at 4°C for 4-h periods against 500 vol. of 50 mM Tris-HCl buffer (pH 8.0 at 20°C) containing 5 mM disodium EDTA.

Reactivation by EDTA

The zinc-treated enzyme remained inhibited after exhaustive dialysis against 50 mM Tris-HCl buffer (pH 8.0 at 20°C). Full reactivation of the enzyme was obtained in the presence of 5 mM disodium EDTA, in the same Tris-HCl buffer. Kinetics of the reactivation can be followed by means of the change in fractional V_{max}, calculated as $(V_{max-I} - \beta V_{max-C})/\beta V_{max-C}$, where V_{max-I} and V_{max-C} are the inhibited and non-inhibited (control) maximal velocities, respectively, and β is the partial inhibition coefficient (in our case, $\beta = 0.50$, Table I). The convenience of this kind of plot of fractional velocities in the analysis of partial inhibitors, has been shown by Yoshino [7]. Reactivation under the indicated conditions occurs with first-order kinetics ($k = 2.6 \cdot 10^{-4}$ s^{-1}); this reaction rate was independent of EDTA concentration in the range of 5–50 mM.

Trapping of Zn^{2+} by allosteric transition

The presence of the allosteric activator completely prevents the reactivation of the enzyme by EDTA; this is shown in the set of experiments depicted in Fig. 1. Samples containing equal enzyme concentrations were incubated and then dialyzed at 20°C under different conditions. Aliquots were then taken from the dialysis bag, which was attached to a plastic tube to allow easy removal of the samples, at various times, and enzyme activity was determined as V_{max}. Enzyme from curve A (Fig. 1) was saturated with 1 mM GlcNAc6P; after 1 min it was added to zinc acetate to obtain a final concentration of 1 μM, then left to stand for 2 h and dialyzed against 250 vol of 50 mM Tris-HCl buffer (pH 8.0) containing 1 μM Zn^{2+} and 1 mM GlcNAc6P. Under these conditions, zinc did not inhibit the enzyme. Curve B represents an experiment in which the enzyme had been exposed to the same ligands as in A, but in the



[illegible]me-course of the modification of glucosamine-6-phosphate
[illegible] activity evaluated as the V_{max} when the enzyme was
[illegible] and then dialyzed under different conditions. All experi-
[illegible]ere carried out at 20 °C, with a 50 nM enzyme solution in 50
[illegible]-HCl buffer (pH 8.0 at 20 °C). As described in the text,
[illegible]nples were incubated with two ligands, the allosteric activa-
[illegible].c6P (1 mM, 1 min), and Zn^{2+} (1 μM zinc acetate for 2 h,
[illegible]times the half-life of the reaction). Samples were then dialyzed
[illegible]50 mM Tris-HCl buffers with different additions. (A) ○,
[illegible]s first incubated with GlcNAc6P, then with Zn^{2+}. Buffer
[illegible]ntained 1 mM GlcNAc6P and 1 μM zinc acetate. (B) ▲,
[illegible] was saturated with Zn^{2+}, then activated with GlcNAc6P.
[illegible]buffer contained 1 mM GlcNAc6P and 5 mM EDTA. (C) ●,
[illegible]s treated as in (B), but the dialysis buffer contained 5 mM
[illegible]r the three experiments, samples were taken for assay at the
[illegible]d times, in the presence of 1 mM GlcNAc6P, to obtain the
[illegible]hyperbolic kinetics (see more details in the text).

[illegible]: order: first it was saturated with Zn^{2+} (1 μM
[illegible]tate for 2 h at 20 °C), then GlcNAc6P was
[illegible]) obtain a final concentration of 1 mM, and
the mixture was dialyzed against a similar Tris-
[illegible]uffer containing 1 mM GlcNAc6P and 5 mM
[illegible]. As shown in the figure, the enzyme presented
[illegible]nibition and was not reactivated by EDTA up to
period. Curve C corresponds to an experiment in
[illegible]e enzyme sample was prepared as in B, but
[illegible] against 50 mM Tris-HCl (pH 8.0) containing 5
EDTA. In this case, the lose of the allosteric
[illegible] produced the complete reactivation of the
[illegible] in 1 h. These results provide evidence that
und sulfhydryls change their environment as a
[illegible]uence of GlcNAc6P binding, and that the allos-
[illegible]vation of the zinc-bound enzyme causes seques-
[illegible] of the metal by the protein.

[illegible]*rse of the modification by* Zn^{2+}

[illegible] binds slowly to the enzyme; the time-course of
[illegible]g can be followed by the change in the fractional
[illegible]; shown in Fig. 2. Enzyme samples were in-
[illegible] with the indicated Zn^{2+} concentrations for
[illegible]nt times, then binding was stopped by adding
[illegible]Ac6P to obtain a final concentration of 2 mM and
[illegible]ssays were run to determine V_{max}. Pseudo-first-
[illegible]inetics were obtained, and the replot of the observed rate constants (k_{obs}) against Zn^{2+} concentration gave a straight line passing through the origin (Fig. 2, inset). This pattern is expected when the rate constant for the dissociation step is very small or almost zero, meaning that Zn^{2+} is very tightly bound or irreversible ligand [8]. The second-order rate constant derived from these data was 820 $M^{-1} \cdot s^{-1}$ (pH 8.0, 20 °C).

Although the zinc ion behaves as a tight, partial inhibitor of the deaminase, the enzyme can slowly lose the metal when treated with EDTA, as discussed earlier. From the first-order rate constants for reactivation by EDTA and the second-order rate constant for zinc inhibition, a dissociation constant for zinc enzyme of $1.46 \cdot 10^{-7}$ M can be estimated (pH 8.0, 30 °C). Such a small constant cannot give an appreciable ordinate intercept in the inset of Fig. 2. The calculated value for the dissociation constant can be valid only if EDTA acts simply by removing Zn^{2+} dissociated from its binding site on the protein, without forming a ternary complex enzyme-Zn-EDTA [9].

Effect of other divalent cations

Similar kinetic changes can be obtained treating the deaminase with Cd^{2+} or Ni^{2+} (data not shown). Other divalent cations (Pb^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+} and Mg^{2+}) assayed at a similar concentration range, did not produce significant kinetic modifications.

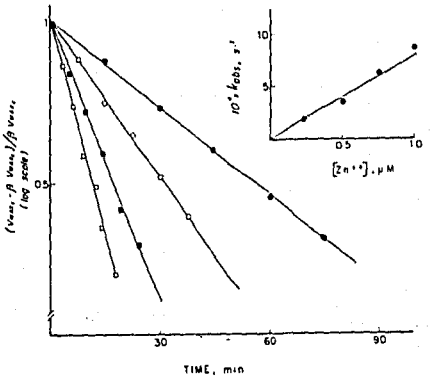


Fig. 2. Semi-logarithmic plot of the time-course of the zinc inhibition, at different concentrations of zinc acetate. Fractional saturation of the enzyme by Zn^{2+} (ordinate) was calculated as described in the text. Enzyme samples (20 nM) were preincubated with the indicated Zn^{2+} concentrations in 50 mM Tris-HCl buffer (pH 8.0 at 20 °C) and aliquots were taken at different times, stopping the zinc-binding reaction with the addition of GlcNAc6P to a final concentration of 1 mM. These aliquots were assayed to determine V_{max} as indicated in Materials and Methods. Zinc concentrations were as follows: ●, 0.25 μM; ○, 0.50 μM; ■, 0.75 μM; and □, 1.00 μM. The inset represents the replot of the pseudo-first-order rate constants as a function of zinc concentration.

It could be of advantage to study, with the aid of nuclear magnetic resonance spectrometry, using $^{113}Cd^{2+}$ as the ligand, the properties described here and the environmental changes produced around the reactive sulfhydryls of the deaminase as a consequence of the allosteric transition. This nuclide may be also a useful spectroscopic probe to detect the possible contribution of atoms, other than sulfur, to the metal binding site.

The possible role of Zn^{2+} as a physiological ligand of *E. coli* glucosamine-6-phosphate deaminase cannot be ruled out, if we take into account the enormous affinity that the enzyme has for zinc ions. In this context, the study of free zinc ion concentration in *E. coli* cells and the correlation with its ability to metabolize amino sugars, could be of interest.

Acknowledgments

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ANEXO III

En el artículo anterior se hace uso de lo que denominamos "cambio en la fracción de V_{max}", para analizar y presentar los resultados de una inhibición parcial. Hemos designado con la letra , al correspondiente coeficiente de inhibición parcial, que en las enzimas modificadas de los artículos II y III, es invariablemente de 0.5. La ordenada de la Fig. 2 del artículo III, representa para una inhibición parcial, lo que la fracción de V_{max} ($V_{max\text{-}I}/V_{max\text{-}c}$) es para una inhibición total, por ejemplo la ordenada de la Fig. 2 del artículo 1.

Esta expresión tiene aplicaciones interesantes, en el caso de los inhibidores irreversibles o fuertemente pegados. Segel[43] propone, la gráfica de V_{max} contra concentración de enzima total, para reconocer un inhibidor irreversible. En ausencia de inhibidor se tiene una recta que pasa por el origen. En presencia de un inhibidor irreversible que "titula" a la enzima agregada a una mezcla que contiene el inhibidor, se obtienen puntos que forman una recta paralela al control que corta la abscisa en el punto de equivalencia. En la Fig. III-1. se muestra una aplicación de esta forma de presentar los datos de inhibición por Zn^{++} a una concentración constante del metal (1 M) y diferentes concentraciones de desaminasa.

El punto de equivalencia de la "titulación" del inhibidor por las cantidades crecientes de enzima, se aprecia como un quiebre en la línea B, o como una intersección en la abscisa en la línea C. Se ilustran otras modalidades en que puede utilizarse el coeficiente beta para corregir y presentar este tipo de datos.

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FIGURA III-1

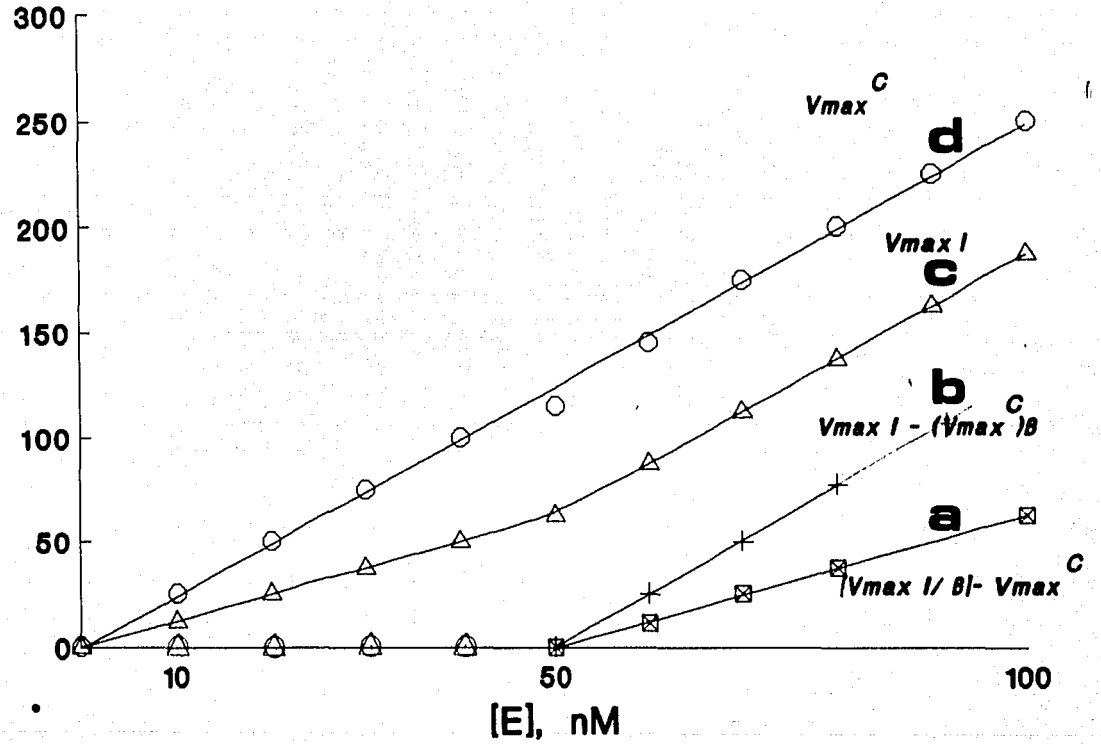
300
250
200
150
100
50
0
10
50
100
[E], nM
Vmax C
d
Vmax I
c
b
Vmax I - (Vmax)B C
a
[Vmax I/ B]- Vmax C

Figura III-1
La ordenada de esta gráfica es diferente para cada curva. En **d**, se ha graficado la Vmax en función de la concentración de enzima. Cada punto proviene de una pequeña serie de 5 concentraciones de G1cN6P utilizada para calcular cada Vmax. Las demás curvas derivan de un mismo experimento, semejante al anterrior, pero realizado en presencia de acetato de Zn 300 nM. El punto de quiebre en las líneas a,b y c tiene una abscisa de 48 nM. Se puede calcular una estequiometría de 300/48 = 6.25 átomos de Zn unidos por molécula de enzima, o sea 1.04/subunidad.

ARTICULO IV

Secondary structure of *Escherichia coli* glucosamina-6-phosphate deaminase from amino acid sequence and Circular Dichroism.

SECONDARY STRUCTURE OF *Escherichia coli* GLUCOSAMINE-6-PHOSPHATE DEAMINASE FROM AMINO ACID SEQUENCE AND CIRCULAR DICHROISM SPECTROSCOPY.

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Running Title: **Secondary structure of glucosamine-6-phosphate deaminase.**

Keywords: **glucosamine-6-phosphate deaminase, circular dichroism spectroscopy, secondary structure prediction.**

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ABSTRACT

The secondary structure of the purified glucosamine-6-phosphate deaminase from *Escherichia coli* K12 was investigated by both circular dichroism (CD) spectroscopy and empirical prediction methods. The enzyme was obtained by allosteric-site affinity chromatography from an overproducing strain bearing a pUC18 plasmid carrying the structural gene for the enzyme. From CD analysis, a 34 % of α-helix, 9 % of parallel β-sheet, 11 % of antiparallel β-sheet, 15 % turns and 35 % of non-repetitive structures, were estimated. A joint prediction scheme, combining six prediction methods with defined rules using several physicochemical indices, gave the following values: α-helix, 37 %; β-sheet, 22 %; turns, 18 % and coil, 23 %. Prediction showed also a considerable degree of alternancy of α and β structures; 64 % of helices are amphipathic and 90 % of β-sheets are hydrophobic. Whole data suggest that deaminase has as dominant motif, an α/β structure.

INTRODUCTION

Catabolism of amino sugars in *Escherichia coli* depends on a group of enzymes encoded by the divergent *nagE- nagBACD* regulon, located at 15.5 min on the chromosome, which has been recently characterized (1-3). The proteins encoded by the genes *nagA* and *nagB*, N-acetyl-D-glucosamine-6-phosphate deacetylase (E.C. 3.5.1.25) and glucosamine-6-phosphate deaminase (E.C. 5.3.1.10), respectively, are necessary for the metabolism of N-acetyl-D-glucosamine (GlcNAc) to fructose-6-phosphate (Fru6P) and ammonia, whereas only *nagB* is necessary for growth on glucosamine (GlcN). Both enzymes are induced during growth on GlcNAc, but only deaminase is induced by growth on GlcN (4,5). Amino sugars are essential components of cell walls and lipopolysaccharides and in the absence of exogenous amino sugars in the medium, glucosamine synthetase, gene *glmS*, is induced to synthesize GlcN from glutamine and Fru6P. Thus, genes *nagB* and *glmS* constitute an example of a classic futile cycle, one degrading, the other synthesizing glucosamine. It is thus obvious that these enzymes must be carefully regulated. For *nagB* this is partly at the level of transcription since the *nag* regulon is controlled by a repressor protein, the product of the *nagC* gene (2,3) which binds to the intergenic *nagE-nagB* region (6). To induce the regulon, GlcNAc or GlcN must enter the cell (5) and GlcNAc6P has been shown to be an inducer *in vitro* (Plumbridge, J.A., unpublished data). This is interesting since the same compound is the allosteric activator of glucosamine-6-phosphate deaminase (7,8), thus regulating both the synthesis and activity of this enzyme suggesting this is indeed the key enzyme in the

control of the utilization of amino sugars.

The recent developments in the study of the *nag* regulon have greatly facilitated research on the enzymes encoded by it. In particular, the DNA sequence has made known the amino acid sequence of the proteins and permitted the construction of strains overproducing them. This has allowed us to obtain higher amounts of these proteins to sustain further enzymological research. Glucosamine 6-phosphate deaminase is the best studied of the*nag* enzymes (7-11), but little attention has been paid to its structural features, although progress in this field will greatly help in understanding its catalytic and regulatory functions. In the absence of crystallographic structural data, but with the knowledge of its amino acid sequence from DNA (1) we obtained some structural information about the deaminase by the use two different and complementary approaches: circular dichroism (CD) spectroscopy and empirical prediction methods from the amino acid sequence. We also report the construction of an *E. coli* strain that overproduces the deaminase and a faster and simpler purification scheme of the enzyme.

MATERIALS AND METHODS

Construction of an overproducing strain.

The gene *nagB* was isolated from pB31-1 as a FspI-ClaI fragment of 1.095 kb which is missing the *nagB* promoter. It was inserted into pUC18 digested with SmaI and AccI, so that the *nagB* gene was downstream of the *lac* promoter. In strain JM101 ($lacI^q$) overproduction is achieved by adding IPTG (1 mM) to exponentially growing cultures. In Δ*lac* strains expression is constitutive.

Glucosamine-6-phosphate deaminase was obtained from an *E. coli* K12 strain in which expression was constitutive to avoid the necessity of IPTG induction. Bacteria were grown in Luria broth containing ampicillin (100 μg/ml) on a 14 l fermentor and harvested during exponential growth phase. Overproduction was estimated to be in the range 30-35% of total bacterial protein, according to densitometric scans of the SDS-electrophoresis gels stained with Coomassie blue.

Enzyme.

The enzyme was prepared by means of a simplified procedure derived from our previously published purification scheme (8). The following modifications were introduced: the extract after centrifugation of sonic-disrupted cells was fractionated with ammonium sulfate (40-55 % saturation) at pH 7.9, 4 °C, and the precipitate dissolved in 0.2 M potassium phosphate buffer (pH 7.5) at a final protein concentration of 5 - 20 mg/ml. The solution was directly applied to a column of N-ε-aminohexanoyl-glucosamine-6-phosphate agarose, prepared as previously described (8). Ten ml of gel were used for an expected yield of 50 mg of pure protein. The column was washed with the same buffer and eluted with six column volumes of a linear concentration gradient of GlcNAc6P (0 to 5mM) prepared in the same phosphate solution. The enzyme elutes as a single protein and activity peak, electrophoretically homogeneous, with a molecular activity for the forward reaction in the range of 1650-1750 s^{-1} at 30°C, pH 7.7. This is the expected value for the pure enzyme. Yields were in the range 80-85 %. Deaminase solution was concentrated by dialysis against 70% glycerol in 10 mM potassium phosphate buffer, pH 7.5

and its final concentration was calculated from the absorbance of the solution at 278 nm and the molar absorptivity $\varepsilon_{278} = 20.0 \times 10^4 M^{-1}cm^{-1}$ (10). Enzyme assays were made as described (8).

Prediction Methods.

Prediction of secondary structures from the amino acid sequence derived from DNA sequence (1) was based on the following methods: Chou and Fasman (12), prediction for β-turns from the same authors (13), Garnier *et al* (14), Gascuel and Goldmard (15), Deléage and Roux (16), and Levitt (17), Conformational preference for total β-strands and for parallel and antiparallel β-structures were also predicted according to Lifson and Sander (18). Several physicochemical parameters were calculated and used to refine the joint prediction: hydrophobicity scale of Miyazawa and Jernigen (19), mean hydrophobicity and hydrophobic moment along the sequence, by the method of Eisenberg *et al.* (20), Hydropathic index of Kyte and Doolittle (21), polarity of Grantham (22), hydrophilicity from Hopp and Woods (23), flexibility from Karplus (24) and surface probability from Emini *et al.* (25). Calculations were routinely performed using two prediction packages developed for PC computers; PREDICT-7, by R.S.Cármenes (26), and SEQANAL, version 1.03, by Antony Crofts, obtained from Biotechnology Center , University of Illinois, USA.

For the Chou and Fasman method, a window of four residues was used to calculate Pα and Pβ values for each residue. The method of Garnier *et al.* was used initially with the simplest decision constant (DC) and run constant (n), being set DC=0; n=1. Taking into account the α/β ratio obtained, a second set of calculations

were then made with decision constants optimized according to Taylor and Thornton (27); in our case, DCα=0, DCβ=20, with n=1. The method of Gascuel and Goldmard was used with the following constants: N[S]α=1.10; N[S]β= 1,545; N[S]coil=1.00. Constants for Deléage and Roux method were optimized as recommended by the authors for α/β class proteins, Levitt's calculations were made taking a nine-residues window size.

Joint prediction of secondary structures was done applying the following rules:

1) Secondary structures were predicted separately by each of the different methods.

2) For a sequence to be predicted as α-helix, it was required that there were at least six sequentially consecutive residues assigned to helical conformation. For β-sheet, the minimum length requirement was set as four residues, and for β-turns, three.

3) Each residue was assigned to the consensus conformation. Discrepancies between different methods about the length of each α-helix segment were corrected by inspection, taking into account the amino acid preferences for location at the ends of helical segments (28).

4) Assignations were also corrected using physicochemical indices. Chain flexibility and hydropathy index were taken as complementary reciprocal indicators of polypeptide segments position with respect to hydrophobic interior of the protein. Segments with high hydropathy and low flexibility were predicted as β-sheets; segments with low hydropathy and high flexibility were taken as loops (29).

5) Helices were predicted as amphipathic when their average hydrophobicity was in the range 0-0.2, with an hydrophobic moment of 0.3-0.4, using an angle of 100 deg. Positive data were correlated with surface probability and hydrophilicity.

6) As general indicators for outside location, reference was made to hydrophilicity (antigenicity), surface probability and polarity indices. Conversely, hydropathy and hydrophobicity were taken as indicators on inside location to classify a segment as "buried" .

Circular dichroism spectra.

CD spectra were recorded in a Jasco J-500A spectropolarimeter calibrated with (+)-10-camphorsulfonic acid. Ultraviolet spectra in the 182-250 nm range were determined on a 0.65 μM deaminase solution (0.115 mg/ml) in 0.1 cm cells, at room temperature. Enzyme was dissolved in 25 mM sodium phosphate buffer, pH 7.5. Spectra were run by duplicate and data averaged. The mean residue ellipticity, [θ], was calculated from ellipticity readings taken each two nanometers and using a mean-residue molecular weight of 112.

To estimate the content of secondary structures, the CD data were analyzed by unconstrained least-squares fitting to the reference spectra of Hennessey and Johnson (30).

RESULTS.

Secondary structure prediction.

The composition and locations of the predicted secondary structures for *E. coli* glucosamine-6-phosphate deaminase obtained by different methods and the joint scheme, are summarized in Fig.

1 and Tables I and II. Although all the methods assigned most of the α-helices, β-strands and turns in similar regions along the sequence, there is a considerable variation in their length and hence in the total amount in each category (Fig.1 and Table I). Prediction of the α-helical content is in good agreement (36-39 %), but there are considerable differences in the β-strand prediction, ranging from 18 to 32 %. Chou and Fasman, and Levitt methods predicted the highest amount of β structure at the expense of a low coil prediction. The joint prediction scheme may be considered more reliable that any single method (31), and we applied it to estimate the secondary structures of the deaminase and to gain some insight into its folding pattern.

Eleven α-helical segments were predicted; the region 235-240 was equally predicted to adopt either a α-helix or β-sheet conformation. We prefered to assign it to a helix because this structure shows a lower dependence on local interactions for stability (32). Seven helices were calculated as amphipathic; three as hydrophobic, and one as hydrophilic. The latter occurs in a region predicted as flexible, polar and hydrophilic (Table II). Conversely, nine β-strands in a total of ten predicted, are hydrophobic. This correlation between hydrophobicity and tendency to form a β-sheet is apparent in data depicted in Fig. 2. Calculation of conformational preferences for β-strands of Lifson and Sander (18) predicted 23 % of total β-structure distributed as 9 % of parallel and 14 % of antiparallel strands.

It is interesting to point out that in ten highest peaks of flexibility, five correspond to segments predicted as β-turns, three as coil segments and one as α-helix.

An overall picture of the secondary structure prediction is presented as a schematic diagram in Fig. 3.

CD Spectroscopy.

The CD spectrum of the glucosamine-6-phosphate deaminase is shown in Fig. 4. In the 182-250 nm range, where the spectrum reflects the structural pattern of the polypeptide chain (33), the CD curve exhibits a positive peak at 192 nm, a negative shoulder around 210 nm and a negative extreme at 219 nm. Analysis of the spectrum by means of the Hennessey and Johnson reference spectra (30) gave the structural coefficients shown in the bottom line of Table I. Since the sum of these coefficients was close to 100 % and no negative contributions from any of the structural types were obtained, the analysis can be considered satisfactory (30, 34). The curve reconstructed from the estimated content of secondary structures fitted well the experimental spectrum (Fig. 4). Therefore, the use of the variable selection method (35) which in some cases improves structural estimation, was not necessary to analyze our CD data.

DISCUSSION

We have compared the contributions to the structure of deaminase of α-helix, β-sheet, β-turns and non-repetitive structures as estimated by various prediction methods with that calculated from CD spectra. Notwithstanding the inherent uncertainty of structure prediction methods, the strong correlation found suggests that reliable information about glucosamine-6-phosphate deaminase has been obtained.

Glucosamine-6-phosphate deaminase gave a GRAVY index (Kyte and Doolittle, 21) of -1.85, that corresponds to a soluble globular protein. The α-helix content was 37 % (prediction) or 34 % (CD); and the β-sheet content was 22 % (prediction) and 20 % (CD); according to Taylor and Thornton (27), proteins having more than 35 % of α structure and more than 15 % of β-sheet usually belong to the class of α/β proteins; The double prediction method from Deleage and Roux (16) also asssigned the deaminase to the α/β family.

A considerable degree of alternancy of β-strands and α-helices is also apparent in predicted structure (Fig. 3); furthermore, two thirds of helical segments were predicted as amphipathic (i.e. hydrophobic on one side and hydrophilic on the other) and 90 % of β-strands as hydrophobic (Table II and Fig. 2). These data suggest a possible folding pattern for deaminase of the α/β type with a central core of β-sheets surrounded by helices with their hydrophilic side exposed to the solvent.

There are other additional indications of a major content of α/β structure in the deaminase molecule. The CD spectrum of the enzyme (Fig. 4) has features considered by Manavalan and Johnson as

characteristic of α/β proteins (33) , such as the intense positive band at 192 nm and two negative bands at 219 and 210 nm, with the first more intense than the second. Amino acid composition of the deaminase is also consistent with this conclusion; calculation of the index proposed by Nishikawa *et al.* (36) to correlate whole protein composition and predominant supersecondary structure gave a distance from origin of 2.71, that fell centrally in the range for α/β structures. Our prediction is also consistent with the observation of Thornton and Chakauya (37), about α/β proteins which usually present a N-terminal β-strand and a C-terminal helical segment.

Our results, both from CD and empirical prediction, indicate that β-sheets are not predominantly parallel or antiparallel, but that there are considerable amounts of both types of strands. It is worthwhile to note that Lifson and Sander prediction gave also good quality data, taking CD as a reference. This distribution of parallel and antiparallel strands may indicate the presence of different types of folding units (38) in this polypeptide chain, in addition to the classical $\beta\alpha\beta$ fold of α/β proteins. If we take also into account the chain length of deaminase (266 residues), the possibility that the polypeptide chain of the enzyme would be folded in two domains with different folding pattern, cannot be ruled out.

It has been shown that two vicinal cysteinyl residues in deaminase molecule, change radically their reactivity with the allosteric transition (10, 11). According to our prediction, only two cysteines are located in segments of high polarity, hydrophilicity, flexibility and positive surface probability.

These are Cys 118, located in the single helical segment predicted as hydrophilic (115-124), and Cys 239, located in segment predicted as a β-turn (239-244). On the other hand, cysteines 219 and 228 are located in a zone predicted as the most hydrophobic of the whole polypeptide chain. Sulfhydryls from Cys 118 and 239 are probably the vicinal pair described by Altamirano *et al.* (11), which exhibits a high affinity for zinc (39). As the accessibility of the sulfhydryl-Zn cluster changes during the allosteric transition, the polypeptide chain around Cys residues 118 and 239 merit more attention in future studies with regard to the dynamics of the allosteric conformational changes.

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LEGEND FOR FIGURES

Fig. 1. Diagrammatic representation of secondary structures of glucosamine-6-phosphate deaminase, predicted from its amino acid sequence, using different methods: **CF**, Chou and Fasman (12, 13); **GOR**, Garnier, Osguthorpe and Robson (14); **GG**, Gascuel and Goldmard (15); **DR**, Deleage and Roux (16); **L**, Levitt (17); **LS**, Lifson and Sander (18); **J**, Joint prediction.

Fig. 2. Comparison of β-structure prediction (joint) with the hydrophobicity scale of Miyazawa and Jernigen (19); window size, 7 residues; relative weight of window edges: 100 %; linear weight variation. Predicted β-strands are denoted as bars.

Fig. 3. Schematic diagram of predicted secondary structure of glucosamine-6-phosphate deaminase by the joint scheme.

Fig. 4. Far UV CD spectrum of glucosamine-6-phosphate deaminase. Continuous line, experimental curve; dotted line, spectrum reconstructed from analysis of the experimental data. Spectra were run by duplicate and data averaged. Bars indicate the deviation of the two measures. See details in the text, under *Materials and Methods*.

FOOTNOTES

Abbreviations: GlcN, D-glucosamine; GlcNAc, N-acetyl-D-glucosamine; GlcN6P and GlcNAc6P, their corresponding 6-phosphate esters; Fru6P, Fructose-6-phosphate; IPTG, isopropyl-β-D-thiogalactoside; CD, circular dichroism.

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TABLE I

Percent of secondary structures of glucosamine-6-phosphate deaminase, from several prediction methods and CD spectroscopy.

METHOD	α-HELIX	β-SHEET	β-TURNS	COIL	α+β
CF	39	32	18	11	71
GOR	39	18	16	27	57
GG	36	20	---- 44----		56
DR	40	22	18	20	62
L	39	30	15	15	69
LS		P: 9 AP: 14 TOTAL: 23			
JOINT	37	22	18	23	59
CD	34	P: 9 AP: 11 TOTAL: 20	15	35	54

[a] Prediction methods are indicated with the same abbrevations as in Fig.1.

TABLE II

Joint prediction of secondary structure of *E. coli* glucosamine-6-phosphate deaminase.

Residue number	predicted structure	lenght.	c o m m e n t s
1 - 7	β	7	High hydropathy. Low flexibility, hydrophilicity and surface probability.
8 - 17	α	10	Amphipathic.
18 - 22	β	5	High hydropathy. Low hydrophilicity and surface probability.
23 - 34	coil	12	Flexibility peak. Medium hydrophilicity. Low hydropathy and hydrophobicity.
35 - 38	β	4	High hydropathy. Low flexibility, hydrophilicity and surface probability.
39 - 45	turn	7	Flexibility peak. Low hydropathy. Maximal value for turn propensity.
46 - 50	β	5	Amphipathic. The single flexible β segment predicted.
51 - 59	α	9	Amphipathic.
60 - 62	coil	3	α-β connection

63 - 69	β	7	Amphipathic
70 - 76	α	7	Amphipathic
77 - 82	coil	6	Peak of maximun flexibility and polarity. Minimal value for hydrophobicity.
83 - 86	turn	4	High polarity and flexibility.
87 - 90	β	4	Low hydrophilicity and flexibility. High hydrophobicity.
91 - 98	α	8	Amphipathic
99 - 107	coil	9	High polarity. Low hydropathy and hydrophobicity.
108 - 111	turn	4	Flexibility peak. High hydrophilicity.
112 - 114	coil	3	Flexibility and polarity peaks.
115 - 124	α	10	Maximun of flexibility and polarity. High surface probability. Hydrophilic helix.
125 - 130	turn	6	High flexibility, polarity and surface probability.
131 - 135	β	5	High hydropathy and hydrophobicity. Low flexibility, hydrophilicity and surface probability
136 - 139	coil	4	Peak of flexibility.

140 - 143	turn	4	High flexibility and surface probability.
144 - 150	turn	7	Probable loop. High flexibility and polarity. Low hydrophobicity.
151 - 156	α	6	Amphipathic.
157 - 161	β	5	
162 - 172	coil	11	High hydropathy. Low flexibility hydrophilicity and surface probability.
173 - 176	turn	4	High flexibility, polarity and surface probability.
177 - 183	coil	7	Flexibility peak
184 - 190	β	7	High hydrophobicity.
191 - 199	α	9	Low polarity, hydrophilicity and surface probability. Hydrophobic helical segment.
200 - 205	β	6	High hydropathy. Low flexibility hydrophilicity and surface probability.
206 - 218	α	13	Low polarity, hydrophilicity and surface probability. Hydrophobic helix.
219 - 222	turn	4	High hydrophobicity and hydropathy. Medium flexibility. Hydrophobic turn.
223 - 226	β	4	High hydropathy. Low flexibility, hydrophobicity and surface probability.
227 - 232	coil	6	High hydrophobicity and hydropathy.

233 - 238	α	6	Overlapped prediction of α & β. Predicted as α, according to Biou (28).
239 - 242	turn	4	Flexibility peak. High hydrophilicity, polarity and surface probability.
243 - 250	α	8	Amphipathic.
251 - 254	turn	4	High flexibility.
255 - 266	α	12	Amphipathic.

FIG. 1

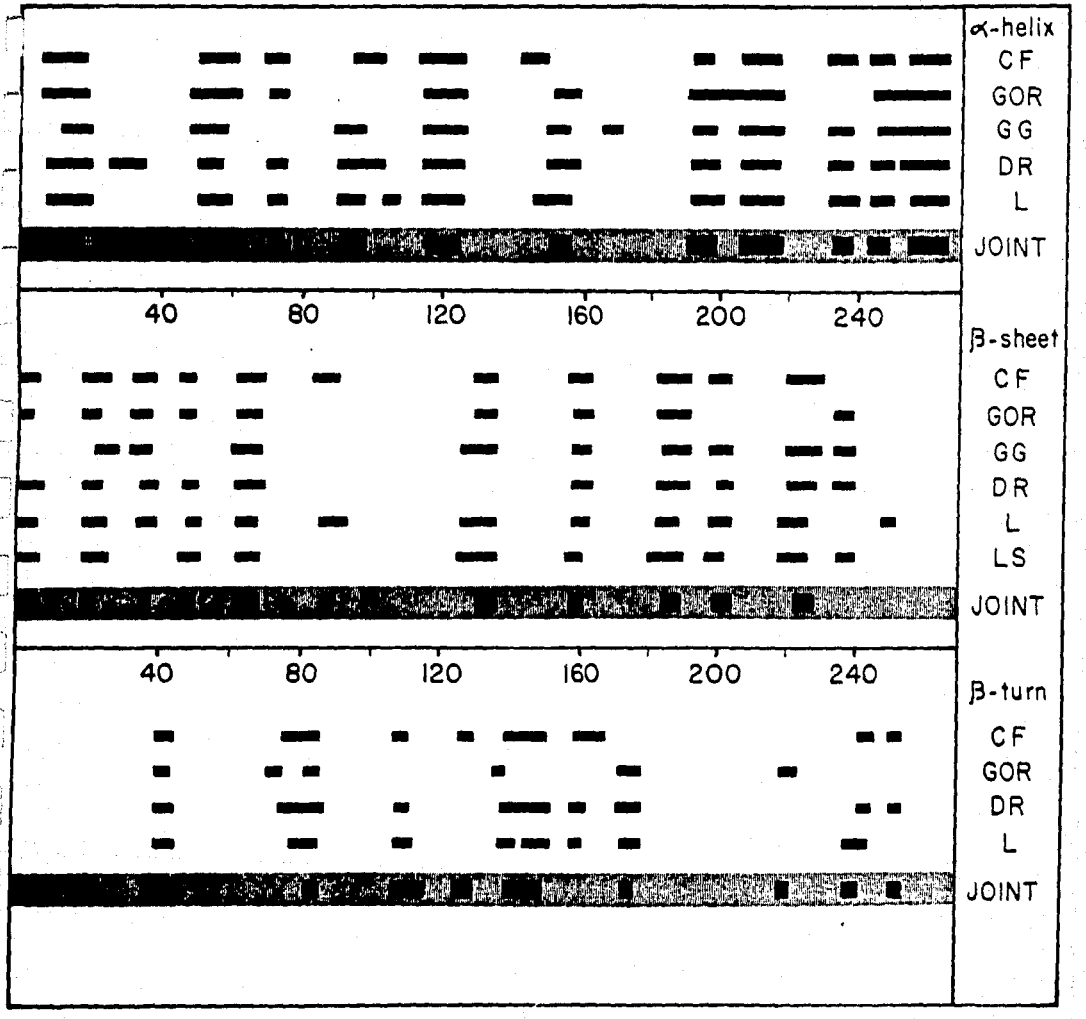
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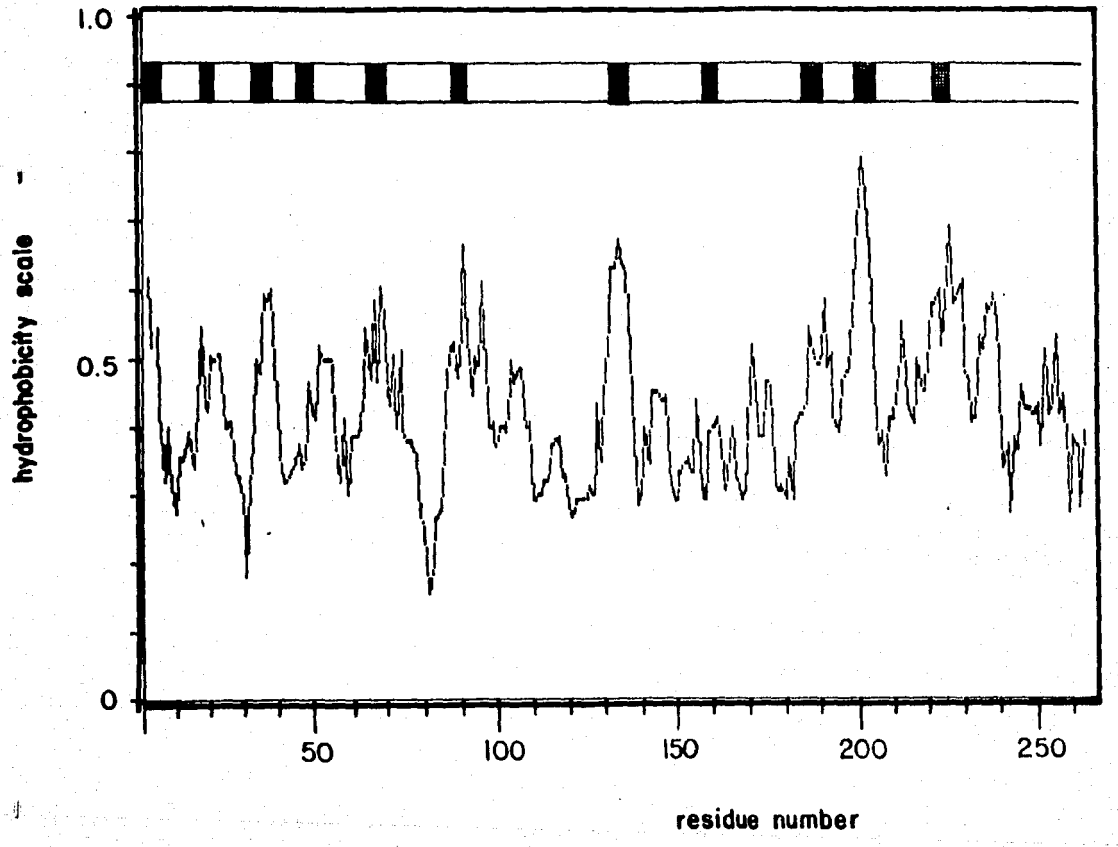
Fig.2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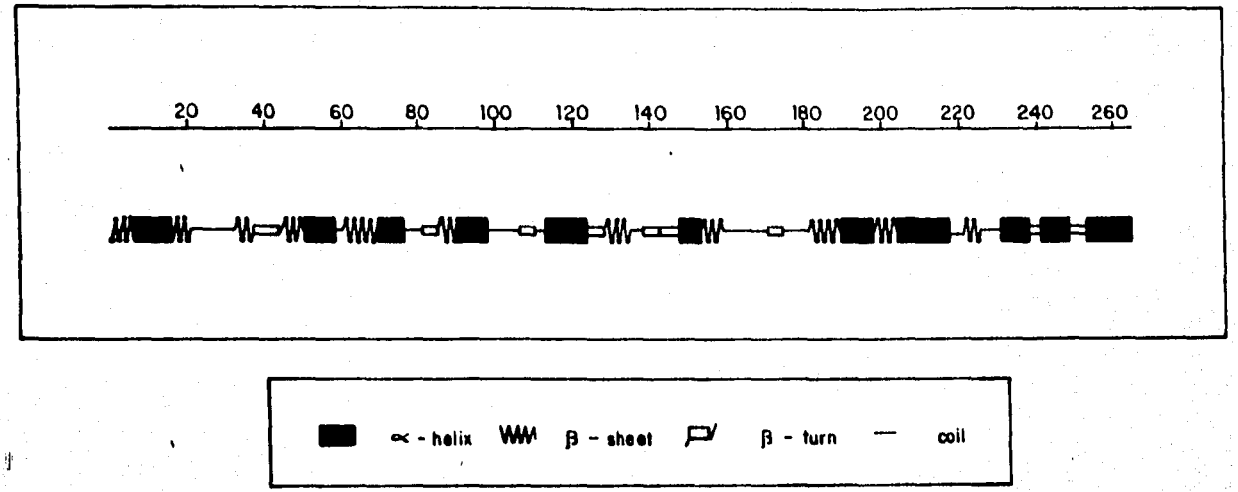
20
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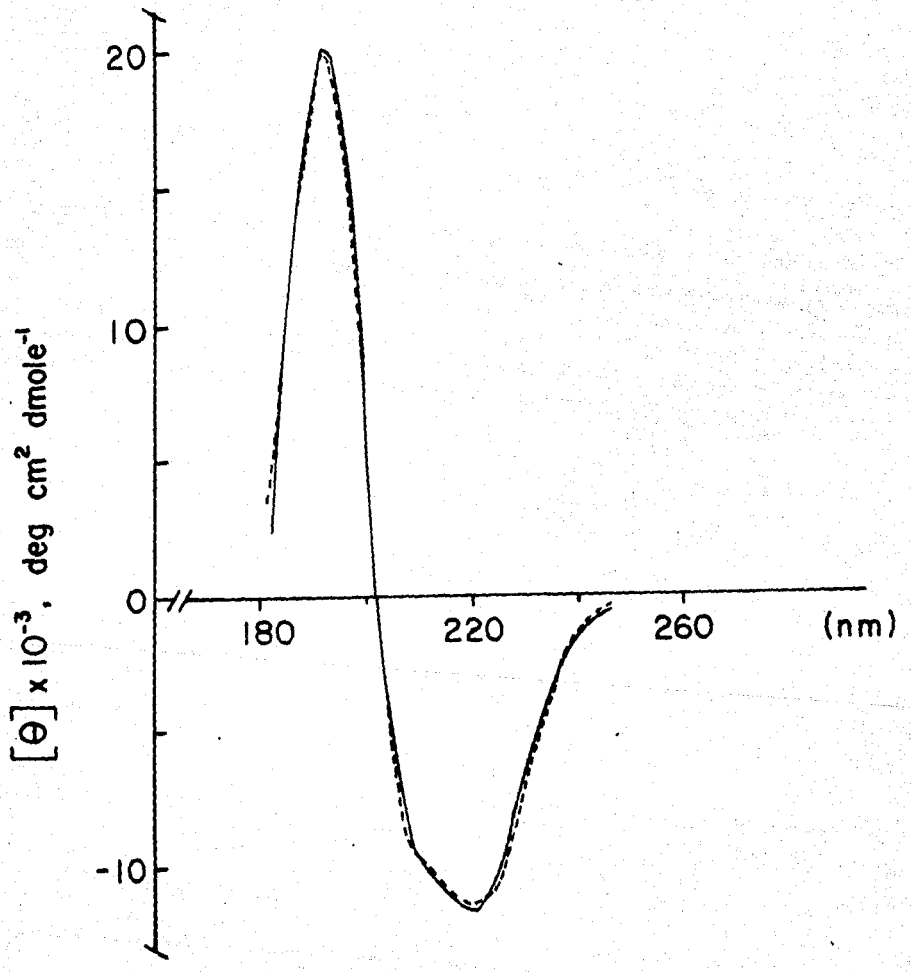
∝ - helix
β - sheet
β - turn
coil

FIG. 3

FIG. 4



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Dear Dr. Calcagno:

We are pleased to inform you that the paper entitled "Secondary structure of *Escherichia coli* glucosamine-6-phosphate deaminase from amino acid sequence and circular dichroism spectroscopy" by Hernandez-Arana, Altamirano, Plumbridge, and yourself has been accepted for publication in *Biochimica et Biophysica Acta*. The typescript has been forwarded to the Publisher and will be included in the section devoted to Proteins and Molecular Enzymology.

Further correspondence regarding this paper should quote the number given above, and be directed to:

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Article no: RPP 023547 Date of Receipt: June 14, 1990
Title : Secondary structure of Escherichia coli glucosamine-6-phosphate deaminase from amino acid sequence and circular dichroism spectroscopy
Author : Dr. Mario Calcagno
Co-Authors: Hernandez-Arana Altamirano M Plumbridge JA

COMMENTS

This is a piece of nice work. The experiments are well planned and the discussion is thorough, although some parts may be somewhat speculative. The authors may wish to consider the following comments and make some minor revisions.

Lack of x-ray diffraction studies of numerous proteins has prompted us to estimate the secondary structure of a protein by sequence prediction methods and analysis of CD spectrum. However, these methods are all empirical. Therefore, there is no justification to list the results in Table I to the first decimal. For instance, a helicity of, say, 36.8% may imply that the value is good to ±0.5%.

According to G.D. Fasman (Prediction of Protein Structure and the Principles of Protein Conformation, Plenum, New York, 1989, pp. 193-301), the methods of Chou and Fasman, Garnier et al., and Lim have been mostly frequently used ones among the more than 20 different methods. The percentage accuracy may vary between 70 and 85%. The authors choose five sequence prediction methods, including Chou and Fasman and Garnier et al., which in turn lead to a joint prediction. Nevertheless, it is difficult to predict its percentage accuracy.

Because it is not known a priori which reference proteins constitute a good basic set for CD analysis, Manavalan and Johnson [Anal. Biochem. (1987) 167:76-85] have now proposed a variable selection procedure added to the method of Hennessey and Johnson to improve the estimation of various conformations in a protein. While the Hennessey and Johnson method used in this paper appears to give an excellent curve fitting (Fig. 5), the authors may care to refer to the Manavalan and Johnson procedure in the discussion.

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En el curso de esta investigación, hemos cambiado de cepa bacteriana. El trabajo se inició con *E. coli* B, cepa ATCC 11313 (Artículos I, II y III), induciendo la expresión de la desaminasa por medio del cultivo de la bacteria en glucosamina, como única fuente de carbono y de nitrógeno. El proceso de purificación era largo y costoso, puesto que para obtener cantidades suficientes de proteína para realizar estudios estructurales, partíamos de una fermentación de 350 litros.

A partir de la colaboración establecida con la Dra. Jacqueline A. Plumbridge, del Institut de Biologie Physico-Chimique de París, disponemos de sus cepas hiperproductoras de la desaminasa en *E. coli K12*, y purificamos la enzima a partir de ellas. Comparando la desaminasa de *E. coli K12* con la de *E. coli B*, hemos obtenido los siguientes datos:

1. La secuencia N-terminal hasta el residuo 32 es idéntica en las dos proteínas.
2. Los electroenfoques entre pH 3 y 9 de ambas enzimas, en su forma nativa o en urea 6 M, dan migraciones idénticas.
3. El estudio cinético de las desaminasas de una y otra cepa, revela iguales parámetros Michaelianos (Km, $V_{max}/(E)$), igual cooperatividad, evaluada por el coeficiente de Hill máximo (h_{max}) y $S_{0.5}$, e igual curva de activación por GlcNAc6P.

Por otra parte, el Dr. Alfried Vogler, nos ha hecho llegar la secuencia del gen nagB de *Klebsiella pneumoniae*, que presenta gran similitud con la de *E. coli*, con sólo 15 sustituciones en 266 residuos. Estas evidencias, permiten suponer que si existe alguna variación en la secuencia de aminoácidos de ambas desaminasas, (lo que es poco probable) ésta es menor o muy conservadora, y carece de repercusiones estructurales y funcionales.

Por lo anterior, una vez demostrada la identidad de las enzimas, decidimos cambiar a *Escherichia coli K12*, empleando fundamentalmente 2 cepas: la JM101, y la IBPC456R, transformadas con plásmidos pUC18 portadores del gen nagB de la desaminasa proveniente de *E. coli* K12, ya sea en su forma silvestre, o con mutantes en sitios específicos. La ventaja del cambio de cepa, consiste en la obtención de mayor cantidad de enzima gracias al empleo de cepas sobreproductoras; esto nos ha

permitido además simplificar el esquema de purificación y facilitado posteriormente la producción de enzimas genéticamente modificadas. La posibilidad de contar con abundancia de proteína es fundamental para emprender estudios que consumen grandes cantidades de enzima como: cristalografía de rayos X, experimentos de desnaturalización y renaturalización de la desaminasa, experimentos de unión de ligandos en equilibrio (diálisis en equilibrio, método de Hummel y Dreyer, etc.), modificación química seguida de fragmentación y análisis de péptidos, estudios de Dicroísmo circular, etc.

En la figura IV-1 se muestra un gel de poliacrilamida con la técnica de Laemmli[44] en el que se observa un extracto crudo de las distintas clonas sobreproductoras de la desaminasa.

[44] Laemmli, U.K. (1970), Nature. 227:680-685.

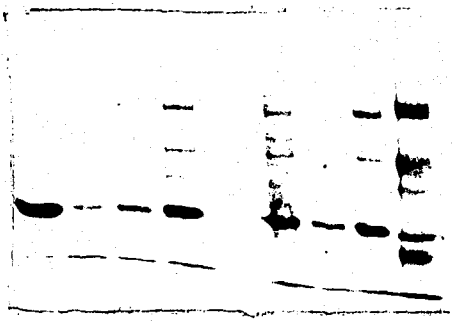

Figura IV-.

Sobreproducción de la glucosamina 6-P desaminasa de Escherichia coli K12. La construcción de la cepa y las condiciones de expresión, se describen en el artículo IV. En el primer carril de la izquierda, se colocó una muestra de desaminasa pura, para referencia. En el último carril de la derecha, aparece una mezcla de calibración (67, 43, 36, 30 y 24 kDa). En los carriles centrales, se corrieron muestras de proteínas totales de Escherichia coli, preparadas disolviendo una muestra de bacterias en el amortiguador de muestra. Cada carril corresponde a una clona diferente, de la bacteria transformada.

ARTICULO V

Vicinal thiols of *Escherichia coli* glucosamine-6-phosphate deaminase: Localization along the sequence, site directed mutagenesis and characterization of mutant enzymes.

VICINAL THIOLS OF ESCHERICHIA_COLI GLUCOSAMINE 6 PHOSPHATE DEAMINASE: LOCALIZATION ALONG THE SEQUENCE, SITE-DIRECTED MUTAGENESIS AND CHARACTERIZATION OF MUTANT ENZYMES

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RUNNING TITLE: Thiol location and mutants of glucosamine-6-phosphate deaminase.

Keywords: Glucosamine-6-phosphate deaminase, site-directed mutagenesis, allosteric conformerization, vicinal sulfhydryls

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SUMMARY

We know from DNA sequencing that glucosamine-6-phosphate deaminase has four cysteinyl residues, at positions 118, 219, 228 and 239. On the other hand, chemical modification experiments showed that the enzyme has a vicinal pair of sulfhydryls, semi-essential for catalysis and allosteric regulation. These groups become protected when the enzyme is activated by homotropic or heterotropic ligands. They are also involved in Zn-binding by the protein. Being these groups the only reactive thiols in the native enzyme in the absence of ligands, we cyanylated them specifically with 5'-nitro-2-thiocyanate benzoate (NTCB) and cleaved the chain at thiocyanoalanine residues, by alkaline hydrolysis (Dégani-Patchornik cleavage) to locate these groups along the polypeptide chain of the enzyme. Fragments were analyzed by SDS-gel electrophoresis. The cleavage pattern indicates that Cys-118 and Cys-239 are the residues bearing the vicinal reactive thiols. Three site-directed Cys→Ser mutants at these positions were then constructed, by the oligonucleotide method. Both single replaced and the double replaced enzymes were obtained. Kinetic studies of the genetically-modified enzymes confirmed the semiessential role of the thiol pairs; single-substitutions had the same effect of double substitution or chemical block, lowering the apparent k cat to the half of its value for wild-type enzyme. The effect of Cys replacement is more complex in relation to cooperative kinetics of the enzyme. Single substitution Cys-118→Ser, did not affect the allosteric behaviour of the enzyme. Substitution at Cys-239, caused a lower homotropic cooperativity, changing the Hill coefficient (h)from 3.0 (wild type) to 2.0. The double substituted enzyme had a minimal homotropic cooperativity (h = 1.25). From Hill plots, it may be seen that the more conspicous change in allosteric behavior, is the reduction of K_T, from 65 mM (wild type) to 8-10 mM in the double replaced protein. These results indicate that Cys residues make a significant contribution to the structure of the T-conformer of the deaminase.

INTRODUCTION

Glucosamine-6-phosphate deaminase from *Escherichia coli*, is an oligomeric protein formed by six identical polypeptide chains (1) which sequence is known from DNA (2). This enzyme catalyzes the reversible conversion of D-glucosamine-6-phosphate (GlcN6P) into D-fructose-6-phosphate and ammonia, and is allosterically activated by N-acetyl-D-glucosamine-6-phosphate (GlcNAc6P) (3,4). The deaminase is encoded by the *nagB* gene, located at 15.5 min in the bacterial chromosome (5); this gene is a component of the divergent regulon *nagE-BACD* which was recently characterized (2,6,7). The amino acid sequence of the enzyme (2) shows four cysteinyls residues, which are residues 118, 219, 228 and 239. Chemical modification studies carried out in the deaminase from *E. coli* B, have demonstrated the presence of two vicinal sulfhydryl groups per polypeptide chain, which react with different thiol reagents only when the enzyme is in its less active allosteric conformation; upon allosteric activation by homotropic or heterotropic ligands, these thiols become completely protected (8,9). They are also involved in Zn^{++} binding by the deaminase and when the Zn^{++}-bound enzyme changes its conformation by allosteric activation, it sequesters the metal ion, which cannot be released unless the enzyme return to the less active conformation (10). The selective chemical block of this pair of sulfhydryls by methylation, oxidation to an intrachain disulfide or reaction with arsenite, produces significant kinetic changes: a lower cooperativity toward GlcN6P and a change of molecular activity to near a half of the value for the native enzyme (9). Binding of some divalent cations as Zn^{++} , Cd^{++} and Ni^{++} had similar effect (10). These results indicate a semi-essential role of vicinal thiols, for both catalysis and allosteric activation. They seem to be located in a region of the polypeptide chain undergoing an important structural change concomitant to the allosteric transition. Changes in cooperativity indicate that this is not simply a passive displacement, having the thiol pair some role in the allosteric equilibrium. The chemical block of these groups makes also the enzyme less catalytically efficient, and its possible that they would be located at the neighbours of the active site. We do not know if these two aspects of vicinal thiols

roleon deaminase mechanism are a consequence of the same kind of interactions, and if in this function both are needed as a vicinal pair. Being both groups equally reactive, the chemical modification approach does not allow to evaluate their separate contribution to catalysis and regulation. In order to pursue this study, we have undertaken the localization of the pair of reactive thiols along the deaminase polypeptide chain; we used then this information to construct the corresponding Cys Ser mutants, which made possible to characterize the functional contribution of each thiol of the vicinal pair. For convenience, these groups will be denominated along this paper as thiols R1 and R2, in their sequence order. The corresponding site-directed Cys Ser mutants will be also referred as mutants 1 and 2,

MATERIALS AND METHODS

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 (11).

Bacteria and enzymes

Wild-type *E. coli* glucosamine-6-phosphate deaminase was obtained from an overproducing strain, as previously described (12). In the first part of this paper, we identify by chemical methods R1 and R2 thiols as Cys-118 and Cys-239, respectively. According to this first evidence, three Cys Ser mutants of the deaminase were constructed: Cys-118 Ser (Mutant 1), Cys-239 Ser (Mutant 2) , and the corresponding double susbtituted enzyme (mutant 1,2). The *E. coli* strain used for transformation, IBPC546R had a kanamycin-resistance cassette in *Pst*1 site in *nagB* gene, to obtain a null mutation at chromosomal level. This strain is D-lac and deaminase is expressed constitutively. Bacteria and enzyme were produced as described (12); this procedure, developed to purify the wild-type protein was also useful to obtain the variantproteins.

Overproduction was controlled by enzyme assays and SDS-polyacrylamide gel electrophoresis in the Laemmli discontinuous system (13). Purity of all enzyme samples used was verified with the same electrophoretical procedure. Glucosamine-6-phosphate deaminase from different mutants was overproduced in the 25-35% range, referred to total bacterial protein, estimated by densitometry of Coomassie G-250 stained gel slabs.

The concentration of wild-type or genetically-modified enzymes were calculated from the absorbance at 278 nm of their solution in 25 mM Tris-HCl buffer, pH 7.8, using the known molar absorptivity for the wild-type protein (9). Mutants at R1 and R2 sites, are not expected to produce absorptivity changes of the enzyme at 278 nm (14).Enzyme assays and kinetic data processing were made as described (8,9). ,Unless otherwise specified, kinetic data were obtained at pH 7.7 and 30°C.

Measures of reactive (R1 and R2) and total SH groups of the deaminase.

Reactive thiols (R1 and R2 groups) were determined with 5-5'-dithiobis-(2-nitrobenzoic) acid, (DTNB), at pH 8.0, 20°C, in the presence of 5 mM EDTA, as described (8). After the completition of the reaction , 10% SDS was added to obtain a final detergent concentration of 1%, and the new formed 2-nitro 5-mercaptobenzoate (NMB) was measured.

Specific cyanylation of R1 and R2 thiols

A 5 1M wild-type deaminase solution was cyanylated under non-denaturing conditions with 2 mM NTCB in 50 mM potassium phosphate buffer pH 7.50, containing 2.5 mM disodium EDTA (buffer A). The course of the reaction was followed spectrophotometrically at 412 nm, against an appropiate blank in a double-beam spectrophotometer.

Cleavage of cyanylated enzyme.

Specific cleavage of deaminase polypeptide chain at R1 and R2 thiols was performed by the procedure of Dégany and Patchornik (15). Aliquots of 2 - 3 nmoles of deaminase in 100 11 samples , were cyanylated overnight under the non-denaturing conditions described, which modifies only R1 and R2 groups. Reaction mixture was then dialyzed against buffer A to get rid of NTCB and NMB, then treated with 50 mM N-ethylmaleimide (NEM) for four hours in 3.5 M guanidinium thiocyanate. This step was introduced as an attempt to block a buried sulfhydryl group, highly reactive upon denaturation (8) which would produce undesirable side reactions with thiocyanoalanine residues in R1 and R2. Samples were then dialyzewd against water; the precipitated protein was dissolved adding solid guanidinium thiocyanate to get a final concentration of 3.5 M, and the pH was adjusted to 9.5 with 0.1 vol of 0.9 M potassium borate buffer, pH 9.5, and some 0.1 M KOH, if neccessary. Samples were left at 37 °C for 24 h, then 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. Some diluted samples were concentrated by liophyllization. In all cases, dialysis were performed in microchambers obtained from transversally cutted 1.4 ml Eppendorf microfuge plastic tubes with a piece of dialysis

membrane trapped under the lid, in a similar way used by Reinard and Jacobsen (16). These dialysis chambers were vigorously stirred in a flask containing the dialysis buffer.

Electrophoretical separation of cleavage fragments.

Deaminase samples after Dégany-Patchornik cleavage at residues R1 and R2, were fractionated by SDS-polyacrylamide gel electrophoresis, using Laemmli discontinuous system (14) and 0.75 mm -thick gel slabs. Stacking gel had 6.5% of total acrylamide concentration with 3.5 % cross-linking. 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 diffussion. These gels yield good separations in the range 30 to 3 kDa. Protein bands were detected with the silver staining procedure of Wray et al. (17). Cyanogen bromide fragments of sperm whale myoglobin, glucosamine-6-phosphate deaminase, trypsinogen (phenyl-methanesulfonyl fluoride-treated), soybean trypsin inhibitor, b-lactoglobulin and a-lactalbumin, were used as molecular weight standards. Myoglobin BrCN fragments, which were prepared according to Gross and Witkop (18) gave the five-band pattern described by Kratzin *et al.* (23).

RESULTS AND DISCUSSION

Selective cyanylation of R1 and R2 sulfhydryls of glucosamine-6-phosphate deaminase

Wild-tipe non-denatured deaminase was cyanylated with NTCB as described under *Materials and Methods*. The reaction followed pseudo-fist-order kinetics, as expected for two equally-reacting groups. A second-order rate constant of $2.1 \times 10^{2} s^{-1} M^{-1}$ was calculated for the reaction at pH 8.0 and 20 °C. The stoichiometry of the reaction, calculated from the NMB anion formed, was 2.07 sulfhydryls per polypeptide chain. According to this result, and the known reactivity of deaminase sulfhydryls, R1 and R2 groups were completely cyanylated. The S-cyanylated enzyme, studied at the deamination direction of the reaction had 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 displays positive cooperativity, with a Hill coefficient of 1.60. These kinetic changes indicate that S-cyano deaminase behaves similarly as other modified forms of the enzyme involving R1 and R2 sulfhydryls (10).

Cleavage at R1 and R2 sulfhydryls

The fragments from Dégani-Patchornik cleavage (15)of glucosamine-6-phosphate deaminase , separated by SDS-polyacrylamide gel electrophoresis , are shown in Fig. 1, lane A. A control sample of non-cyanylatede enzyme, submitted to all subsequent steps described under *Materials and Methods*, was run in lane B. Four cleaved fragments were found of 27, 17 , 13 and 3.5 kDa. The latter is usually faint, and probably most of this component is lost in the fixation and staining steps. A considerable amount of enzyme tremains uncleaved. This may due to incomplete hydrolysis,but it may be also a consequence of side reactions, mainly b-elimination (15). Other possible interference, is the loss of cyano group from thiocyanoalanine residues by reaction with buried sulfhydryls exposed on denaturation. This cause of loss of S-cyano groups at R1 and R2, was kept low by adding the guanidinum salt in the presence of a high concentration of NEM. Incomplete cleavage, giving chain fragmenmts split at only one modified cysteine, is evident from Fig. 1, and the schematic interpretation shown in Fig. 2. In this figure, the segments between cysteinyl residues are designated with letters from A to E and their calculated molecular weight is given. The fragment of 17 kDa may be only BCDE wich calculated molecular weight is 16,636. This proves that Cys-118 is one of the cleavage sites and identical to R1. The presence of a peptide pof 27 kDa suggests that the other cleavage, corresponding to R2, may be close to C-terminal end of the chain. Then, the most probable location for R2 is the residue 239, if we identify the 27 kDa fragment with ABCD (26,574 Da) and the 3 kDa band with segment E. According to this interpretation, the 13 kDa band observed in Fig.1 contains peptide fragments A (13,210 Da) and BCD (13,364 Da), which were not resolved under our experimental conditions.Fig. 2 summarizes this information, and shows the position of cysteinyl residues corresponding to R1 and R2 sulfhydryls.

Titration of thiol groups from Cys→Ser mutants.

Mutants 1, 2 and 1,2, were constructed, and the corresponding genetically-modified versions of the enzyme were purified, as described under *Materials and Methods*. In these variant proteins, serine was introduced to replace cysteine, as change thar may be considered as structurally conservative. The three modified proteinsobtained from mutants, were active and allosteric deaminases, and the affinity chromatography step withan immobilized analog of the

activator, worked similarly with wild-type and mutant proteins.

Sulfhydryls were titrated in the three genetically-modified enzymes, under native and denaturing conditions, and the results are summarized in table I. Data from the wild-type enzyme are also included for comparison. Enzymes from mutants 1 and 2, lack one reactive sulfhydryl, and both are absent in the double substituted enzyme. This results confirms the identification of Cys-118 and Cys-239 as the residues corresponding to the vicinal pair R1-R2. There is a buried cysteinyl residue, reacting almost instantaneously after denaturation. The fourth cysteinyl residue is not titrable by the thiol-disulfide exchange reaction with DTNB, and it may be oxidized, forming an interchain disulfide bond.

It would be expected that cysteinyls 118 and 239 be located at a superficial, polar and flexible segments of the polypetide chain of the enzyme. From circular dichroism spectroscopy and a combination of predictive algorithms from amino acid sequence, we have proposed a secondary structure and a folding pattern for glucosamine-6-phosphate deaminase (23). According to this model, Cys-118 appears in the single hydrophillic a-helical segment, which was also predicted as a flexibity peak, and Cys-239 is located in a predicted b-turn, giving high scores of surface probability and flexibility. On the other hand, Cys-219 and Cys-228 are located in hydrophobic segments predicted as as buried.

From chemical reactivity experiments, we know that Cys-118 and Cys-239 are close placed in the three-dimensional structure of the enzyme, because their thiols behave as vicinal groups. Their cross-linkage these residues by oxidation to disulfide or by arsenite or Zn^{2+} binding (9,10) did not produce an important conformational distortion of the enzyme. Indeed, cross-linked deaminases between residues 118 and 239 behave kinetically as the enzyme having these cysteines modified with monofunctional substituents.

Kinetics of genetically-modified glucosamine-6-phosphate deaminases.

The kinetic behaviour of the three modified deaminases, from mutants 1, 2 and the double mutant 1,2 is summarized in table II. Data from wild-type enzyme were also included for reference. The double mutation presents the most marked kinetic changes; the apparent catalytical constants, $V_{max}/[E]$, i.e. the microscopic catalytical k_{cat} times the number of sites, changes from 1800 s^{-1} to 900 s^{-1} in the

genetically-modified enzyme, while Km values change from 2.0 to 0.8 mM. Both single mutants present similar changes in their kinetic behaviour, as shown in table II. Being glucosamine-6-phosphate deaminase an allosteric enzyme of the K-type (1), these data were obtained under hyperbolic kinetics, obtained of a saturating concentration of the allosteric activator, GlcNAc6P. In the absence of this ligand, the enzyme from the double mutant displays positive cooperativity, but lower than the wild-type enzyme, with a maximun Hill coefficient of 1.5. The enzyme obtained from mutant 1 displays an unmodified cooperative behaviour, when compared with the natural protein. On the other hand, the enzyme from mutant 2, is distinctly less cooperative, having a Hill coefficient one unit lower.

Kinetics changes produced by the double substuitution of Cys-118 and Cys-239 by serine residues, are essentially the same as those induced by several kinds of chemical reactions modifying the corresponding thiols. This result points out that these kinetic changes are due to the lack of the free thiol pair and are no depending on steric factors or produced by the presence of modifyed chemical groups. Both sulfhydryls, from Cys-118 and Cys-239 are necessary for the full catalytic capacity of the R conformer of the deaminase, but their precise role must be established.

We have already shown that the allosteric transition is interferred by the chemical block of the pair of reactive thiols. The observed change in the value of Hill coefficient, is due to a considerable reduction of the K_T, suggesting that the thiols may have some function in the stabilization of the T-conformer (9,10). Present experimental evidence points out that this function must be attributed mainly to Cys-239, because the . Cys-118 Ser replacement did not affected the allosteric behaviour of the deaminase.

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T A B L E I

Sulfhydryls groups reacting with D'NB , per mole of deaminase subunit.

	NA'IVE		DENA'URED	
Wild-type	2.04	0.09	3.10	0.11
Mutant 1 Cys-118-Ser	0.96	0.08	2.02	0.05
Mutant 2 Cys-239-Ser	1.05	0.13	1.89	0.07
Mutant 1,2 Cys118-Ser:Cys-239-Ser	0.11	0.04	0.96	0.06

TABLE II

KINETIC PARAMETERS OF Cys-Ser MUTANTS OF GLUCOSAMINE 6-PHOSPHATE DEAMINASE

	K_m (mM)		k_{cat} (s^{-1})		h	
Wild-Type	1.92	0.14	1810	45	3.09	0.07
Mutant 1 Cys-118-Ser	0.75	0.02	900	62	3.03	0.08
Mutant 2 Cys-239-Ser	0.85	0.07	846	57	2.10	0.20
Mutant 1,2	0.79	0.03	896	48	1.24	0.12

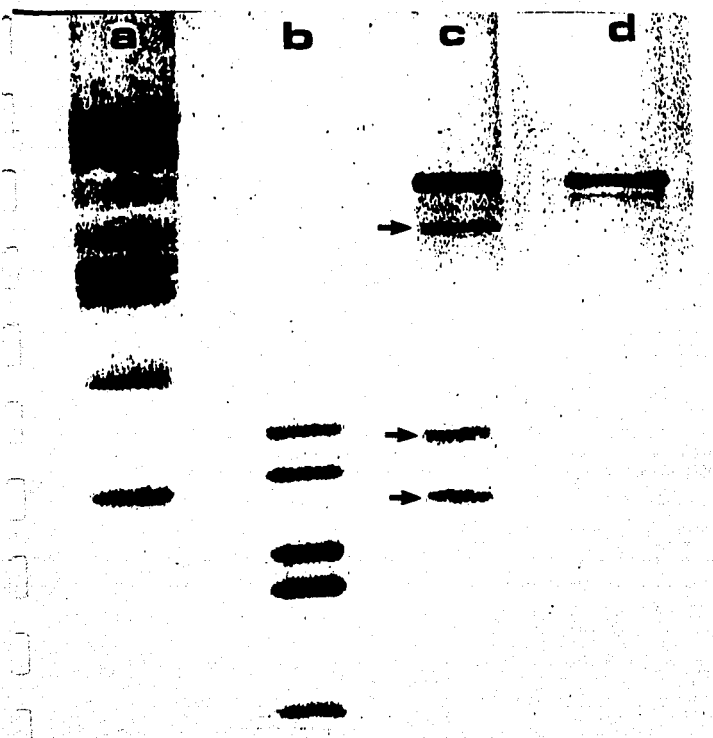
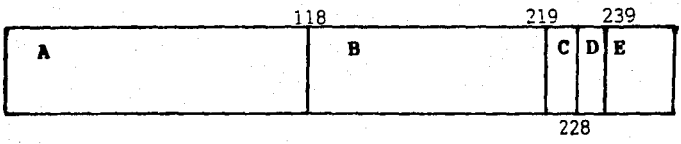


FIGURE !

Figure 2



Mr = 29,846

A = 13,210

B = 11,021

C = 1,090

D = 1,253

E = 3,272

Cleavage fragments

A	13,210	(13 kDa)
BCDE	16,636	(17 kDa)
ABCD	26,574	(27 kDa)
BCD	13,664	(13 kDa)
E	3,272	(3.5 kDa)

Figure 1

Electrophoretical separation of polypeptide chain fragments of E. coli glucosamine-6-phosphate deaminase, obtained by clevage at the selectively cyanylated sulfhydryl groups. Arrows indicate fragments of 27, 17 and 13 kDa.

Figure 2
Schematic representation of the polypeptide chain of E. coli glucosamine-6-phospahate deaminase. The position of cysteinyl residues was indicated, and the fragments between them were designated with letters from A to E, in the sequence order. Calculated molecular weights of the segments is also given. Molecular weights indicated between parenthesis, are estimated values from electrophoresis gels.

ANEXO V

Llamará la atención que en el artículo 1, mencionamos la existencia de 5 residuos de cisteinilo por cadena, mientras que en la enzima proveniente del gen nagB clonado por la Dra. Plumbridge, se encuentran sólo 4. Hemos repetido las titulaciones de tioles en condiciones nativas y reductoras, con las enzimas de ambas cepas, encontrando idénticos resultados: dos grupos SH reactivos en la forma R, y uno oculto, sumamente reactivo en la proteína desnaturalizada. El balance a 4 o 5 cisteinilos, implica la suposición de que existen uniones S-S intra o intercatenarias. Por reducción y diálisis, en 1987 habíamos titulado 4.6 SH totales por cadena, cifra que redondeamos a 5, tomado en cuenta que en la composición de aminoácidos de esta enzima reportada por Calcagno *y col.*[21], se da también esta cifra. Para verificar la existencia de uniones disulfuro, hemos hecho la titulación de Thannhausser *y col.*[45] que revela a la vez grupos SH y S-S. El resultado fue igual en las desaminasas de *E. coli* B y K12: tres cisteinilos reducidos y uno oxidado por cadena, en un total de cuatro. Se trata pues de un error de redondeo en el artículo I.

Estos datos de titulación de disulfuros se complementaron con los de las mutantes Cys Ser en los otros dos residuos de cisteinilo, descartados como componentes del par vecinal, es decir Cys-219 y Cys-228. Nuestros resultados preliminares indican que el primero de estos residuos es el que aparece oxidado, formando una unión entre cadenas. Hemos podido aislar el dímero correspondiente, por electroforesis en gel de poliacrilamida. Esperamos completar próximamente este estudio con la desnaturalización térmica de la enzima nativa y las mutantes de los cisteinilos ocultos.

[45] Thannhauser, T., Konishi, Y. and Scheraga, H. in "Methods in Enzymology" (1987). Vol. 143:115-119.

DISCUSION Y CONCLUSIONES GENERALES

Los principales resultados de este trabajo se refieren a la localización y caracterización funcional de un par de sulfhidrilos de las cisteínas 118 y 239. Los experimentos presentados en esta tesis permiten concluir que se encuentran en posición vecinal en el plegamiento tridimensional de la proteína. Los estudios de dicroísmo circular y diferentes algoritmos de predicción de estructura secundaria de la desaminasa, situán estos residuos, en regiones flexibles, muy hidrofílicas y superficiales (*Artículo IV*).

Conocer el papel de los SH superficiales en la función de la enzima, es una de las preguntas que nos planteamos en casi todos los artículos de esta serie. Si bien no hemos completado la elaboración de un modelo preciso, podemos afirmar los siguientes puntos:

1. No se localizan en el sitio activo, ni participan en la unión de la porción éster fosfato del sustrato, como se ha reportado para la enzima fosfogluconato deshidrogenasa de *Candida utilis* y otras enzimas glucolíticas[46] o de la vía del fosfogluconato. Sin embargo este par de tioles parece encontrarse en las proximidades del sitio activo, ya que su bloqueo con grupos polares voluminosos inactiva la enzima (*Artículo I*).

2. Su condición semiesencial para la actividad de la enzima se relaciona con su carácter de par vecinal. El efecto de su bloqueo con grupos pequeños: monofuncionales (-CH3, -CN), bifuncionales (arsenito, cationes divalentes), su oxidación a disulfuro o su reemplazo por serinas, no interfiere con la unión de los ligandos al sitio activo, puesto que no se modifica significativamente el Km para la GlcN6P, pero sí afecta el mecanismo catalítico, ya que la kcat,

[46] Rippa, M., Bellini, T., Signorini, M y Dalocchio, F. (1981). J. Biol. Chem. 256:451-455.

disminuye a exactamente a la mitad con respecto a la enzima control *(Artículos I, II, III, V)*. Si bien no se ha establecido con certeza el mecanismo cinético de la enzima, existen evidencias a favor de un mecanismo de equilibrio rápido al azar [47]. En ausencia de datos termodinámicos, esto permite manejar las Km como indicadores de la afinidad de los sitios. Es posible entonces que estos grupos sulfhidrilo participen en el mecanismo de la enzima en forma indirecta, jugando un papel semiesencial en los cambios conformacionales durante su ciclo catalítico.

3. Con respecto a su participación en la conformerización alostérica, la interpretación es más compleja. Se detecta también una función semiesencial, ya que tanto su bloqueo químico, como su reemplazo por serina, disminuye la cooperatividad homotrópica con respecto a la GlcN6P, y por lo tanto, el margen de activación por GlcNAc6P, *(Artículos II, III)*. Este mismo efecto se manifiesta, en la doble mutante y en la mutante simple del residuo 239. El cambio por serina de la cisteína 118, no produce cambios homotrópicos significativos *(Artículo V)*. Esto indica que en esta función, ambos residuos no son equivalentes.

Un posible modelo, podría incluir un papel predominante de la cisteína 239 en la estabilidad del confórmero alostérico menos activo (forma T, en la nomenclatura de Monod), y un efectoparitario de ambos grupos en la forma R, en algún estado conformacional asociado al ciclo catalítico, e independiente del equilibrio alostérico. Un argumento a favor de la participación de la cisteína 239 en la estabilidad del confórmero T, es la disminución del Kt en la enzima modificada, como si ésta quedase en un estado intermedio entre R y T, sin poder alcanzar plenamente la conformación menos activa. Sin embargo es importante no perder de vista que los cambios más sobresalientes en la cooperatividad se observaron cuando se bloquean ambos tioles. Como si

[47] Calcagno, M .y col. datos no publicados.

la Cys 118 por si sola no pudiera producir una interacción estabilizadora del confórmero T, pero contribuyera a esta estructura cuando está presente el tiol de la Cys 239.

Los tioles de ambos cisteínilos constituyen un buen marcador de la transición alostérica. En el tercer artículo, se aprovecha esta propiedad para estudiar la interacción con el zinc. Es precisamente la gran afinidad que tienen estos grupos SH por el zinc lo que nos lleva a considerar que, el efecto del bloqueo de estos grupos sulfhidrilo vecinos, no es simplemente un hallazgo experimental, sino que podría ser un mecanismos de regulación de la actividad de la enzima, a través de su interacción con el zinc. Un hecho en favor de esta posibilidad, es el siguiente: cuando no conocíamos la existencia de estos grupos vecinales, y la enzima no se ensayaba en presencia de EDTA, el coeficiente de Hill, variaba de un lote de enzima a otro, y era frecuente encontrar que reactivos quelantes de metales como el ADP y el ATP, aumentaban la cooperatividad. A partir de la introducción del EDTA en la mezcla de reacción para ensayo de la desaminasa, la cooperatividad homotrópica no varía, y el papel de los nucleótidos como posibles moduladores alostéricos negativos, ha sido descartado.

Una interpretación unificadora de los cambios cinéticos observados cuando se modifican los tioles vecinales, se discute en el artículo II, ésta se refiere a la posibilidad de que el bloqueo de tioles produzca un cambio en la enzima que sea a su vez la causa de un comportamiento conocido como "cinética de medios sitios" (half of the sites reactivity)[48], que ha sido observado en algunas proteínas oligoméricas. Una manifestación de este comportamiento son las curvas de saturación bifásicas, que revelan la existencia de sitios de diferente afinidad. También se han observado cinéticas de modificación químicade residuos ubicados en sitios ligantes con reactividad bifásica, y en algunos casos basta modificar la mitad de los grupos

[48] Hertzfeld, J., Ichiye, T y Jung, D., (1981). Biochemistry 20: 4936-4941.

para inactivar por completo a la enzima[49]. Este comportamiento se ha observado en tetrámeros y hexámeros, y revela una asimetría preexistente en la molécula. En la desaminasa, esta asimetría podría ser generada como consecuencia del bloqueo de los grupos SH vecinales, si el número de sitios considerado de por lo menos 6 en la enzima nativa, disminuye a 3 en las proteínas modificadas. En esta situación la constante catalítica aparente, disminuiría a la mitad sin que sea necesario postular un cambio en la kcat microscópica. Esta cinética de medios sitios inducida por el bloqueo de los tioles, puede explicar por si misma el cambio en la cooperatividad homotrópica. La dependencia de la cooperatividad con respecto al número de sitios es evidente, y puede verificarse por simulación. Con los parámetros alostéricos de la desaminasa nativa que se presenta en el artículo 2, (Kr = 2 mM; Kt = 65 mM, y seis sitios). hemos simulado la curva de velocidad específica en función de la concentración específica de GlcN6P, obteniendo una curva de cooperatividad positiva, con un coeficiente de Hill máximo del orden de tres. Este coeficiente cambia a 1.5, cuando se simula la ecuación con los mismos valores de Kr y Kt, pero con la mitad de los sitios. Estos resultados son congruentes con los obtenidos experimentalmente con las enzimas modificadas que tienen un coeficiente de Hill entre 1.5 - 1.6.

Sólo tenemos evidencias cinéticas de un número de sitios activos igual a seis y no hemos verificado directamente este dato en la enzima nativa y en las modificadas. Sin embargo el modelo de la mitad de los sitios no deja de ser atractivo, porque representa una interpretación única de los dos tipos de cambios cinéticos, generados por el bloqueo de los tioles.

4. La predicción de estructura secundaria y los estudios de dicroísmo circular, aportan resultados que en conjunto permiten ubicar taxonómicamente a la desaminasa, en la familia de las proteínas α/β, *(Artículo IV)*. Destacan en primer lugar, la alternancia de segmentos α

[49] Macquarri, R. y Bernard, S.(1971). J. Mol. Biol. 55:181-192.

con segmentos E, el carácter hidrofóbico de las hojas beta plegadas, y el carácter anfipático de un gran porcentaje de hélices alfa. Es interesante destacar la localización de los grupos SH de la desaminasa, en estructura secundaria obtenida en esta predicción. Sólo dos cisteínas la 118 y 239, se encuentran en la superficie de la proteína, mientras que la 219 y la 228, se localizan en regiones muy hidrofóbicas y predichas como ocultas. Estos resultados teóricos se correlacionan con los obtenidos experimentalmente en el artículo V en el que se demuestra que las cisteínas 118 y 239, son las que forman el par de tioles vecinales.

Las sustitución de los tioles vecinales de las cisteínas 118 y 239 podrían producir un cambio de conformación que afecte algún paso intermediario del mecanismo catalítico. Tomando como referencia el mecanismo de Rose[20] (Fig. 3), podríamos preguntarnos si en las mutantes cambia la accesibilidad al agua de las formas protonadas intermedias o la capacidad de la proteína para estabilizar la *cis*-enolamina intermediaria.

PERSPECTIVAS

El estudio de los grupos sulfhidrilo de la glucosamina 6 fosfato isomerasa no es un capítulo cerrado. Existen aún preguntas importantes que permanecen sin respuesta, y que vale la pena intentar resolver.

1. El cambio en la cooperatividad homotrópica, y la disminución de la constante catalítica de la desaminasa, ¿son la consecuencia de un hecho único, como por ejemplo la cinética de medios sitios, o son dos fenómenos independientes?

2. Si son fenómenos independientes, ¿sólo una de las cisteínas es importante en la estabilización del confórmero tenso?

3 ¿ Cuáles son los grupos que interaccionan con los sulfhidrilo en el confórmero menos activo?.

4. ¿Cuál es el microambiente en el que queda atrapado el zinc cuando se produce la transición alostérica?

Para responder estas preguntas se proponen los siguientes experimentos:

Utilizar las mutantes de los grupos sulfhidrilo, para continuar el estudio cinético iniciado en el artículo IV, en especial para comprobar si únicamente la mutante 239 es la que influye en el cambio de cooperatividad homotrópica. También Estudiar el posible cambio en la cooperatividad heterotrópica de las enzimas modificadas genéticamente por medio del estudio de la unión del activador alostérico.

Aprovechar la capacidad de estos grupos tioles de unir metales divalentes para realizar estudios de resonancia magnética nuclear, con Cadmio 113, para conocer el microambiente de esta región a través de las interacciones del metal y al mismo tiempo utilizar estas señales para monitorizar la transición alostérica.

Cuantificar directamente por diálisis en equilibrio el número de sitios activos en la enzima nativa y en la doble mutante, para verificar o descartar la hipótesis de una cinética de la mitad de los sitios inducida por el bloqueo de los tioles.

Realizar un estudio semejante al de Rose[20], de cinética de intercambio de Tritio con el agua, para valorar los pasos intermediarios en el ciclo catalítico, y su posible modificación en las mutantes.

Por otra parte la posibilidad de bloquear reversiblemente estos grupos sulfhidrilo, permitirán realizar estudios de otros residuos, con reactivos que reaccionan inespecíficamente con los SH. La hélice alfa en la que se localiza la cisteína 118 resulta una zona de la proteína muy interesante para futuros estudios. Es la única hélice hidrofílica y flexible de la molécula, cuyos residuos interaccionan todos con el medio. Probablemente al igual que las cisteínas, estos aminoácidos tengan un papel funcional definido bien puedan utilizarse como monitores de los cambios conformacionales de la enzima.

Cada uno de nosotros no es más que un hombre, un intento, alguien a medio camino. Pero debe estar a medio camino en la dirección de lo perfecto, debe tender al centro, no a la periferia.
Recuérdalo: se puede ser un lógico estricto o un gramático y, al mismo tiempo, estar colmado de fantasía y de música. Se puede ser músico o jugador de abalorios y, contemporáneamente, estar entregado por entero a la ley y a la regla. El hombre que imaginamos y queremos, que es nuestra meta llegar a ser, debería poder cambiar todos los días su ciencia o su arte por otro cualquiera, dejaría resplandecer en el juego de abalorios la lógica más cristalina y en la gramática la fantasía más ricamente creadora. Así tendríamos que ser, tendríamos que poder ser colocados a cada hora en distinto lugar, sin que nos opusiéramos o nos confundiéramos.

Magister Musicae,
en El Juego de Abalorios
Hermann Hesse.