GLUCOSAMINA-6-FOSFATO DESAMINASA DE RIÑON DE PERRO:

PROPIEDADES CINETICAS Y MOLECULARES.

T E S I S

QUE PARA OBTENER EL GRADO DE

MAESTRO EN CIENCIAS BIOMEDICAS

(BIOQUIMICA)

presenta

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PRESENTACION.

Este trabajo ha sido estructurado de la siguiente manera: un resumen, una sección introductoria en la que se detallan los antecedentes, la importancia y los objetivos del trabajo, con un especial énfasis en la purificación de la enzima. Posteriormente se incluye el manuscrito del artículo enviado para su publicación a la revista *Biochimica et Biophysica Acta,* y que actualmente se encuentra en revisión. En él se incluyen los aspectos metodológicos empleados en el trabajo, los resultados y la discusión de los mismos, así como las figuras con sus leyendas y la bibliografía. En el siguiente apartado se presentan una discusión general y las conclusiones de la tesis, seguidas de las perspectivas de desarrollo futuro de este trabajo. Finalmente se encuentra una sección de anexos en los que se detallan aspectos metodológicos no incluidos en el artículo, pero que fueron desarrollados durante la tesis, así como la deducción de la ecuación para construir un gráfico que nos permitió determinar la constante de disociación del activador alostérico de la enzima, derivada del modelo concertado de Monod. Se encuentra también un anexo en donde se detalla la obtención del peso molecular de la enzima, y finalmente, se incluyen dos artículos publicados colateralmente durante los estudios de la maestría.

RESUMEN.

En este trabajo describimos el primer procedimiento de purificación que permite obtener, en forma pura y homogénea, la enzima glucosamina-6-fosfato desaminasa (E.C. 5.3.1.10) a partir de un tejido animal, en este caso, corteza renal de perro. Se demostró la pureza de la enzima obtenida mediante diversos procedimientos. La estrategia de la purificación se basó principalmente en dos pasos bioespecíficos de cromatografía de afinidad, uno de ellos usando N-ε-amino-n-hexanoil-D-glucosamina-6-fosfato inmovilizada en agarosa, como un análogo del ligando alostérico de la enzima, y el otro mediante la unión de la enzima a fosfocelulosa, la cual se comportó como una cromatografía de afinidad por el sitio activo de la enzima.

La proteína es una molécula hexamérica, con peso molecular alrededor de 180 kDa, compuesta de subunidades de 30.4 kDa; su punto isoeléctrico fue 5.7. El coeficiente de sedimentación fue de 8.3S, y con una relación friccional 1.28. La enzima mostró cooperatividad homotrópica positiva hacia la D-glucosamina-6-fosfato (*hmax* = 2.1, a pH 8.8). La cooperatividad fue completamente abolida mediante concentraciones saturantes de N-acetil-D-glucosamina-6-fosfato; este modulador alostérico activó la reacción con un efecto K típico. Bajo condiciones de cinética hiperbólica, obtuvimos un valor de K$_M$ de 0.25 ± 0.02 mM, para la D-glucosamina-6-fosfato. Suponiendo seis sitios catalíticos por molécula, el valor de la *kcat* es 42 s^{-1} por sitio. Los datos de

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velocidad en función de la concentración de sustrato tuvieron un buen ajuste al modelo alostérico de Monod, para el caso de fijación exclusiva, tanto para el sustrato como para el activador, con dos sitios interactuantes para el sustrato. Se determinó el valor de la K_{DIS} para el activador N-ε-acetil-D-glucosamina-6-fosfato, el cual fue de 14 μM.

SUMMARY

Glucosamine-6-phosphate deaminase (E.C. 5.3.1.10), from dog kidney cortex, was purified to homogeneity, as judged by several criteria of purity. The purification procedure was based on two biospecific affinity chromatography steps, one of them using N-ε-amino-n-hexanoyl-ᴅ-glucosamine-6-phosphate agarose as an immobilized analog of the natural allosteric ligand, and the other by binding the enzyme to phosphocellulose, which behaved as an active-site affinity chromatography. The enzyme is an hexameric protein of about 180 kDa, composed of subunits of 30.4 kDa; its isoelectric point was 5.7, and its sedimentation coefficient was 8.3S. The frictional ratio was 1.28, indicating that dog deaminase is a globular protein. The enzyme displays positive homotropic cooperativity towards ᴅ-glucosamine-6-phosphate ($hmax$ = 2.1, pH 8.8). Cooperativity was completely abolished by saturating concentrations of N-acetyl-ᴅ-glucosamine-6-phosphate; this allosteric modulator actived the reaction with a typical K-effect. Under hyperbolic kinetics, a K_M value of 0.25 ± 0.02 mM for ᴅ-glucosamine-6-phosphate was obtained. Assuming six catlytic sites per molecule, $kcat$ is 42 s^{-1}. Substrate-velocity data were fitted to the Monod's allosteric model for exclusive-binding case for both substrate and activator, with two interacting substrate sites. The K_{DIS} for N-acetyl-ᴅ-glucosamine-6-phosphate was estimated in 14 μM.

INTRODUCCION.

INTRODUCCION.

La purificación de una proteína, especialmente cuando se trata de una enzima, puede ser abordada desde varios puntos, sin embargo, los procedimientos tradicionalmente usados suelen ser muy lentos, por lo que no solamente consumen tiempo, sino que exponen a la proteína que nos interesa a la acción de factores adversos, principalmente las proteasas endógenas. En este sentido, la cromatografía de afinidad ha contribuido en una forma significativa para lograr procedimientos de purificación sencillos y rápidos. La rapidez en un procedimiento de purificación es esencial, ya que nos permite confiar en que la proteína que estamos obteniendo es muy parecida a cómo se encontraba dentro de la célula, sin modificaciones que alteren significativamente sus propiedades cinéticas y moleculares. Esta es la razón por la cual muchas enzimas, que son demasiado inestables durante el proceso de purificación, no han podido ser estudiadas y conocidas de una manera más detallada. En esta situación se encontraba la glucosamina-6-fosfato desaminasa de tejidos animales, la cual es objeto de estudio en el presente trabajo.

La enzima glucosamina-6-fosfato isomerasa desaminasa, (2-amino-2-desoxi-ᴅ-glucosa-fosfato cetol isomerasa, desaminante, E.C. 5.3.1.10), que cataliza la conversión reversible de la glucosamina-6-fosfato (GlcN6P) en fructosa-6-fosfato (Fru6P) y amonio (ver más adelante), fue descubierta hace casi 40 años por

Leloir y Cardini[1] en corteza renal de cerdo. A pesar de ello, la desaminasa ha sido mejor estudiada en microorganismos, en especial en *Escherichia coli*[2,3,4]. En esta bacteria el gen *nag B*, que codifica para la desaminasa, se encuentra formando parte de un regulón divergente *nagE-nagBCD*[5,6,7,8]. Este regulón codifica para varias proteínas relacionadas con el transporte y el catabolismo de la D-glucosamina (GlcN) y la N-acetil-D-glucosamina (GlcNAc), así como una proteína represora del propio regulón. El marco de lectura abierto (ORF) denominado *nagD*, carece de función conocida. La secuenciación del gen de la desaminasa de *E.coli*, ha permitido profundizar más en el conocimiento de la enzima de esta bacteria, por lo que actualmente se cuenta ya con una información estructural y funcional muy amplia de la desaminasa de esta especie[9].

Se sabe también que la desaminasa es una enzima clave en el metabolismo de los aminoazúcares, y que en bacterias, esta enzima

[1] LELOIR, L. F. AND CARDINI, C. E. (1956). BIOCHIM. BIOPHYS. ACTA, 20:33-42.

[2] COMB, D. G. AND ROSEMAN, S. (1958). J. BIOL. CHEM. 232:807-827.

[3] MIDELFORT, C. AND ROSE, I. A. (1977). BIOCHEMISTRY, 16:1590-1596.

[4] CALCAGNO, M., CAMPOS, P.J., MULLIERT, G. AND SUASTEGUI, J.,(1984) BIOCHIM. BIOPHYS. ACTA, 787:165-173.

[5] WHITE, R.J. (1968) BIOCHEM J. 106:847-853.

[6] ROGERS, N,J., OGHI, T., PLUMBRIDGE, J. AND SOLL, D. (1988) GENE, 62:197-207.

[7] VOGLER, A.P. AND LENGELER, J.W. (1989) MOL. GEN. GENET.,219:97-105.

[8] PLUMBRIDGE, J.A. (1989) MOL. MICROBIOL., 23:505-515.

[9] ALTAMIRANO, M. M., PLUMBRIDGE, J.A., HERNANDEZ-ARANA, A. AND CALCAGNO, M.L. (1991) BIOCHIM. BIOPHYS. ACTA, 1076:266-272.

está sujeta a mecanismos de control tanto genéticos como alostéricos[3,6], siendo moduladores alostéricos positivos la N-acetil-D-glucosamina-6-fosfato (GlcNAc6P)[2,4], y el ión H^{+}[10].

Aun cuando la desaminasa está presente en varios órganos o tejidos animales[11,12], antes del presente trabajo, la enzima no había podido ser obtenida en forma completamente pura y homogénea de fuentes no microbianas. Hasta la fecha, solamente se han dado a conocer algunas purificaciones parciales de esta enzima a partir de tejidos animales: riñón de cerdo[2,13], cerebro humano y de rata[14,15], riñón de rata[16], asi como de moscas[17,18]. Una de las razones de esta situación es quizá la gran inestabilidad de la proteína durante la purificación[16]. A pesar de ello, en estos trabajos de purificaciones parciales, pudo determinar que la desaminasa de animales, al igual que la de la mayoría de los microorganismos, es una enzima alostérica, activada también por la GlcNAc6P;

[10] ALTAMIRANO, M.M. Y CALCAGNO, M. EN PREPARACION.

[11] NOLTMAN, E. A. (1972) IN THE ENZYMES, 3 RD EDN. (BOYER, P.D. ED.) VOL 6, PP 314-318, ACADEMIC PRESS, NEW YORK.

[12] SUKENO, T., KIKUCHI, H., SAEKI, H., AND TSUIKI, S. (1971) BIOCHIM. BIOPHYS. ACTA 244,19-29.

[13] LELOIR, L. F. AND CARDINI, C.E. (1962) METHODS IN ENZIMOL. 5,418-422.

[14] PATTABIRAMAN, T. N. AND BACHHAWAT, B. K. (1961) BIOCHIM. BIOPHYS. ACTA. 54, 273-283.

[15] FAULKNER, P. AND QUASTEL, J. H. (1956) NATURE 177,1216-1218.

[16] KIKUCHI, K. AND KIKUCHI, H. (1979) SCI. REP. RES. INST. TOHOKU UNIV.-C. 26, 92-97.

[17] BENSON, R. L. AND FRIEDMAN, S. (1970) J. BIOL. CHEM. 245, 2219-2228.

[18] ENGHOFER, E. AND KRESS, H. (1980) DEP. BIOL. 78, 63-75.

sinembargo, no se conocen con certeza las características moleculares y fisicoquímicas (peso molecular, punto isoeléctrico, etc.) de la enzima de fuentes animales. Tampoco contamos con información confiable acerca de las constantes cinéticas de la enzima, y por lo tanto, la función que ésta pueda desempeñar en los tejidos animales. No obstante, a este respecto, y a pesar de que el equilibrio de la reacción favorece al sentido catabólico (ver más adelante), en algunos de los trabajos antes citados se ha señalado, que la desaminasa puede jugar un papel biosintético dentro del metabolismo de los aminoazúcares en los tejidos de los animales, (como Pattabiraman en cerebro humano y de rata[14], Benson y Friedman en moscas adultas[17], y Enghofer y Kress en pupas de *Drosophila*[18]). Los argumentos que se presentan para adjudicar una función biosintética a la desaminasa son dos: primero, no se ha detectado actividad de la GlcN6P sintetasa en los tejidos estudiados, solamente encuentran actividad de la desaminasa. En segundo lugar, la constante de equilibrio indica que la desaminación es facilmente reversible si la siguiente reacción de la vía es exergónica, (como podría ser el caso de la reacción de acetilación de la GlcN6P por medio de acetil coenzima A, catalizada por la GlcN6P acetil transferasa, E.C. 2.3.1.3.)[19].

El problema entonces está delimitado: no contamos con datos suficientemente claros o confiables acerca de la desaminasa de

[19]DAVIDSON, E. A., BLUMENTHAL, H. J. AND ROSEMAN, S. J. BIOL.CHEM. (1957) 226,125.

animales para poder establecer con certeza sus propiedades y las implicaciones fisiológicas de la actividad de esta enzima.

Finalmente, la gran mayoría de las purificaciones de la desaminasa de animales publicadas son ya antiguas, y los procedimientos empleados resultan actualmente obsoletos e inoperantes, por ejemplo, la precipitación con sulfato de amonio, que implica el empleo de diálisis o columnas de Sephadex para eliminar las sales, procedimientos en los que la proteína puede sufrir alteraciones por proteólisis, debido al tiempo prolongado que requieren estos procedimientos, así como también las precipitaciones con acetona, tratamientos térmicos etc., que han sido utilizados en los intentos para purificar la desaminasa de tejidos animales, y que sin duda, producen algún tipo de modificación en la proteína.[20]

Tomando en cuenta estos antecedentes, y el hecho de que en nuestro laboratorio tenemos amplia experiencia con la desaminasa de *E. coli*, particularmente en su purificación por cromatografía de afinidad, nos propusimos purificar la glucosamina-6-fosfato desaminasa de corteza renal de perro, diseñando una ruta de purificación que evite el daño de la enzima durante el desarrollo del proceso. Una enzima pura y libre de modificaciones, (como daño proteolítico), permitirá un estudio estructural y cinético en condiciones rigurosas, lo cual no se ha podido hacer hasta ahora

[20] SCOPES, R.K. PROTEIN PURIFICATION, PRINCIPLES AND METHODS. SECOND EDITION, SPRINGER-VERLAG, NEW YORK, 1987.

con una desaminasa de origen animal.

Este estudio nos permitirá aclarar varios de los puntos anteriormente mencionados acerca de la enzima de animales, en este caso de mamíferos; también nos permitirá vislumbrar el posible papel fisiológico que pudiera estar desempeñando esta enzima en el riñón. Su predominio en este órgano, en comparación con otros[1] (tabla A), nos permite plantear que la expresión de esta proteína en la corteza renal, señala al riñón como un órgano central en el catabolismo de los aminoazúcares.

También resulta atractiva la comparación de la desaminasa de riñón con la desaminasa de *E. coli*, hasta ahora mucho mejor conocida. Este punto resulta particularmente interesante en relación con la regulación de este paso metabólico.

Finalmente, la gran importancia del presente trabajo radica en el desarrollo de una estrategia de purificación adecuada, que pueda ser tomada como base para la futura purificación de otras enzimas relacionadas estructural y funcionalmente con la desaminasa, y con la que comparten algunos ligandos. Cabe recordar que la N-acetil-ᴅ-glucosamina 6-P desacetilasa está en un estado semejante a la desaminasa en cuanto a su purificación de animales, y que se sabe muy poco de las N-acetil transferasas que convierten la GlcN6P en GlcNAc6P.

T A B L A   A .

Determinación de actividad de la desaminasa en diferentes órganos.

O R G A N O	ACTIVIDAD UNIDADES/MG
Riñón	10.0
Cerebro	2.2
Intestino	1.0
Hígado	0.5
Pulmón	0.4

Ubicación de la enzima dentro del metabolismo de los aminoazúcares.

En los animales, los aminoazúcares pueden encontrarse en diversos órganos[21,22]. Su importancia radica en que son precursores de polímeros estructurales como los proteoglicanos, o de las cadenas de oligosacárido de los glicolípidos. Los aminoazúcares participan de esta forma en la composición de sustancias importantes como la mielina en el sistema nervioso[23,24].

Por otro lado, los aminoazúcares están presentes en muchas glicoproteínas, las cuales pueden cumplir funciones diversas[25,26,27]. También son precursores del ácido siálico, y son constituyentes de la heparina.

Las vías metabólicas en relación con los aminoazúcares han sido establecidas desde hace ya mucho tiempo. Los progresos

[21] BRANTE, G. IN D. RICHTER, METABOLISM OF THE NERVOUS SYSTEM, (1957) PERGAMON PRESS, LONDON, P. 112.

[22] ISUMI, K., SATO, M., HAYASHI, K., TAMURA, M., AND YAMASHINA, I. (1963) J. BIOCHEM. 54, 530-.

[23] BRANTE, G., IN F. BRUCKE, BIOCHEMISTRY OF CENTRAL NERVOUS SYSTEM, (1959) PERGAMON PRESS, LONDON, P. 291.

[24] GUHA, A., NORTHOVER, B. J., AND BACHHAWAT, B. K. (1960) J. SCI. IND. RESEARCH 19-C, 287.

[25] WATKINS, W. M. IN GOTTSCHALK, A. GLYCOPROTEINS PART B, (1972) ELSEVIER PUB. CO. AMSTERDAM, P 832.

[26] JUTISZ, M., AND DE LA LLOSA, P., IN GLYCOPROTEINS PART B (1972) ELSEVIER PUB. CO AMSTERDAM, PP 1019-1039, AND 1039-1056.

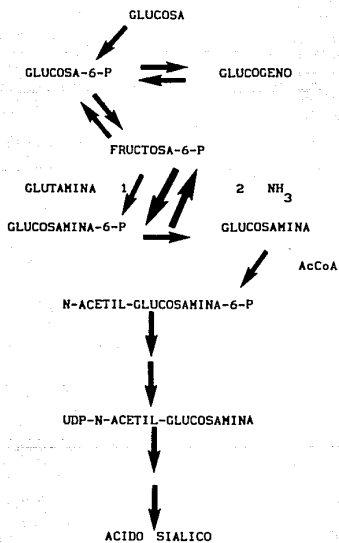[27] MARTIN, D. W.Jr EN BIOQUIMICA DE HARPER, (1986) 10a ED, EL MANUAL MODERNO, MEXICO, P 471.

recientes han consistido princialmente, en la descripción de la organización de los genes en *Escherichia coli*, por parte de Jacqueline Plumbridge, del IBPC, en París, los estudios sobre la desaminasa por parte de nuestro grupo, y de la glucosamina-6-P sintetasa, por parte del grupo de Bernard Badet, en en Instituto Curie de París, todos estos avances se refieren también a la bacteria *Escherichia coli*. Los progresos en relación con eucariotes, particularmente mamíferos, han sido escasos. Las enzimas correspondientes han resultado muy difíciles de purificar, debido a que generalmente son más inestables que las de fuentes bacterianas; por otra parte, la presencia de isoenzimas en tejidos animales, puede complicar el aislamiento de cada enzima en forma homogénea.

Actualmente sabemos que los aminoazúcares derivan de la glucosa, que es desviada del flujo glucolítico hacia la vía metabólica de los aminoazúcares para formar glucosamina, de la cual a su vez derivan todos los aminoazúcares. La glucosamina (GlcN) se forma a partir de la Fru6P, como el éster fosfórico en C-6, a través de dosreacciones distintas: (1 y 2 fig. I)[28]. La primera es irreversible, y es catalizada por la enzima glucosamina-6-fosfato isomerasa (dependiente de glutamina), también conocida como glucosamina-6-fosfato sintetasa (E.C.5.3.1.19) (antes: Glutamina-Fructosa 6-P amidotransferasa,

[28] WARREN, L., IN GOTTSCHALK, A., GLICOPROTEINS PART B (1972) ELSEVIER PUB. CO. AMSTERDAM, P 1099.

F I G U R A 1 .

Esquema general del metabolismo de los aminoazúcares.


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                        GLUCOSA
                          |
                          v
      GLUCOSA-6-P   ⇌    GLUCOGENO
          ⇅
      FRUCTOSA-6-P

 GLUTAMINA   1          2   NH₃
                ⇅ ⇅
GLUCOSAMINA-6-P   ⇌   GLUCOSAMINA
                            |
                            v   AcCoA

      N-ACETIL-GLUCOSAMINA-6-P
                |
                v

      UDP-N-ACETIL-GLUCOSAMINA
                |
                v

           ACIDO SIALICO
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E,C. 2.6.1.16). Esta enzima usa un grupo amino donado por la glutamina.

La segunda reacción (2, fig. I), catalizada por la glucosamina-6-fosfato desaminasa, es reversible y el amonio es el donador del grupo amino; el equilibrio favorece al sentido desaminante de la reacción[4]. Esta enzima se localiza en una encrucijada metabólica con la glucólisis (fig, I), con la cual comparte un metabolito común, la Fru6P. Se puede observar que en los organismos que sintetizan aminoazúcares, también existen procesos metabólicos que permiten utilizar los carbonos de estos sustratos como fuente energética.

Sin embargo, el destino del amonio, producto de la reacción en el sentido catabólico de la desaminasa no está del todo claro. En relación con este aspecto, se sabe que la regulación renal del equilibrio ácido-base mediante la excreción de amonio está basado en el metabolismo renal de la glutamina, la cual es extraida del plasma por el riñón, y constituye la principal fuente de amonio que amortigua los iones hidrógeno excretados hacia la orina[29]. La enzima que participa mayoritariamente en esta producción renal de amonio, es la glutaminasa I.

Resulta entonces interesante considerar el sentido desaminante de la reacción catalizada por la desaminasa renal, ya que puede desempeñar algún papel en este aspecto de la función renal.

[29] PITTS,   R.   F.,   PILKINGTON,   L.   A.,   MacLEOD,   M.   B.   AND   LEAL-PINTO, E. (1972) J. CLIN. INV. 51,557-565.

Reacción catalizada por la enzima.

La reacción catalizada por la glucosamina-6-fosfato desaminasa es la siguiente:
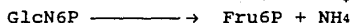
$$GlcN6P \longrightarrow Fru6P + NH_4^+$$

La constante de equilibrio, definida como $(Fru6P)(NH_4)/(GlcN6P)$, es de $0.18M$[4].

Mecanismo Químico.

En relación al mecanismo químico de la reacción anteriormente mencionada, se han postulado una serie de pasos, los cuales se muestran en la fig. II, y que corresponden al mecanismo de las isomerasas cetoaldólicas propuesto por Rose[30].

Al iniciar el ciclo catalítico, el amonio pierde un protón, y el NH_3 ataca al carbono carbonílico de la Fru6P. Se forma así un aminoalcohol intermediario que se deshidrata. Esto resulta en la enolización de la Fru6P que formando una enolamina intermediaria, (probablemente de configuración *cis*). Dicha enolización puede ponerse de manifiesto por medio del intercambio isotópico entre un tritio en posición $1(R)$ en la $1-^3H-Fru6P$ y el agua, lo que necesita la presencia en el sistema de NH_4^+. La enolamina es la

[30] ROSE, I. (1975) ADV. ENZYMOL. 43,491-502.

forma enólica del producto, es decir la GlcN6P.

Cuando se incuba Fru6P y NH4Cl en agua tritiada en presencia de la enzima, se recupera una Fru6P con una cantidad prácticamente estequiométrica de tritio incorporado, lo cual significa que la enzima actúa en forma estereoespecífica para el intercambio de uno solo de los dos hidrógenos del C-1 de la Fru6P. Cuando se hace actuar la glucosa 6-fosfato isomerasa (E.C. 5.3.1.9) sobre la Fru6P marcada de esta manera, la marca se pierde completamente hacia el agua.

Ya que la estereoespecificidad de esta enzima es conocida, podemos inferir que la especificidad de la desaminasa produce la movilización del mismo hidrógeno sustituyente en C-1. que se identifica con el H pro R. Este hidrógeno, es por lo tanto proquiral.

La estereoquímica de la reacción en el C-1 de la Fru6P, en relación con la especificidad estereoquímica en R para la posición C-2, es la típica de estas isomerasas cetoaldólicas.

Mecanismo cinético.

En cuanto al mecanismo cinético de la enzima, sólo existen estudios cinéticos con la enzima de *E. coli*. Las dificultades técnicas son importantes, porque es necesario medir las velocidades de reacción en el sentido biosintético (formación de GlcN6P), y no disponemos de un procedimiento satisfactorio para hacerlo. A pesar de estas limitaciones, se han reunido evidencias

figII
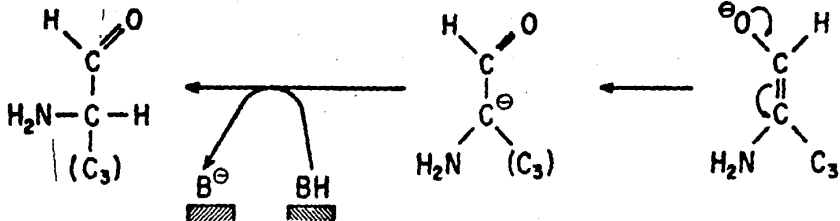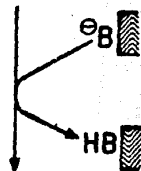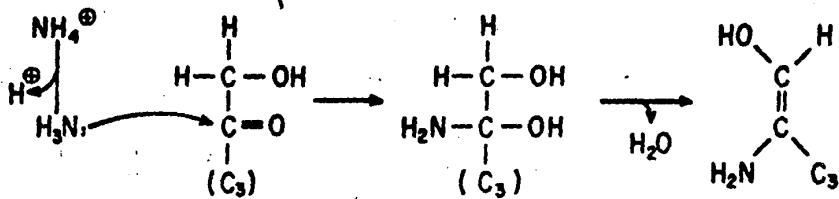
cinéticas que sugieren que se trata de un mecanismo del tipo
"equilibrio rápido al azar"[31].

[31] MULLIERT, G. Y CALCAGNO, M.L., RESULTADOS NO PUBLICADOS

A R T I C U L O .

MANUSCRITO ENVIADO A B.B.ACTA

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Ref # : RPP 025299

Dear Dr. Calcagno:

On behalf of the Managing Editors of *Biochimica et Biophysica Acta*, we would like to thank you for submitting the article entitled "Purification and characterization of glucosamine-6-phosphate deaminase from dog kidney cortex" by Altamirano, Lara-Lemus, Libreros-Minott, and yourself.

We have received the enclosed comments from the reviewers. Reviewer I points to the need for further evidence concerning the kinetic studies. If this can be provided and other criticisms of the reviewers met, we will consider a revised version of the manuscript. We ask that the manuscript be edited prior to any resubmission by a colleague skilled in the use of written English (preferably as a mother tongue).

An original and two copies of any amended version should be returned. The changes and alterations that have been made should be indicated in red ink on one copy of the revised manuscript. In your covering letter please give a point-by-point response to the reviewers' comments and detail the changes made. The top copy and a reviewer's annotated copy of your paper is enclosed.

Yours sincerely,

Cori Beychok

Cori Beychok
Editorial Secretariat

COMMENTS

This paper would probably be acceptable, in this reviewers opinion, providing some key points are clarified and the quality of its English improved, particularly the Discussion section.

My major criticism of this work is the lack of evidence that the kinetics are based on initial velocity determinations. It appears that only single time point values were used in developing the data. Do the authors have evidence that these points are on the linear portion of the velocity curve? When a single time assay is used enzyme stability could be a contributing factor to the inferred initial velocity values. There is no point in presenting an involved kinetic analysis to provide Hill constants and allosteric effects unless the data is based on accurate initial velocity measurements.

A more minor point is concerned with the enzymes purification. It would be easier to repeat the authors' purification scheme if volumes of solution were given at each step.

Numerous literary corrections are required, some of which are indicated below.

p. 9, line 7 from bottom - to remove ligands, instead of to get rid of.

p. 12, line 13 from bottom - replace the first procedures with for the first time. Line 10 from bottom - omit the. Line 8 and 9 from bottom - which takes advantage of enzyme binding to an... Line 5 from bottom - but it did not yield the dog kidney...

p. 13, line 2 - the involvement, not involving. Line 3 - bound at its... Line 4 - catalytically not catalitically. Line 17 - exposure not exposition. Line 20 - its stabilization not to stabilize it. Line 25 - consisting of not formed by. Line 26 - evidence yet not yet evidences.

p. 14, line 1 - more acidic the dog deaminase (a word is missing here). Line 8 - obtention is not an English word. Line 11 - fit well not fitted. Line 4 from bottom - omit and. Lines 2 and 3 from bottom - is similar, being around...

p. 15, line 11 - ...their structurally similar ligands. Line 12 - ...appears to be involved in substrate binding. Line 16 - omit best known. Line 17 - is not was.

Fig. 3, legend - concentration symbols not given.

Fig. 4 - open and closed circles not designated.

If your report extends beyond this page, please continue on a separate sheet

Article no: RPF 025299   Date of Receipt: July 25, 1991

Title   : Purification and characterization of glucosamine-6-phosphate
                 deaminase from dog kidney cortex

Author   : Dr. Mario Calcagno

Co-Authors: Altamirano M   Lara-Lemus R   Libreros-Minott

COMMENTS

   This manuscript describes the purification and characterization of glucosamine-6-phosphate deaminase from dog kidney. The authors have successfully adopted and modified the crucial affinity chromatography procedure which has been used in the purification of the same enzyme from E. coli.

   The enzyme has been purified to homogeneity as judged by several methods of electrophoresis, and a comprehensive study of the physical and kinetic properties of the enzyme is described.

   The kinetic studies are of interest for understanding the regulation of metabolism of amino sugars. For proper evaluation of kinetic data the authors should provide greater details of how the data are obtained. For example, the stability of $GlcNH_2$-6-phosphate at pH 8.8 (since the compound is unstable above neutral pH) and the percentage of substrate being used during kinetic experiments.

   Minor corrections are as marked in the manuscript and suggested as follow:

   (1) The references are out of order after number 9.

   (2) Symbols are missing from legend for fig. 3.

   (3) Fig. 4(A) and 4(B) are not labelled.

PURIFICATION AND CHARACTERIZATION OF GLUCOSAMINE-6-PHOSPHATE

DEAMINASE FROM DOG KIDNEY CORTEX

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Running Title: Dog kidney glucosamine-6-phosphate deaminase

SUMMARY

Glucosamine-6-phosphate deaminase (E.C. 5.3.1.10), from dog kidney cortex, was purified to homogeneity, as judged by several criteria of purity. The purification procedure was based on two biospecific affinity chromatography steps, one of them using N-ε-amino-n-hexanoyl-D-glucosamine-6-phosphate agarose as an immobilized allosteric ligand analog, and the other by binding the enzyme to phosphocellulose followed by substrate elution, which behaved as an active-site affinity chromatography. The enzyme is an hexameric protein of about 180 kDa, composed of subunits of 30.4 kDa; its isoelectric point was 5.7. The sedimentation coefficient was 8.3S, and its frictional ratio was 1.28, indicating that dog deaminase is a globular protein. The enzyme displays positive homotropic cooperativity towards D-glucosamine-6-phosphate ($h_{max} = 2.1$, pH 8.8). Cooperativity was completely abolished by saturating concentrations of N-acetyl-D-glucosamine-6-phosphate; this allosteric modulator activated the reaction with a typical K-effect. Under hyperbolic kinetics, a K_M value of 0.25 ± 0.02 mM for D-glucosamine-6-phosphate, was obtained. Assuming six catalytic sites per molecule, k_{cat} is 42 s^{-1}. Substrate-velocity data were fitted to the Monod's allosteric model for the exclusive-binding case for both substrate and activator, with two interacting substrate sites. The K_{DIS} for N-acetyl-D-glucosamine-6-phosphate was estimated in 14 μM.

Glucosamine-6-phosphate isomerase deaminase (2-amino-2-deoxy-D-glucose-6-phosphate ketol isomerase, deaminating, (E.C. 5.3.1.10), also known as glucosamine-6-phosphate deaminase, the enzyme catalyzing the reversible conversion of D-glucosamine-6-phosphate (GlcN6P) into D-fructose-6-phosphate (Fru6P) and ammonia has been mostly studied in microorganisms, in particular from *Escherichia coli* (1-4). In this species, the gene *nagB*, encoding deaminase is part of the divergent regulon *nagE-nag*BACD (5-8), which encodes a set of proteins involved in the transport and catabolism of amino sugars (N-acetyl-D-glucosamine and D-glucosamine), and its regulation. Deaminase is a key enzyme in amino sugar metabolism, and both fundamental types of regulation, *i.e.,* allosteric modulation of enzyme activity and the transcriptional control of gene expression, are known to be operative in the control of amino sugar utilization in bacteria (1,3). This avoids the operation of the futile cycle that would be produced by the synthesis of GlcN6P from Fru6P and glutamine, catalyzed by glucosamine-6-phosphate synthetase, and its conversion back to Fru6P, by the deaminase. Although glucosamine-6-phosphate deaminase is also present in several animal organs (*review*, 4), the enzyme has not been obtained pure from non-bacterial sources. Several purification schemes have been published, giving partial purified deaminases from hog kidney (1,9), human brain (10), rat kidney (11), and flies (12,13). Deaminase is especially active in kidney cortex,

and it was in pig kidney that the enzymatic conversion of GlcN6P into Fru6P was initially described by Leloir and Cardini (9). Conversely, glucosamine-6-phosphate synthetase activity is low in kidney and higher in liver (14). The role of kidney in amino sugar metabolism seems to be mainly catabolic. Although the deaminase reaction is reversible (K_{eq} = [Fru6P] [NH$_3$]/[GlcN6P] = 0.18 M) (9,3), the *E. coli* enzyme does not normally play a biosynthetic role *in vivo*. A mutation, *GlmX*, which favours the reversibility *in vivo* has recently been described in *E. coli* (15). Nevertheless, several authors consider that the reverse, biosynthetic reaction may be functional in amino sugar synthesis in some animal tissues. Evidence suggesting that the reaction can be reversible has been pointed out by Benson and Friedman for adult houseflies (12), by Enghofer and Kress for *Drosophila* pupae (13), and by Pattabiraman for human brain (10).

In this research we report the first total purification of glucosamine-6-phosphate deaminase from an animal source, and the initial molecular characterization of the pure protein. A better knowledge of kidney deaminase, and its comparative study with the bacterial enzyme, will be of value to understand the function and regulation of this enzyme in animal tissues.

MATERIALS AND METHODS

Kidneys. Kidneys from adult dogs were a gift from the Department of Biochemistry, National Institute of Cardiology, SSA, Mexico. They were received frozen and stored at -45°C until use.

Reagents. Biochemical reagents, enzymes, proteins used as molecular weight standards, and cellulose phosphate were purchased from Sigma Chemical Co., (St. Louis, MO). Electrofocusing ampholytes and polybuffer for chromatofocusing were from Pharmacia-LKB Biotechnology, Sweden. GlcNAc6P was prepared by acetylation of GlcN6P, and purified by ion-exchange chromatography according to Leloir and Cardini (14). N-ε-aminohexanoyl glucosamine-6-phosphate agarose, the gel for allosteric site affinity chromatography, was prepared following a procedure previously described (3). Other chemicals were reagent-grade products.

Enzyme assays. Glucosamine-6-phosphate deaminase was routinely assayed in the direction of Fru6P formation, with a stop-time colorimetric procedure. A typical reaction mixture of 200 μl contained 50 mM Tris-HCl buffer (pH 8.8 at 30°C), 1 mM disodium EDTA, 1 mM GlcNAc6P, and variable amounts of the substrate. The reaction was started by the addition of the enzyme, and incubated for a fixed time at 30°C. Reaction was stopped by the addition of 2.0 ml of 10 M HCl; Fru6P formed was determined using the resorcinol reaction of Roe (16), as described by Davis and Gardner (17). In the presence of interfering substances (*i.e.*, samples from a sucrose gradient or when Fru6P was used in elution

buffers), enzyme activity was measured in the direction of GlcN6P formation, also with a fixed time colorimetric method. Reaction mixture (100 μl) contained 0.5 mM GlcNAc6P, 1 mM disodium EDTA, 10 mM potassium phosphate buffer (pH 7.5 at 30°C), 50 mM ammonium chloride, and 5 mM Fru6P; GlcN6P formed in a fixed time was determined using the Elson-Morgan reaction, (18), as described by Levy and McAllan (19). All data corresponding to hyperbolic kinetics were fitted by the procedure of Wilkinson (20). Under homotropic cooperative kinetics, V_{MAX} and $S_{0.5}$ were calculated according to Atkins (21), and maximal value of the Hill coefficient (h_{max}), from the slope of the central linear portion of the Hill plot, by least-squares fit. The program ENZFITTER for non-linear regression analysis (R.J. Leatherbarrow, Elsevier Biosoft, Cambridge, UK, 1987) was also used to fit data to different equations. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of one micromole of Fru6P in 1 min at 30° C and pH 8.8.

Protein determinations. The method of Bradford (22) with bovine serum albumin as standard was used as reference during purification. The concentration of pure enzyme was estimated using the $E_{1\%}$ of 11.2 at 278 nm, the value reported for the deaminase purified from *E. coli* (23).

Chromatofocusing. Chromatofocusing was performed at room temperature in a 7 x 170 mm column packed with PBE 94. Starting buffer was 25 mM imidazole-HCl (pH 7.4) and the elution was run with 90 ml of polybuffer 74 diluted 1:10 in deionized water.

Electrophoresis and Isoelectric Focusing. Electrophoreses of the native protein were performed in 80 x 80 x 2.7 mm polyacrylamide gel slabs with a linear pore gradient obtained by varying the monomer concentration from 4 to 30 g/dl. Cross-linkage was constant, at 4%. Gels were prepared in 90 mM Tris base and 80 mM boric acid, pH 8.3, containing 2.5 mM EDTA. The same buffer was used to run electrophoreses at 125 V for 16 h. Gels were stained with Coomassie blue R-250 and destained by diffusion. Several proteins were used as molecular weight standards.

Electrophoreses under denaturing conditions were made according to Laemmli (24) in 180 x 140 x 0.7 mm gel slabs (T = 11.4 % , C = 2.6 %), running simultaneously several proteins as chain weight standards.

Analytical isoelectric focusings in polyacrylamide gel were run in 125 x 125 x 2 mm gel slabs (T = 6% , C = 4%), using a horizontal electrophoresis apparatus (Multiphor, LKB, Sweden) at 25 W (constant power) for 3 h at 4 °C. Ampholyte ranges were 3-9 or 5-8 pH units (Pharmalyte, Pharmacia-LKB Biotechnology) diluted 1:15 in deionized water.

Gel filtration chromatography. The pure deaminase was chromatographied in a 16 x 700 mm calibrated column of Sephadex G-150, equilibrated with 0.1 M potassium phosphate buffer and 1 mM EDTA, pH 7.5 (Buffer A). Elution profiles were obtained from the continuous recording of the eluate absorbance at 280 nm. Several proteins having known physical constants were used as standards. Stokes radii and diffusion coefficient were obtained according to Kulbe (25), using data from Laurent and Killander (26) and

Potschka (27) for reference proteins. Deaminase molecular radius was calculated using the classical equation (28):

$$r = \left[\frac{3 M r \bar{v}}{4 \pi N} \right]^{-1/3}$$

A partial specific volume of 0.737 ml g^{-1}, the value reported for the deaminase from *E. coli* (3), was used in this calculation. Frictional ratio was obtained from Stokes and molecular radii.

Sedimentation in the ultracentrifuge. Ultracentrifugation of the pure deaminase was made in 5-20% sucrose density linear gradients, prepared in buffer A and run in a Beckman SW50.1 swinging bucket rotor at 290 000 x g for 12 h at 5 °C. Cellulose nitrate centrifuge tubes containing several protein standards were included in the same run. The content of the tubes was analyzed by puncturing its bottom and collecting the fractions. Sedimentation coefficients were calculated with reference to standards. Deaminase molecular weight was estimated from sedimentation coefficient using the equation of Martin and Ames (29) with reference to two standards, and taking the average of both data.

R E S U L T S

Enzyme purification. All procedures were carried out at room temperature, except homogenization, dialysis, and centrifugation, which were performed at 4°C. The frozen kidneys were dissected to separate the cortex. Cortical tissues were suspended in purified

water at a ratio of 3 ml per gram of wet weight, and homogenized with a Janke & Kunkel Ultra-Turrax homogenizer for 30 s at maximum speed. The homogenate was centrifuged at 25,000 x g for 20 min and the supernatant was diluted with the addition of three volumes of purified water. Then, the pH was brought to 5.9 by the careful addition of 0.1 M acetic acid. Immediately, wet phosphocellulose was added, at a ratio of 20 ml of packed resin for each gram of total protein; the pH was kept at 5.9 and the slurry was gently stirred for 30 min. To increase the enzyme adsorption, GlcNAc6P was added to obtain a final concentration of 0.1 mM. Then, phosphocellulose containing the adsorbed deaminase was collected and used to pack a 15 x 300 mm column. It was then washed with 0.1 M potassium acetate solution containing 1 mM EDTA, adjusted at pH 6.2. Elution was performed with a linear concentration gradient of KCl (0 to 1 M) prepared in the same acetate solution. Deaminase was eluted at about 0.4 M KCl; fractions containing the enzyme were pooled and its pH was adjusted to 7.5 by the careful addition of 50 mM KOH. The preparation was immediately applied to a 7 x 20 mm column packed with the allosteric affinity gel, N-ε-aminohexanoyl-ᴅ-glucosamine-6-phosphate agarose previously equilibrated with buffer A. The enzyme was eluted with a 5 mM GlcNAc6P solution prepared in the same buffer. Fractions containing the enzyme were pooled and applied to a small phosphocellulose column equilibrated with 20 mM potassium phosphate buffer, pH 5.9 containing 1 mM EDTA. The column was washed with this buffer and the enzyme was eluted with a solution containing 10 mM potassium Fru6P, 0.1 mM potassium GlcNAc6P, and 20 mM potassium phosphate, pH 6.20. Elution was specific for

Fru6P; KCl at an equivalent ionic strength or fructose 1,6 bis-phosphate at the same concentration did not release the enzyme. When elution was performed with Fru6P alone, a higher ligand concentration was required and protein emerged from the column as a broader peak (not shown). A single protein and activity peak emerged from the phosphocellulose column, with a specific activity of 85 units per milligram of protein. This corresponds to a k_{cat} of 42 s^{-1}, assuming six catalytic sites. The enzyme was dialyzed against buffer A to change the pH and get rid of the ligands; then it was concentrated by dialysis against 20 volumes of 50% glycerol in buffer A, and stored at -20 $^{\circ}$C. The purification procedure is summarized in Table I.

Enzyme stability. Enzyme samples were incubated overnight at 4 $^{\circ}$C, at different pH values in the range 5.0 to 9.0. Buffer salts were potassium acetate, imidazole-HCl and Tris-HCl. The enzyme was then assayed at pH 8.8. Specific activity remained essentially unchanged in all pH ranges.

Criteria of purity and isoelectric pH. The protein obtained gave a single band on SDS-polyacrylamide electrophoresis, polyacrylamide pore-gradient electrophoresis, and isoelectric focusing in polyacrylamide gel under native conditions (Fig. 1). Chromatofocusing gave a single symmetrical protein and activity peak, with a nearly constant specific activity over the whole profile (Fig. 2). Both methods, isoelectric focusing and chromatofocusing, gave an isoelectric pH of 5.7 for the pure deaminase.

Molecular properties and subunit composition. From SDS-polyacrylamide gel electrophoreses, a molecular weight of 30,400 ± 400 was estimated for the enzyme subunit. Molecular weight of the native enzyme was determined by several methods (Table II) and found to be near 180,000 Da, indicating the hexameric nature of the enzyme. Sedimentation coefficient was 8.3S; other physico-chemical constants for the native protein, determined by different methods, are listed on Table II.

Allosteric activation. Dog kidney glucosamine-6-phosphate deaminase displayed positive homotropic cooperativity with respect to GlcN6P *i.e.* in the deaminating direction of the reaction, as shown in Fig. 3A. Glucose-6-phosphate, reported to be an allosteric effector of housefly deaminase, did not affect the kinetics of the dog enzyme (data not shown).

When initial velocities were measured as a function of substrate concentration at different fixed activator levels, curves shown in Fig. 3B were obtained. Hill coefficients, at pH 8.8, varied from 2.1, in the absence of GlcNAc6P, to 1.0 in the presence of a saturating concentration (1 mM) of the allosteric activator (Fig. 3B, inset). Under these conditions, kinetic data fit well to hyperbolic kinetics, with a Km of 0.25 ± 0.02 mM and a molecular activity of 255 s^{-1}, calculated for the hexameric oligomer. Kinetic data were fitted to Monod *et al.* equation (30) for the exclusive-binding case. An allosteric constant, L, of 620 and a n value (number of GlcN6P sites) of 2 were obtained from the data in absence of GlcNAc6P. When GlcNAc6P was varied in the range 0-100

μM, and data were plotted according to Horn and Börnig (31), a set of parallel lines resulted. The replot shown in the inset of Fig. 3C, used to calculate K_{DIS} for GlcNAc6P from these data, was derived from the following expression:

$$L^{\bullet} = \frac{L}{(1 + [GlcNAc6P]/K_{DIS})^m}$$

where $L^{\bullet}$ is the apparent allosteric constant, at different GlcNAc6P concentrations, for the exclusive binding case of the allosteric activator, and m is the number of GlcNAc6P sites; better fits were obtained with $m = 3$. From the inset of Fig. 3C, we can calculate the dissociation constant for GlcNAc6P, which was 14 μM.

Variation of Km and Vmax with pH. The enzyme was assayed at different pH values, in the range 6.0 to 10.0, in the presence of 1 mM GlcNAc6P. Constant ionic strength (I = 0.05) buffers of MES-Tris and Tris-CAPS were used (32). The plot of log V_{max} against pH (Fig. 4A) had two segments, with slopes of +1 and 0, with the break on the ordinate at pH 8.8. The plot of log (V_{MAX}/K_m), as a function of pH, gave a more complex profile, with breaks at pH 6.5, 7.0, 8.1, and 8.5. When data were corrected for substrate ionization (pK_2 = 8.1), taking as the true ligand the protonated ammonium species, the corresponding break at this pH disappears (Fig. 4B).

DISCUSSION

In this article we describe the first procedure to obtain a pure and homogeneous glucosamine-6-phosphate deaminase from an animal source, in this case, dog kidney cortex. The purification scheme includes two affinity chromatography steps; the chromatography on N-ε-amino-n-hexanoyl-ᴅ-glucosamine-6-phosphate agarose, takes advantage on the binding of the enzyme to an immobilized analog of its allosteric activator and its elution with GlcNAc6P. This affinity matrix is the key step in the purification of deaminase from *E. coli* (3,33), but it was insufficient to obtain the dog kidney enzyme in a completely homogeneous form. A second type of affinity interaction was then introduced into the purification scheme, the use of phosphocellulose binding, combined with substrate elution. Indeed, phosphocellulose was reported to be a good affinity material for enzymes specific for sugar-phosphate ligands (34). The involvement of the active site in this step is apparent because: *a*) deaminase is bound above its isoelectric pH; *b*) binding was more efficient in the presence of the allosteric activator, GlcNAc6P, and *c*) better elution conditions were obtained when GlcNAc6P was added to the substrate-containing elution buffer. This favorable effect on binding and elution steps reflects the increase in affinity of the deaminase active site for its ligands (Fru6P or phosphoglucosyl residues in phosphocellulose fibers). These observations not only support the idea that the interaction of deaminase with phosphocellulose occurs mainly at the active site, but also proves the existence of an enzyme-Fru6P binary complex in the catalytic cycle. Fru6P was preferred for

elution because it does not form a catalytically active complex and it is less expensive and more stable in solution at the working pH, than GlcN6P. The reported purification procedure, which can be completed in two days, avoids excessive exposure of the deaminase to renal tissue proteolytic activity; moreover, the use of deaminase ligands and specific interactions in most steps, helps to stabilize it during purification.

The enzyme obtained was found to be homogeneous, by different purity criteria. Its specific activity was 85 μmoles mg^{-1} min^{-1}, one order of magnitude lower than *E. coli* deaminase. Glucosamine-6-phosphate deaminase from dog kidney is a hexameric enzyme, formed by 30.4 kDa subunits. In this aspect, it is similar to the *E. coli* deaminase, although we do not yet have proof that it is a homopolymeric protein, like the bacterial deaminase. The isoelectric pH for both enzymes is slightly acid, and that for dog kidney is the more acidic. As judged from its frictional ratio, which is close to unity, dog deaminase should also fall into the class of globular proteins.

Like most glucosamine-6-phosphate deaminases so far described, the dog enzyme is an allosteric protein, activated by GlcNAc6P. Activation can be described as a classical K-effect; saturating concentrations of the allosteric activator induce hyperbolic kinetics without effect on V_{max}. Michaelis kinetics is observed at saturating GlcNAc6P concentrations and this proves that it is an exclusive-binding ligand. The data of initial velocities at different GlcN6P concentrations, fitted well to the Monod *et al.* equation (30) for the case of substrate exclusive-binding; the fit gave two interacting substrate sites per molecule. This may be an

indication that the enzyme is functionally a trimer of dimers, with the dimer as the unity of the allosteric transition. The nearest integer value for m, the number of allosteric sites, which gave the best fit in replots as depicted in the inset of Fig. 3C, was three. Using this value, a K_{dis} of 14 μM, for GlcNAc6P was found, at pH 8.8. This value is consistent with the observed profile of h_{max} as a function of GlcNAc6P concentration, shown in the inset of Fig. 3B.

Dog kidney deaminase has a Km value for GlcN6P one order of magnitude lower than the *E. coli* enzyme (0.25 and 2.0 μM, respectively) but a k_{cat} nearly seven times lower (42 s^{-1} and 300 s^{-1}, respectively, assuming six catalytic sites per molecule) (3). The catalytic efficiency (k_{cat}/K_m) for both enzymes is thus similar, and around 1 x 10^6 M^{-1} s^{-1}. On the other hand, the dissociation constant for GlcNAc6P is somewhat lower. This higher apparent affinity for GlcN6P and its regulation by low concentrations of its N-acetyl derivative is consistent with a catabolic role of kidney deaminase. Activity of the enzyme increases with pH, as shown in Fig. 4A. The unprotonated form of a group dissociating with a pK_a near 8.8 seems to be essential for catalysis. It may be a general base participating in proton abstraction in the catalytic process (2). Data in Fig. 4B indicates that the protonated form of the substrate of the 2-deoxy-2-amino group is the true ligand of the enzyme. This is interesting, because it explains how active and allosteric sites differentiate between their structurally very similar natural ligands. In Fig. 4B, two groups, with pK values near 7.0 and 8.5, involved in substrate binding, are apparent.

Most of the molecular and kinetic properties of glucosamine-6-phosphate deaminase purified from dog kidney cortex are similar to those of the best characterized deasminase, that of *E. coli* deaminase. This emphasizes that these proteins were highly preserved in evolution, both structurally and functionally.

ACKNOWLEDGMENTS

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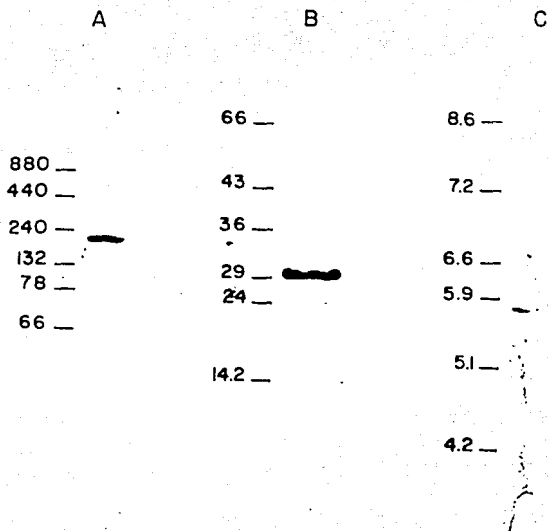30. Monod, J., Wyman, J. and Changeux, J-P. (1965) *J. Mol. Biol.*

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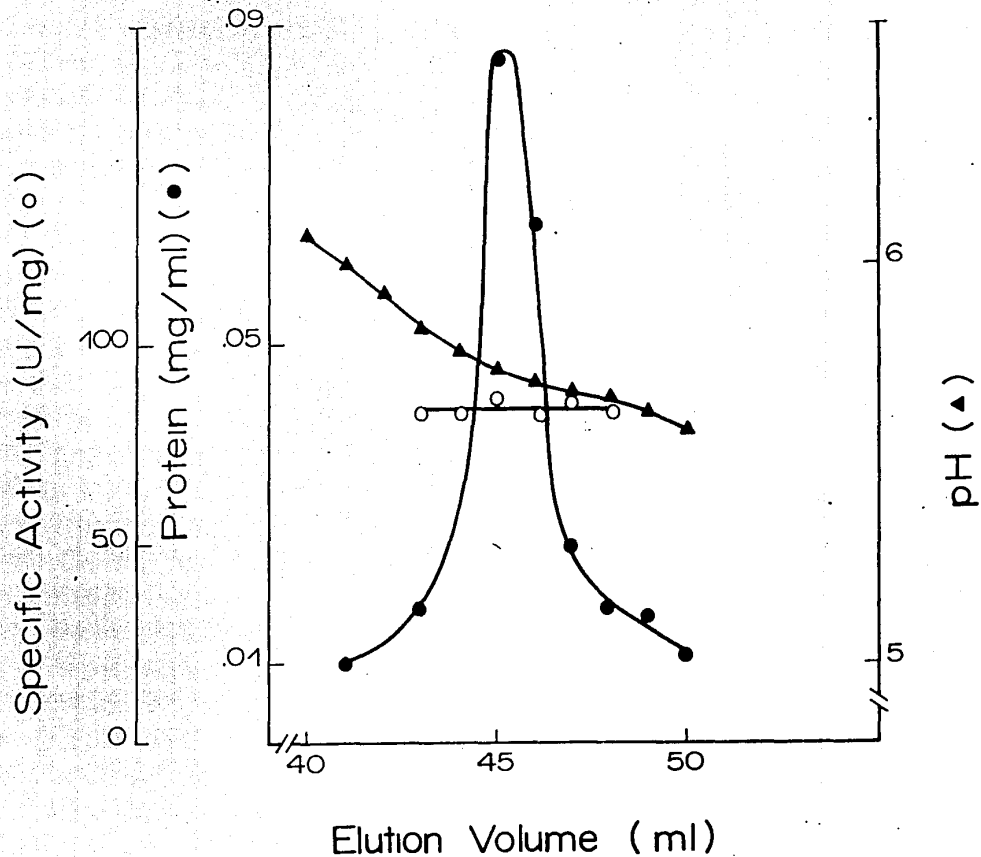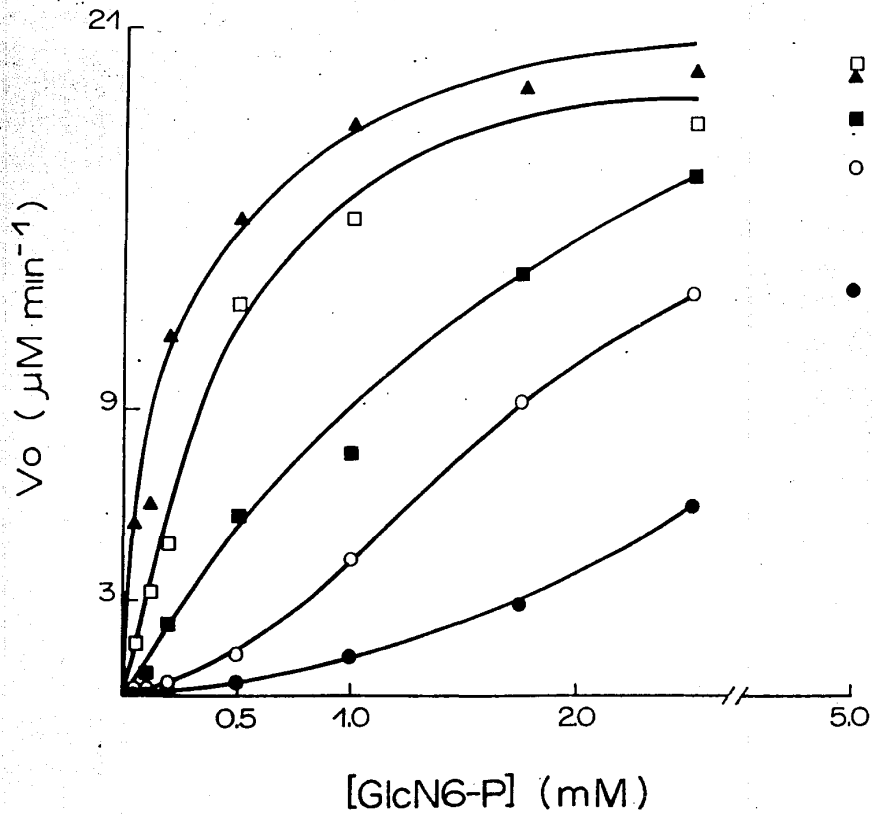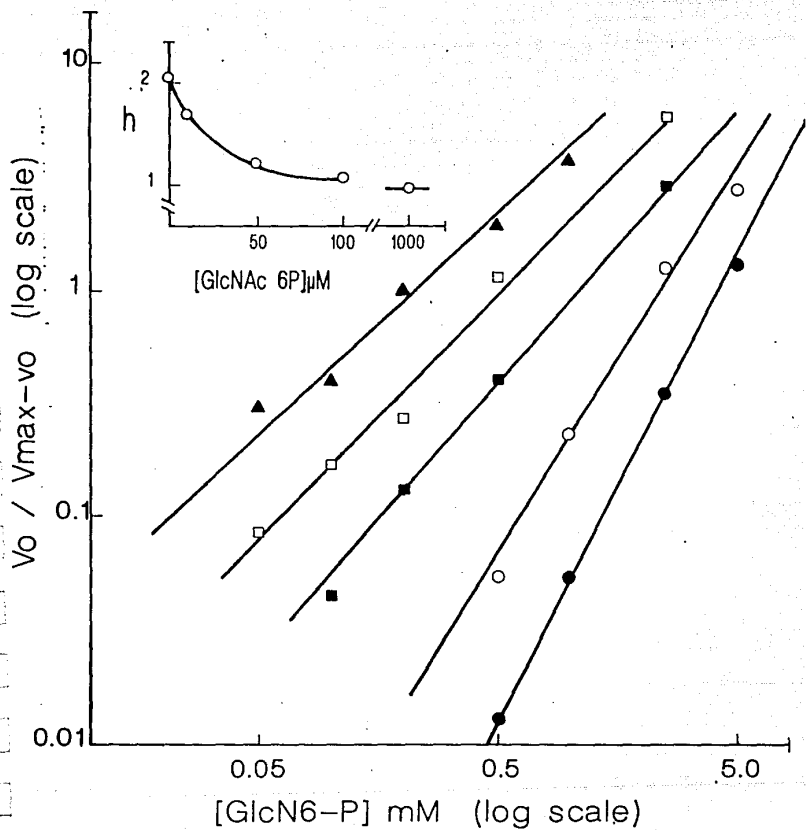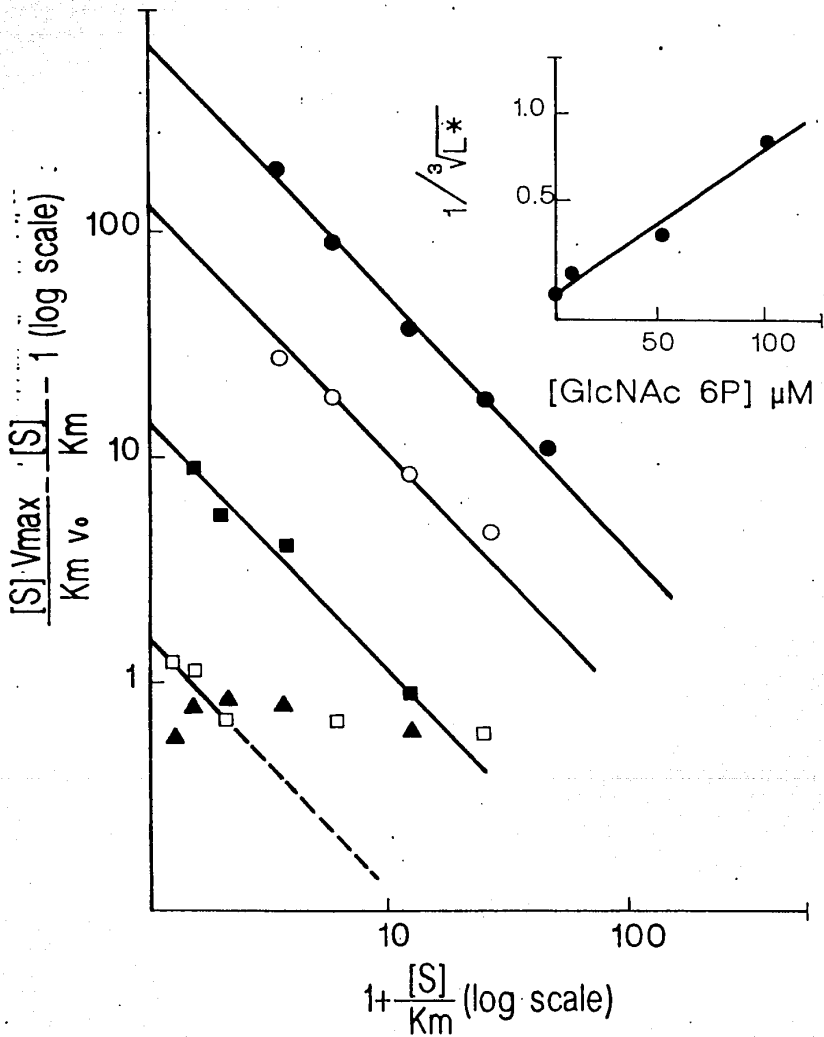
fig 1

fig 2

fig 3   a

[GlcN6-P] (mM.)

b

Vo / Vmax-vo (log scale)

10

1

0.1

0.01

h

2

1

50   100   1000

[GlcNAc 6P]μM

0.05   0.5   5.0

[GlcN6-P] mM (log scale)

C

$\dfrac{[S]}{Km} \cdot \dfrac{Vmax}{v_o} - 1$ (log scale)

100

10

1

$1 + \dfrac{[S]}{Km}$ (log scale)

10   100

Inset:

$1/\sqrt[3]{L*}$

1.0

0.5

50   100

[GlcNAc 6P] µM
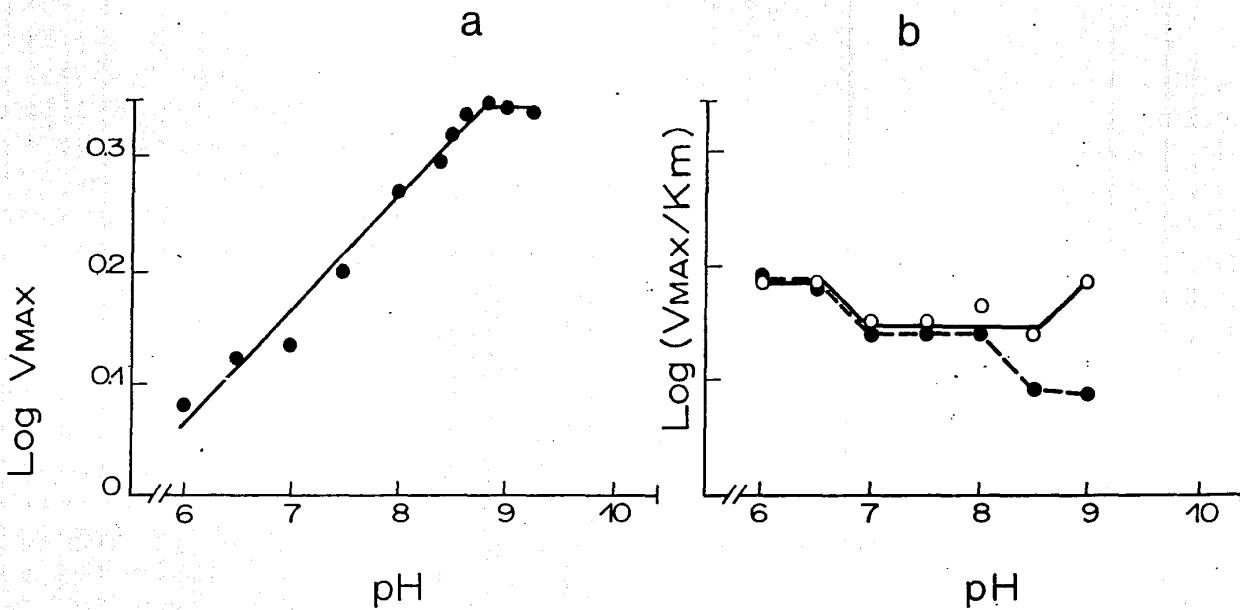
fig 4

Fig. 1

Electrophoretical purity of dog kidney glucosamine-6 phosphate deaminase. *Lane A*: Pore-gradient polyacrylamide gel electrophoresis (T, linear from 4% to 30%; C = 3.6%). Coomassie blue R-250 staining. A sample of 10µg was applied. *Lane B*: Polyacrylamide gel electrophoresis under denaturing conditions, according to Laemmli (24). Homogeneous gel (T = 11%, C = 2.6%); Coomassie blue R-250 staining. A sample of 10 µg of protein was applied. *Lane* C: Isoelectric focusing under non denaturing conditions on polyacrylamide 4% gel; 60 µg of the enzyme were run. Staining: Coomassie brilliant blue R-250

Fig. 2

Chromatofocusing of dog kidney glucosamine-6 phosphate deaminase. The experimental conditions are described under *Materials and Methods*. A sample of 0.25 mg of the pure deaminase was loaded, and eluted with PBE buffer 74 (Pharmacia Biotechnology).

● : Protein concentration, calculated from absorbance at 280 nm, using an E $^{1\%}$ of 0.9 ml mg^{-1} cm^{-1}. o : deaminase specific activity (µmoles of Fru6P formed per min per mg of protein). ▲ : pH of each fraction.

Fig. 3

A. Initial velocity of the reaction of dog kidney glucosamine-6-phosphate deaminase, plotted *versus* the substrate concentration. Curves were obtained at the following GlcNAc6P concentrations: (●) none; (o) 10 μM; (■) 50 μM; (□) 100 μM and (▲) 1 mM. Velocities were measured at pH 8.8, 30°C.

B. Hill plot of the data presented in A. Slopes (Hill coeffcient, *h*) plotted against GlcNAc6P concentration, are shown in the inset.

C. Plot of the data of the previous figures, according to Horn and Börnig (31). This plot is a linearization of the Monod *et al.* equation (30) for the exclusive-binding case of substrate and activator. *Inset*: replot of the reciprocal values of cubic root of the apparent allosteric constants, $\overset{*}{L}$, as a function of GlcNAc6P concentration. The abscissa intercept corresponds to *Kdis* for the activator.

Fig. 4

pH dependence of the kinetic parameters for the deaminating direction of the reaction of dog kidney glucosamine-6-phosphate deaminase. The enzyme was assayed under hyperbolic kinetics, obtained in the presence of 1 mM GlcNAc6P, as described under *Materials and Methods*. MES-Tris or CAPS-Tris buffers with fixed ionic strenght (32) were used, at a final concentration of 0.1 M.

A. Maximum velocity pH profile. B. V_{max}/K_m pH profile.

TABLE 1

Purification Scheme for Glucosamine 6-Phosphate Deaminase from Dog Kidney Cortex [a]

Procedure	Specific Activity (μmol min^{-1} mg^{-1})	Total Protein (mg)	Total Units (μmol min^{-1})	Purification factor	Yield (%)
Supernatant 30 000 x g	0.061	1620	98	- -	100
Cellulose-phosphate Chromatography	1.3	62	81	22	83
Allosteric-site affinity Chromatography	28	2	63	463	64
Cellulose-phosphate Chromatography	85	0.615	52	1390	53

[a] The Results presented here are for a typical purification starting with 15g of Renal Cortex.

T A B L E   II

Summary of physico-chemical properties of Glucosamine 6-phosphate
Deaminase from Dig Kidney Cortex

Molecular weight (oligomer)	180 000 Da
Molecular weight (monomer)	30 500 Da
Isoelectric pH	5.7
Diffusion Coefficient	44.7 $\mu m^2 s^{-1}$ **a** 42.4 $\mu m^2 s^{-1}$ **b**
Sedimentation Coefficient $S_{20.W}$	8.3×10^{-13} S
Molecular radius	3.75 nm
Stokes radius	4.8 nm
Frictional ratio ($f/_{f_0}$)	1.28

a Value obtained by using Stokes-Einstein equation

b Value obtained graphically

D I S C U S I O N   G E N E R A L

Y   C O N C L U S I O N E S .

DISCUSION GENERAL Y CONCLUSIONES.

1.- En este trabajo describimos el primer procedimiento de purificación que permite obtener, en forma pura y homogénea, la enzima glucosamina-6-fosfato desaminasa a partir de un tejido animal, en este caso, corteza renal de perro.

El procedimiento de purificación descrito se basa en el uso consecutivo de dos tipos de cromatografía de afinidad; la unión de la enzima a la N-ε-amino-hexanoil-ᴅ-glucosamina-6-fosfato agarosa, aprovecha la capacidad de la enzima para unirse a un análogo de su activador alostérico, inmovilizado en agarosa. Esta matriz resultó ser insuficiente como paso único para purificar la enzima de riñón de perro. El segundo tipo de cromatografía de afinidad, usando celulosa fosfato como un ligando análogo al sustrato $Fru6P^{32}$, permitió lograr este objetivo. Cabe mencionar que tampoco la celulosa fosfato por sí sola logra rendir una enzima pura, resaltando entonces la necesidad del uso combinado de los dos tipos de cromatografía de afinidad para lograr la purificación.

2.- Con este procedimiento de purificación logramos, además, el propósito de realizar una purificación rápida, ya que puede ser completado en dos días de trabajo, sin necesidad de diálisis prolongadas, evitando así la exposición excesiva de la desaminasa

[32] CHILLA, R., DOERING, K. M.,DOMAGK, G.F., AND RIPPA, M. (1973) ARCH. BIOCHIM. BIOPHYS. 159,235-239.

a la actividad proteolítica del tejido renal. De igual manera, el uso de ligandos y de interacciones específicas en todos los pasos de la purificación, contribuye a proteger a la enzima. Esto se refleja en el alto rendimiento logrado así como en la actividad específica de la enzima pura (85 μmoles mg^{-1}min^{-1}).

3.- Llama la atención, sin embargo, que la actividad específica obtenida sea un órden de magnitud más baja que la publicada para la enzima de *E.coli*.[4]. Actualmente no contamos con evidencias experimentales que pudieran aclarar la razón de esta diferencia. Una posibilidad podría ser que existiera algún activador fisiológico de la desaminasa renal, que actuara aumentando la V_{max} de la enzima (activador V clásico). La naturaleza de este activador bien podría ser algún catión divalente, ya que se ha descrito que tanto la desaminasa bacteriana como la de tejidos de mamíferos, son afectadas por cationes[16], especialmente el zinc para el caso de la desaminasa de *E.coli*[33].

4.- En relación con las características moleculares de la desaminasa de corteza renal de perro encontramos que se trata, al igual que la desaminasa de *E. coli*, de una proteína hexamérica; aunque el peso molecular de la subunidad de la enzima renal es ligeramente mayor al de la desaminasa de *E.coli*. El punto isoeléctrico de ambas proteínas, también es muy parecido. Es decir encontramos un parecido muy estrecho entre las características de

[33]ALTAMIRANO, M. M. AND CALCAGNO, M. (1990) BIOCHIM. BIOPHYS. ACTA 1038,291-294.

ambas. Con respecto a las demás constantes fisicoquímicas, es pertinente aclarar que la relación friccional, así como el coeficiente de difusión, son datos que no se han obtenido para la enzima bacteriana.

5.- Consideraremos ahora el comportamiento y propiedaes cinéticas de la desaminasa de riñón de perro.

Al igual que la mayoría de las desaminasas con excepción de la de *Candida albicans*[34], la enzima de perro es una proteína alostérica activada por la GlcNAc6P. Esta activación puede ser descrita como un sistema K clásico, ya que la enzima, a concentraciones saturantes de GlcNAc6P, adquiere una cinética hiperbólica, sin aumento de la *Vmax*.

Los valores de velocidad inicial a diferentes concentraciones de sustrato (GlcN6P), tienen un buen ajuste a la ecuación de Monod y cols.[35] para el caso de fijación exclusiva para el sustrato. Debido al buen ajuste observado de los datos experimentales a este modelo alostérico, el más simple disponible, consideramos conveniente evaluar los distintos parámetros cinéticos en base al mismo. El valor de n (número de sitios interactuantes) fue de 2, lo cual podría parecer poco congruente con el carácter hexamérico de la enzima. Este valor, sin embargo, puede reflejar que la unidad de la transición alostérica podría ser en realidad un

[34]DAS, M. AND DATTA, A.(1982) BIOCHEM. INT.5,735-741.

[35]MONOD, J., WYMAN, J. R., AND CHANGEUX, J. P. (1965) MOL. BIOL. 12,88-118.

dímero, conformado a su vez de tres subunidades; es decir, un dímero de trímeros. En este sentido encontramos una diferencia importante con respecto a la desaminasa de *E. coli*, en donde se han determinado 6 sitios interactuantes, uno por sub-unidad[36]. Esto puede ser una explicación de la menor cooperatividad homotrópica observada con la enzima de perro.

Con los datos presentados, es muy difícil para nosotros poder determinar con exactitud si el valor de *n* es de 2, o de 3, ya que el método por el cual lo determinamos es poco preciso, sin embargo es indudablemente menor de 6.

Por otra parte, el regráfico de la figura 3c del artículo, obtenido a partir de la ecuación de Monod para el caso de fijación exclusiva, permite obtener la constante de disociación para el activador (K_{dis}). Como se puede observar, el valor obtenido es de alrededor de 14 μM. Este valor es más bajo que el descrito para la desaminasa de *E. coli*; es decir, la desaminasa renal tiene una afinidad aparente mayor por su ligando alostérico. La razón por la cual se ajustó la unión del activador alostérico a este modelo, es que la saturación de la enzima con activador, induce la abolición de la cooperatividad de la enzima, adquiriendo el comportamiento de una enzima que sigue la cinética de Michaelis, tal como corresponde a una activación producida por la unión de un ligando alostérico en forma exclusiva.

El número obtenido de sitios interactuantes para el activador,

[36] RESULTADOS NO PUBLICADOS

es decir la raíz de la constante alostérica aparente L^*, fue de 3. Este valor se consideró como el más aproximado, ya que el ajuste de los datos a una línea recta con la raíz cúbica de L^* era el mejor.

Observamos, así. una diferencia entre el valor de n para el sustrato y para el activador; esta discrepancia no puede ser explicada a partir de los datos presentados en la tesis, quedando el recurso de establecerlo mediante experimentos en los que se mida la unión de la GlcNAc6P por un método directo, como por ejemplo, diálisis en equilibrio.

6.- En la última parte del trabajo, en la que analizamos la variación de la actividad de la enzima con respecto al pH, observamos que la actividad de la desaminasa de riñón de perro se incrementa con el pH, (Fig 4a). De esto se deduce que la forma no protonada de un grupo que se disocia con un pK cercano a 8.8, parece ser esencial para la catálisis. Dicho grupo podría ser una base del sitio activo que participa en la reacción eliminando un protón en el proceso catalítico.

Por otra parte, los datos que se muestran en la figura 4b, indican que la forma protonada del sustrato (en el grupo 2-desoxi-2-amino), es el ligando real de la enzima. Esto resulta interesante, ya que podría explicar cómo el sitio activo y el alostérico, diferencian sus respectivos ligandos, GlcN6P y GlcNAc6P, que son tan parecidos estructuralmente.

Finalmente, también observamos a partir de esta figura que dos grupos con valores de pK cercanos a 7.0 y 8.5 participan de alguna manera en la unión del sustrato a la enzima.

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Comparando los valores de Km y de "pH óptimo" publicados para varias de las desaminasas purificadas parcialmente[11], observamos una relativa congruencia entre la desaminasa renal pura, y los de la mayoría de las desaminasas animales purificadas parcialmente. Sin embargo es obvio que la actividad específica obtenida por nosotros es más alta que la descrita en purificaciones parciales.

7.- Cuando comparamos la desaminasa renal con la de *E. coli* la mayoría de las propiedades moleculares y cinéticas son muy parecidas, evidenciando que al menos la funcionalidad de ambas, ha sido conservada en la evolución.

No obstante, algunas de las diferencias entre ellas son interesantes, y representan fenómenos dignos de estudiarse en forma más profunda, ya que su comprensión podría aportar más información acerca de la función y regulación de esta enzima en tejidos animales. Algunas de ellas son: *a)* el mayor peso molecular de la subunidad, lo cual implica la existencia de un mayor número de residuos, o bien la presencia de aminoácidos modificados, lo que abrire nuevas posibilidades en cuanto a su regulación. *b)* La baja cooperatividad de la desaminasa renal en comparación con la enzima de *E. coli*, aunado a una mayor afinidad aparente de la primera, tanto para el sustrato como el activador. Esto podría representar simplemente la función catabólica de la desaminasa en el riñón.

8.- Así, el papel catabólico de la desaminasa en el riñón, queda sugerido en el presente trabajo por la alta afinidad que esta enzima exhibe por su sustrato GlcN6P, así como la regulación de la actividad de la enzima mediante concentraciones muy bajas de su activador alostérico (GlcNAc6P).

El funcionamiento del ciclo improductivo que se generaría entonces, entre la actividad de la gucosamina-6-fosfato sintetasa que formaría glucosamina-6-fosfato, y la actividad de la desaminasa que la hidrolizaría con un consumo de ATP, queda evitado en los mamíferos, por la localización diferencial de ambas enzimas en dos órganos distintos[37,38], la sintetasa en el hígado, donde la desaminasa está en bajas concentraciones, y en la corteza renal, donde las proporciones de las dos se invierten (tabla B).

Esta distribución está en relación con las funciones específicas de estos órganos: el hígado, que es el sitio principal de síntesis de las glicoproteínas plasmáticas[39,40], (y sitio primario de síntesis de la glucosamina presente en el suero[41]) y la corteza renal, que parece ser el sitio de catabolismo de los aminoazúcares provenientes de las glicoproteínas del suero[42].

[37] KIKUCHI, K. AND TSUIKI, S. (1979) BIOCHIM. BIOPHYS. ACTA 584,246-253.

[38] KIKUCHI, H., KOBAYASHI, Y., TSUIKI, S. (1971) BIOCHIM. BIOPHYS. ACTA 237,412-421.

[39] SARICONE, E. J. (1962) BIOCHEMISTRY 1,1132.

[40] SARICONE, E. J. (1962) ARCH. BIOCHEM. BIOPHYS. 100,516.

[41] SPIRO, R. G. (1959) J. BIOL. CHEM. 234, 742.

[42] MOSCARELLO, M. A., SUTHERLAND, L. AND JACKSON, S. H. (1967) CAN. J. BIOCHEM. 45, 136-141.

T A B L A   B .

Actividades de la sintetasa y desaminasa en riñón e hígado.

ENZIMA	ACTIVIDAD (UNIDADES/MG)	
	RINON	HIGADO
GlcN6P sintetasa	1.5	56.8
GlcN6P desaminasa	660	67.0

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P E R S P E C T I V A S .

PERSPECTIVAS.

Aun quedan muchos aspectos por explorar acerca de las propiedades de esta enzima; el presente estudio es la primera ventana abierta desde donde puede verse un panorama muy amplio respecto al conocimiento de esta proteína. Algunos de estos aspectos incluyen un estudio comparativo más completo con la desaminasa de *E. coli*, destacando los siguientes puntos:

1.- Estudio cinético detallado de la cooperatividad de la desaminasa renal en función del pH. El objetivo es determinar el papel de los protones en cuanto a la activación o inhibición de la actividad de la enzima. Para lograr este propósito, se realizarán experimentos empleando amortiguadores de fuerza iónica constante en un intervalo de valores de pH similar al empleado en lo realizado en el artículo, midiendo la actividad de la enzima en ausencia del activador alostérico.

2.- Determinar la composición de aminoácidos de la proteína. Esto permitirá comparar la desaminasa renal con la de *E. coli*, ya que de esta última se conoce incluso la secuencia de aminoácidos. Para realizar este propósito se requerirá la colaboración de alguna institución que tenga actualmente la infraestructura necesaria. En cuanto a la obtención de enzima, no requeriremos de gran cantidad, ya que para la determinación de aminoácidos por HPLC se necesita un mínimo de proteína. Para el análisis comparativo usaremos

algoritmos de comparación de composiciones globales, como el de Cornish Bowden[43].

3.- Con base en la composición de aminoácidos, sabremos cuantos residuos de cisteína posee esta enzima. El estudio de las cisteínas es importante, ya que al menos en la enzima bacteriana estos residuos tienen que ver con la transición alostérica. En este sentido hay que mencionar que también se podrían determinar cisteínas mediante su titulación espectrofotométrica con DTNB. Sin embargo, la cantidad de proteína requerida para este tipo de experimentos está fuera de nuestras posibilidades, dada la poca cantidad de enzima que podemos obtener a partir del riñón.

Es pertinente mencionar que ya hemos realizado experimentos encaminados a determinar si esta desamina tiene grupos SH implicados en su actividad, para ello, la enzima fue incubada durante varias horas con N-etil maleimida y con arsenito de sodio. Los resultados que obtuvimos indicaron una inactivación total de la enzima tratada con NEM, y sólo una ligera inhibición con el arsenito, lo cual indica que esta enzima tiene por lo menos un SH reactivo, aparentemente esencial para la catálisis, pero no grupos tiol vecinales capaces de formar un complejo con el arsénico trivalente.

4.- El estudio del papel fisiológico de esta enzima en la corteza renal implica la realización de experimentos con animales o con órganos mantenidos funcionales fuera del animal. En este punto

[43]CORNISH-BOWDEN, A. (1983) METHODS IN ENZYMOL. 91,60-75.

también existe una limitación ya que no contamos con acceso a animales ni con el equipo necesario, por lo que, aunque es muy interesante explorar qué sucede con la enzima y con los aminoazúcares en los riñones de animales acidóticos, probablemente este tipo de experimentos queden como una opción secundaria.

A N E X O S .

ANEXO UNO.

Metodología Empleada No Incluída en el Manuscrito del Artículo.

Durante el presente trabajo se desarrolló una metodología para tratar de realizar diálisis sin daño significativo de la proteína. Este método consistió en incluír dentro del tubo de diálisis, una resina con la cual interactúe la enzima de nuestro interés. En nuestro caso, utilizamos celulosa fosfato o la matriz de afinidad alostérica, aunque, también probamos con resinas de intercambio iónico (DEAE-Sephacel), asi como con otras proteínas (ovoalbúmina). La idea es que a medida que se dializa, por ejemplo, sulfato de amonio, al bajar la concentración de iones la proteína comenzará a unirse al intercambiador iónico; o bien, en el caso de interacciones por afinidad, al disminuír la concentración del ligando, la enzima se une al análogo inmovilizado. Lo único que se requiere para que funcione adecuadamente, es dializar con un amortiguador de baja concentración y con un pH adecuado para cada propósito.

Con este procedimiento de "diálisis con inmovilización", se protege a la proteína que se desea mediante su interacción con una matriz[20], a la vez que se ahorra tiempo, ya que durante la diálisis se efectúa también la adsorción de la proteína, quedando lista para empacar la columna correspondiente.

En lo que se refiere a la desaminasa de riñón, la recuperación de enzima fue superior al 90%, al usar este método.

ANEXO DOS.

Determinación del peso molecular de la enzima por medio de diversos métodos.

En este apartado señalamos la determinación del peso molecular de la enzima tanto del oligómero como de la subunidad.

El peso de la proteína oligomérica determinado por ultracentrifugación en gradiente lineal de sacarosa (5% a 20%) fue de 180 000 Da. Figura:

No. de Fracción
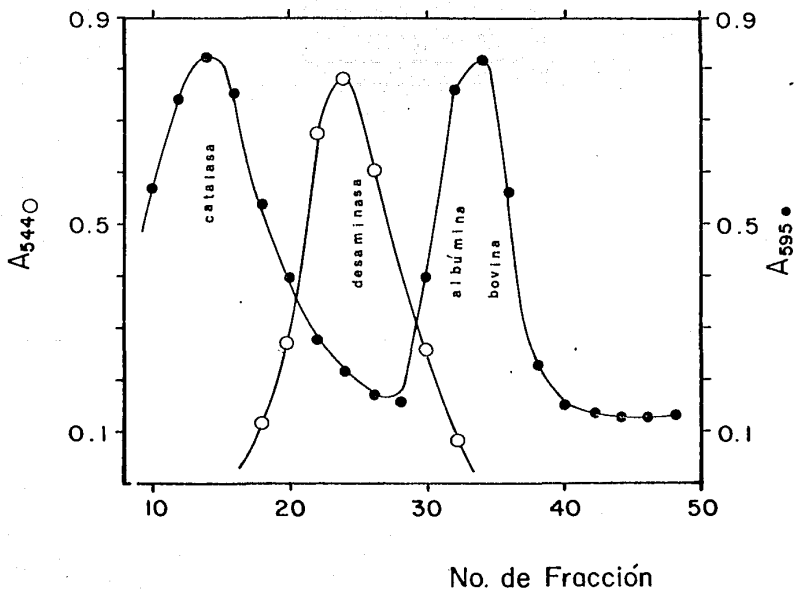
Sin embargo el peso del oligómero obtenido por cromatografía de filtración molecular fue de 160 000 Da. Por otra parte la determinación del peso de la enzima en geles de poliacrilamida en gradiente de poro (4% a 30%), en condiciones no desnaturalizantes arrojó un peso de 230 000 Da. (ver figura 1 del artículo)

ANEXO TRES

Deducción de la Ecuación Empleada para Determinar la K_{dis} del Activador.

$$L' = \frac{L}{\left(1 + \dfrac{[A]}{K_a}\right)^n}$$

$$\frac{1}{L'} = \frac{\left(1 + \dfrac{[A]}{K_a}\right)^n}{L}$$

si $n = 3$:

$$\frac{1}{\sqrt[3]{L'}} = \frac{1 + \dfrac{[A]}{K_a}}{\sqrt[3]{L}}$$

$$\frac{1}{\sqrt[3]{L'}} = \frac{1}{\sqrt[3]{L}} + \frac{[A]}{K_a \sqrt[3]{L}}$$

$$\frac{1}{\sqrt[3]{L'}} = \frac{1}{\sqrt[3]{L}} + [A] \frac{1}{K_a \sqrt[3]{L}}$$

cuando: $\qquad \dfrac{1}{\sqrt[3]{L'}} = 0 \quad \Rightarrow \quad [A] = -K_a$

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ANEXO IV

Artículos publicados colateralmente durante los estudios de Maestría.

Artículo 1.

pHMETRICAL DETERMINATION OF THE GLUCOSAMINE-6-PHOSPHATE
ISOMERASE DEAMINASE REVERSE REACTION

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Summary: In the reverse direction, the reaction catalyzed by glucosamine 6-phosphate isomerase deaminase consumes ammonia and forms GlcN6P. As a consequence of the formation of a product with a lower pK than the substrates, a measurable pH drop in the reaction medium is produced. This property can be used to follow potentiometrically the course of the reaction. The usefulness of the method is demonstrated obtaining the inhibition pattern by GlcN6P when Fru6P is the varied substrate.

INTRODUCTION

The reaction catalyzed by the enzyme glucosamine 6-phosphate isomerase (deaminase) (2-amino-2-deoxy-D-glucose-6-phosphate ketol isomerase (deaminating), E.C. 5.3.1.10) is reversible, and the equilibrium constant, defined as [Fru6P] [NH$_3$Cl]/[GlcN6P], has been estimated in 0.22 M, at 30°C, pH 7.7 (1). Initial velocities of the reverse reaction were generally determined by measuring colorimetrically the GlcN6P formed in a fixed time by means of the Elson-Morgan reaction or some of its modifications (2,3).

Employing this procedure, some kinetic data have been published (1,4), although it is always difficult to maintain a reasonably low reaction progress to obtain good initial velocity measures at different substrate concentrations.

As part of our studies on this enzyme from *Escherichia coli*, we were interested in determining the product inhibition patterns of the enzyme reaction in the glucosamine-forming direction under conditions of hyperbolic kinetics. This study requires a different assay method, compatible with

Abbreviations: GlcN6P, D-glucosamine 6-phosphate; GlcNAc6P, N-acetyl-D-glucosamine 6-phosphate; Fru6P, D-fructose 6-phosphate.

(*) To whom all correspondence should be addressed.

variable concentrations of GlcN6P and saturating concentrations of GlcNAc6P, the allosteric activator of the enzyme. Both amino sugars interfer with the colorimetrical assay of the enzyme. The kinetics measurements in the presence of the reaction product, in the thermodynamically unfavorable direction of the reaction, also require a sensitive method to obtain reliable initial velocity values.

In the course of the reaction, catalyzed by glucosamine 6-P isomerase, an ammonium ion (pK = 9.3) disappears from the reaction mixture, and a 2-amino group with a pK = 8.1 (5) is formed on the sugar-phosphate. On the other hand, the pK of the phosphoric acid ester at C-6 in both sugar-phosphates remains practically unchanged; indeed, values of 6.08 and 6.11 have been reported for GlcN6P and Fru6P, respectively (5). The change in the buffer capacity of the incubation mixture produced by the enzyme reaction determines a measurable release of protons. In this paper, we describe an enzyme assay that takes advantage of this fact to obtain a continuous and sensitive record of the reaction, using a potentiograph. We also demonstrate the value of the method to obtain the product inhibition pattern, when Fru6P is the variable substrate.

MATERIALS AND METHODS

Materials. Fru6P and GlcN6P were products of Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Other reagents were of the best quality available. GlcNAc6P was prepared by chemical acetylation of GlcN6P, according to Leloir and Cardini (6), and the purity of the product was controlled by thin layer chromatography on microcrystalline cellulose plates, developed in ethyl acetate: acetic acid: water: ammonia, 3:3:2:1.

Glucosamine-6-phosphate isomerase deaminase was purified from *E. coli* B (ATCC 11303) as already described (1), omitting the protamine sulfate step.

Ensyme assay. The activity of glucosamine-6-P-isomerase deaminase was measured in standard 1.5 ml polypropylene microcentrifuge tubes under continuous magnetic stirring in a contolled-temperature bath at 30°C. Changes of pH were recorded with a Metrohm E-536 potentiograph, using a small combined glass calomel electrode. The span of the recorder was used at the maximal sensitivity of the instrument, i.e., 0.04 pH unit/cm.

The reaction mixtures contained the substrates, (Fru6P and NH_4Cl), an excess (2.5 mM) of the allosteric activator, GlcNAc6P, and when indicated, variable amounts of the reaction product, GlcN6P. Each mixture was titrated to a pH near 7.7 with 0.1 M KOH, and the volume was completed to 490 µl with water. The reaction was started with the addition of 10 µl of enzyme solution in water, usually 0.2 - 0.8 µM. The reaction was recorded for a few minutes, and the velocities were calculated from the H^+ concentration difference between the first and second minute. The buffering capacity of each reaction mixture was measured by means of a similarly prepared sample,

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to wich 2.5 µl of 40 mM HCl was added instead of the enzyme. The pH change was recorded to calculate the actual release of hydrogen ions during the enzyme reaction.

Treatment of kinetic data. Michaelis-Henri kinetics was fitted and the corresponding parameters calculated according to Wilkinson (7); inhibition data were also processed according to Canela (8). In both cases, a Commodore 128 microcomputer was used, with the necessary modifications of the original programs.

RESULTS AND DISCUSSION

Fig. 1 A shows a typical potentiograph record for the glucosamine-6-phosphate isomerase deaminase reverse reaction. The initial rates were read at the recorded curve over a pH range smaller than 0.1 unit. We have already reported (1) that K_m values for Fru6P and ammonium are nearly constant for this enzyme over the pH range 7.2 to 7.7; the maximal velocity for the reaction is also constant over a wider range. So, the measures allow the determination of the kinetic parameters without a significant effect of the pH drop during the reaction.
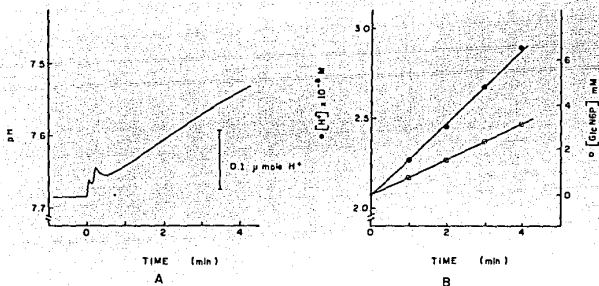

Figure 1. A: A typical potentiograph record of the glucosamine-6-phosphate isomerase reverse reaction. The reaction mixture contained 10 mM Fru6P, 50 mM NH₄Cl, 2.5 mM GlcNA6P and 30 nM enzyme. The vertical line indicates the pH change produced by the addition of a calibrating amount of HCl solution. B: (●) Data from the same experiment expressed in actual H⁺ concentration. Reaction rate is 2.13×10^{-9} M min⁻¹, and corresponds to a release of H⁺ ions of 8.05×10^{-5} M min⁻¹, according to the buffer capacity determined from calibration. (o) Time course of the same reaction, determined by the simultaneous colorimetrical assay of GlcN6P, as described in Methods. (vo = 7.92×10^{-4} M min⁻¹).

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The linearity of the time course of the reaction is apparent when hydrogen ion concentration (the actual concentration measured, or the calculated amount of H^+ ions released per liter) is plotted as a function of time. (Fig. 1 B).

The value of the H^+/GlcN6P ratio was determined experimentally in a reaction mixture containing 10 mM Fru6P and 50 mM NH_4Cl, under conditions similar as described in Methods. Simultaneously with the pH recording, 10 μl samples of the reaction mixture were taken every 30 seconds for colorimetric analysis of GlcN6P according to Levy and Mc Allan (3). (Fig. 1B). This experiment gave a H^+/GlcN6P ratio of 0.11 (average of two separate determinations).

Taking into account the concentration change of GlcN6P and ammonia, its pK values and the pH change, a theoretical H^+/GlcN6P ratio of 0.10 was calculated for the same extent of reaction.

When the proton release rate was measured as described at fixed substrate and allosteric activator concentrations, first-order kinetics with respect to the enzyme concentration over a wide range was obtained. (Fig. 2).
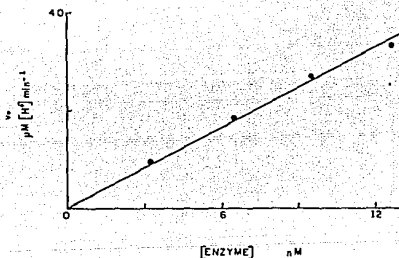
Figure 2. Initial reaction rates (expresed in concentration of released H^+ ions per minute) as a function of enzyme concentration. The reaction mixture contained 10 mM Fru6P, 50 mM NH_4Cl, 2,5 mM GlcNAc6P and variable enzyme concentrations, as indicated. From these data and a H^+/GlcN6P ratio of 0.10 (see the text), a molecular activity of 447 s^{-1} can be calculated.

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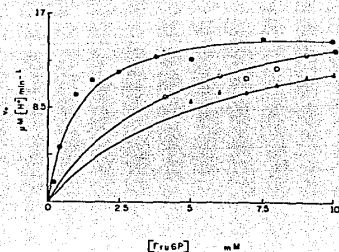-oꞁ ₂r kinetics with respect
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[Fru6P]   mM

Figure 3.   Inhibition pattern by GlcN6P with variable concentration of Fru6P, 50 mM NH₄Cl and 2.5 mM GlcNAc6P. Reaction rate is expressed in concentration of released H⁺ ions per minute. (●) without GlcN6P; (o) 0.5 mM GlcN6P; (▲) 0.75 mM GlcN6P. The inhibitor concentrations were respectively 30 and 20% less than the equilibrium concentrations calculated with the lowest concentration of Fru6P used. The values of the parameters, evaluated according to Canela (8), were: (●) Vm = 16.38 ± 0.77 µM/min, Km = 0.96 ± 0.18 mM; (o) Vm = 18.10 ± 0.22 µM/min, Km = 1.13 ± 0.12mM, Ki = 0.16 ± 0.04 mM (▲) Vm = 16.50 ± 0.72 µM/min, Km = 1.06 ± 0.19 mM, Ki = 0.17 ± 0.09 mM. Points represent experimental data; lines were drawn with the parameters obtained by the statistical procedure.

The GlcN6P inhibition pattern with variable Fru6P and fixed ammonium concentrations is show in Fig. 3. The Km value for Fru6P is 1.07 mM. The inhibition by GlcN6P is competitive, according to the statistical procedure of Canela (8); apparent Ki is 0.17 mM.

The procedure described in this article is simple, reliable, and has the additional advantage of providing a continuous record of the reaction. Its main value resides in that it allows measuring the enzyme activity in the presence of considerable amounts of GlcN6P or GlcNAc6P.

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Artículo 2.

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 $(1,10\text{-phenanthroline})_3\text{-Cu(II)}$ 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. © 1989 Academic Press, Inc.

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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[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; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); SDS, sodium dodecyl sulfate.

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 × 10⁴ 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.
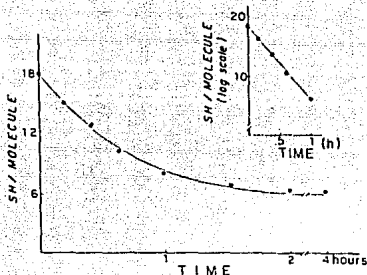
Reaction rate measurements. Enzyme assays were

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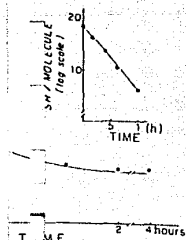
made in the forward (deaminating) direction of the reaction, by means of a stop-time colorimetric determination of Fru6P with the resorcinol-hydrochloric acid reaction (4).

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
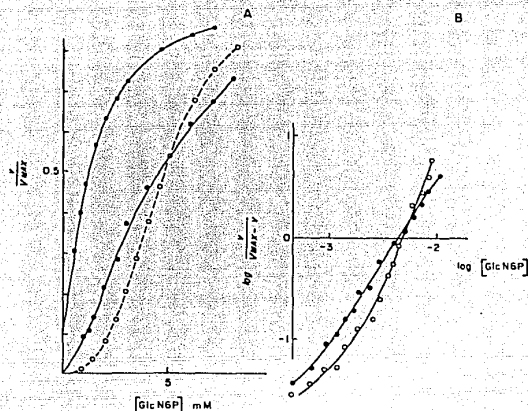
FIG. 2. Kinetics of glucosamine-6-phosphate deaminase modified by methylation. Experimental conditions are described under Materials and Methods. Data plotted are averages from two separate experimental series. (A) Squares: in the presence of 2.0 mM GlcNAc6P (K_m 1.63 mM); full circles: in the absence 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.

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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	4.53 ± 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-

h_{max}	$s_{0.5}$
3.0 ± 0.02	4.83 ± 0.40
1.7 ± 0.10	4.59 ± 0.35
1.67 ± 0.12	5.60 ± 0.25
07	4.80
1.50	8.50

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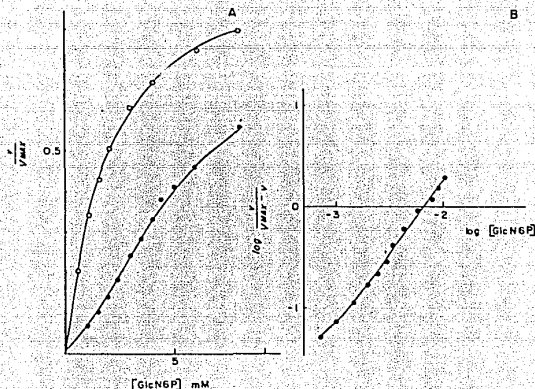
FIG. 3. Kinetics of arsenite-treated glucosamine-6-phosphate deaminase. Experimental conditions are described under Materials and Methods; plotted data are averages from three separate experimental series. (A) Open circles: assays in the presence of 2.0 mM GlcNAc6P (K_m 1.63 mM); full circles: in the 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-
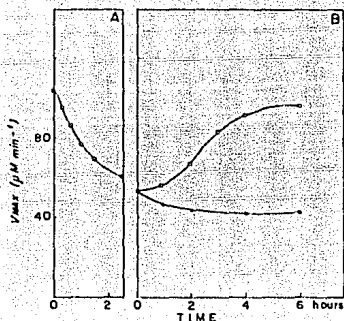
FIG. 4. (A) Time course of arsenite modification of glucosamine-6-phosphate deaminase, evaluated by means of the V_{max}; 1500 μl of a solution containing 0.1 μM glucosamine-6-phosphate deaminase, 1 mM sodium arsenite, 20 mM Tris-HCl buffer (pH 8.0, 20°C), and 5 mM EDTA was incubated at room temperature; 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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*Sin duda alguna, Sancho, que
ya debemos de llegar a la segunda region del aire, adonde se
engendra el granizo, las nieves; los truenos, los relampagos y los
rayos se engendran en la tercera region; y si es que desta manera
vamos subiendo, presto daremos en la region del fuego...*

Don Quijote