Centro de Investigación en Alimentación y Desarrollo, A.C.

ESTUDIOS CINÉTICOS Y CONFORMACIONALES DE LA BETAÍNA ALDEHÍDO DESHIDROGENASA DE RIÑÓN DE CERDO

Por:

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TESIS APHOI3ADA POI LA

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COMO I EQUISITO PAHCIAL PAHA OBTENER EL GI ADO DE

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CONTENIDO

RESUMEN

Como parte del mecanismo concentrador de orina, las células de la médula renal están expuestas a un ambiente hiperosmótico, conteniendo altas concentraciones de iones inorgánicos y urea. La síntesis glicina betaína (GB) es crucial para el ajuste osmótico de las células renales. Para que esta síntesis se lleve a cabo, la betaína aldeíhldo deshidrogenasa (BADH) debe de mantener su actividad y estabilidad bajo dichas condiciones ambientales. Se encontró que la BADH se inactiva completamente a 45°C, formando agregados de muy alto peso molecular. La fuerza iónica dada por cationes monovalentes (K+), incrementa la termoestabilidad de la enzima. La desnaturalización térmica de la enzima analizada mediante fluorescencia indica que ésta se lleva a cabo en dos etapas. El K+ estabiliza significativamente a la enzima durante la primera etapa de transición, no así en la segunda, donde la temperatura es extrema para la BADH. Altas concentraciones de urea inducen la agregación de la BADH, siendo ésta revertida a fuerza iónica fisiológica y alta, lo que significa que bajo condiciones fisiológicas renales, la BADH puede mantener su estabilidad y por tanto, su actividad bajo condiciones de alta concentración de urea. Concentraciones fisiológicas de urea inhiben de manera competitiva a la BADH con respecto a NAD+, y no-competitiva con respecto a BA, bajo condiciones saturantes de los sustratos. Las constantes de afinidad aparentes para NAD+ se ven poco afectadas por urea; sin embargo para BA, se incrementan un orden de magnitud bajo estas condiciones. A condiciones subsaturantes, la inhibición por urea es mixta con respecto a NAD+, y no-competitiva con respecto a BA. El efecto de la urea sobre la KmBA se incrementa 3 veces, es menos drástico bajo estas condiciones.

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El dlsulflram es un fármaco que Inhibe a las aldel1ldo deshldrogenasas y es usado en el tratamiento del alcoholismo. A pH 7.0 y bajo condiciones de pseudo prlrner orden, concentraciones por debajo de 30 1,1M de DSF inhiben a la 13ADH de rif'ión de cerdo. Concentraciones fisiológicas de betrilna alde1·1ldo y glicina betalna protegen parcialmente a la enzima contra la lnactivación, mientras que las de NAD' y NADH la pl'()teglm compkitmnente. Este r,fecto protector de NAD' es dependiente de la concentración y los datos obtenidos sugieren que la coenz.irna induce cambios conforn1acionales que protegen tl la BADH de la inactivación por disulfirarn.

La regulación d(la actividad de la l.3ADH renal está directamente influenciada por el medio ambiente en el cual se encuentra. L.a fuerza iónica y las concentraciones de NAD' dese111pef1an papelt s irnportanl.es en dicha rc;gulación.

SINOPSIS

Aldehído deshidrogenasas

La betaína aldehído deshidrogenasa (EC 1.2.1.8 betaína aldehído: NAD^+ oxidorreductasa) cataliza la reacción de oxidación de la betaína aldehído a glicina betaína y pertenece a la super familia de las aldehído deshidrogenasas (ALDH) (Yoshida *et al.*, 1998).

La BADH ha sido purificada a homogeneidad a partir de animales, plantas y microorganismos. En plantas se ha purificado a partir de *Spinacia oleracea, Amaranthus hypochondriacus, Amaranthus palmieri* (Weretilnyk y Hanson, 1989; Valenzuela-Soto y Muñoz-Clares, 1994; Figueroa-Soto y Valenzuela-Soto, 2001). Estudios filogenéticos muestran que la BADH de animales y algunas de bacterias pertenecen a la familia de las ALDH9, mientras que las de plantas a la ALDH10 (Vasiliou *et al.*, 1999).

Localización de la BADH

Plantas. En plantas de espinaca, la enzima está localizada en las hojas, mayoritariamente en los cloroplastos y en mucha menor cantidad en el citoplasma (Weigel *et al.*, 1986). Por otro lado, se ha demostrado la localización de la BADH en los peroxisomas de las hojas de plantas de arroz (Nakamura *et al.*, 1997).

Animales. En mamíferos, se ha reportado que la BADH está presente en el hígado, el cerebro, la glándula adrenal, el páncreas, el músculo esquelético, el músculo cardiaco y el riñón (Chern y Pietruszko, 1995; Pietruszko *et al.*, 1996; Izaguirre *et al.*, 1997; Guzmán-Partida y Valenzuela-Soto, 1998). En riñón de cerdo la BADH está localizada en la corteza y la médula (Figueroa-Soto *et al.*, 1999). En la médula interna, la BADH se encuentra específicamente en las células que rodean los túbulos (Figueroa-Soto *et al.*, 1999). Chern y Pietruszko (1999) demostraron que la BADH de hígado de rata está localizada en la matriz mitocondrial.

Características cinéticas de la BADH

Especificidad por substratos. La BADH de mamíferos puede usar aldehídos alifáticos o aromáticos como sustratos, por ejemplo: el aminobutiraldehído, el γ-trimetilaminobutiraldehído, el acetaldehído, el gliceraldehído, el benzaldehído, y la *N,N* – dimetilglicina; sin embargo, la enzima muestra una marcada preferencia por la betaína aldehído (BA). Las BADH de plantas tienen una mayor especificidad por BA.

Por otra parte, se ha visto que la BADH puede usar como coenzima, tanto al NAD^+ como al $NADP^+$, dependiendo de la fuente de la enzima. En general, todas las BADH estudiadas prefieren al NAD^+ como coenzima, en contraste, algunas otras, como la de *P. aeruginosa*, prefieren utilizar al $NADP^+$.

Mecanismo cinético. Aún cuando la BADH se ha purificado de varias fuentes, y su gene ha sido clonado y secuenciado de varios organismos, existen escasos estudios cinético sobre esta enzima. Se han reportado tres mecanismos cinéticos para la BADH dependiendo de la fuente de donde fue aislada. La enzima de *E. coli* sigue un mecanismo Ping Pong (Falkenberg *et al.*, 1990), mientras que en *P. aeruginosa* presenta un mecanismo al azar con preferencia para NAD^+ como el primer sustrato que se une a la enzima (Velasco-García *et al.*, 2000); en amaranto y en riñón el mecanismo es Iso Bi Bi Ordenado (Valenzuela-Soto y Muñoz-Clares, 1993; Figueroa-Soto y Valenzuela-Soto, 2000).

Características estructurales de la BADH

La BADH se puede encontrar como homotetrámero presentando masas moleculares de 213-219 kDa en bacterias y animales (Falkenberg y Strom, 1990; Guzmán-Partida y Valenzuela-Soto, 1998; Velazco-García *et al.*, 1999). En plantas se encuentra como dímero de 125 kDa (Valenzuela-Soto y Muñoz-Clares, 1994). Cada subunidad de BADH tiene tres distintos centros: un centro de unión de la coenzima, un centro catalítico y un centro de oligomerización (Johansson *et al.*, 1998).

Regulación de la Betaína Aldehído Deshidrogenasa

Cationes mono y divalentes. Varias enzimas requieren cationes para su máxima actividad, pues afectan las constantes cinéticas de la reacción y/o tienen un papel en la estabilización de la conformación activa de la enzima (Ahmad *et al.*, 1995; McQuenney y Markham, 1995).

Se ha propuesto que la BADH de *E. coli*, hojas de amaranto, *Bacillus subutilis*, riñón de cerdo y cangrejo son activadas en algún grado por K^+ (Falkenberg y Strom, 1990; Dragolovich y Pierce, 1994; Valenzuela-Soto y Muñoz-Clares, 1994; Boch *et al.*, 1997; Guzmán-Partida y Valenzuela-Soto, 1998).

En plantas de amaranto, el K^+ a baja concentración (50 mM) aumentó 100% la actividad de la BADH, mientras que una concentración de 150 mM aumentó la actividad un 150% a concentraciones subsaturantes de sustrato. Altas concentraciones de KCl (0.4 M) no afectaron de manera importante la actividad de la enzima. (Valenzuela-Soto y Muñoz-Clares, 1994)

La BADH de riñón de cerdo tiene tolerancia hacia Na^+ y K^+ manteniendo cerca del 50% o más de la actividad máxima a una concentración de 0.4 M de estos cationes a concentraciones saturantes o subsaturantes de substratos. El NH_4Cl y el Mg^{++} a concentraciones saturantes de sustratos inhiben más del 60% de la actividad de la enzima (Guzmán-Partida y Valenzuela-Soto, 1998).

Recientemente se encontró que la BADH de *P. aeruginosa* requiere estrictamente iones K^+ para el mantenimiento de su conformación activa; no

obstante, la identificación del mecanismo por el cual estos iones pueden regular a la enzima aún permanece sin estudiar (Valenzuela-Soto *et al.*, 2003). Por otra parte, se ha encontrado que la BADH renal requiere de cierta fuerza iónica (dada por cationes monovalentes) para mantener su estructura tetramérica (Valenzuela-Soto *et al.*, 2003).

El manganeso también inhibe la actividad de la BADH renal (Ruiz-López *et al.*, 2006).

Con base en la revisión de la literatura disponible sobre esta enzimas, en este trabajo se planteó la siguiente hipótesis:

La estructura y/o conformación de la BADH renal se modifica en función de la fuerza iónica, así como en presencia de otros efectores fisiológicos. Estos en conjunto afectan su actividad catalítica.

Para contrastarla, se propuso como objetivo general el determinar las características cinéticas y estructurales de la betaína aldehído deshidrogenasa de riñón bajo condiciones de fuerza iónica fisiológica y en presencia de otros efectores fisiológicos, como son la urea, la temperatura, el pH y los reactivos específicos con grupos sulfhidrilos.

En primera instancia, se abordó el estudio sobre el requerimiento de fuerza iónica fisiológica dada por un catión monovalente, el cual se presenta en el capítulo 1. Mediante ensayos de inactivación por temperatura se

comprobó que el ión K^+ protege a la enzima hasta 40°C. Adicionalmente, se determinó la importancia del K^+ para el mantenimiento de la conformación activa de la enzima. El efecto protector del K^+ ó de los cationes monovalentes es específico, dado que esto no se observó al adicionar Mg^{++}. El análisis de fluorescencia intrínseca del triptofano permitió evidenciar que el K^+ aumenta la afinidad de la enzima por la coenzima.

En animales, el producto más abundante del catabolismo de las proteínas es el nitrógeno, el cual es excretado en la orina en forma de urea. La concentración de urea en la orina puede ser de 20 a 100 veces mayor que en la sangre de humanos (5-10 mM) (Yang y Bankir, 2005). La acumulación pasiva o activa de la urea en el fluido intersticial de la médula renal aumenta al máximo la concentración de orina que puede formar el riñón (Levinsky y Berlinder, 1959). Un número significativo de estudios han demostrado que la urea es un desestabilizador de macromoléculas e inhibidor de funciones, tales como la unión de ligandos (Yancey, 2005). Sin embargo, existen pocos estudios cinéticos sobre el efecto de la urea en enzimas.

En el capítulo 2, se presentan los resultados sobre el efecto de urea sobre la actividad y estabilidad de la BADH, y cómo este efecto varía con la fuerza iónica. Los estudios sobre el efecto de la urea en la actividad de la BADH mostraron que a concentraciones fisiológicas de urea (entre 0.3 y 1 M), no existen cambios en su actividad, mientras que a concentraciones mayores a 1 M de urea, se observa un efecto inhibitorio marcado. La enzima

se inactiva al adicionar 1 M de urea, a 37°C y la presencia de fuerza iónica fisiológica evita que la enzima sea inactivada por tal concentración de urea. Se encontró que la urea ejerce su efecto sobre la agregación de las subunidades de la BADH, disminuyendo la concentración del tetrámero, que es la conformación activa de esta enzima. No obstante, esta agregación puede ser revertida por la presencia de fuerza iónica fisiológica.

Pocos estudios de investigación se han enfocado al estudio cinético del efecto de concentraciones bajas de urea en las proteínas. Esta información es de relevancia, pues proporcionaría información de lo que podría estar pasando *in vivo*. Dado que las células del riñón de los mamíferos normalmente se encuentran bajo condiciones hipertónicas debido al mecanismo concentrador de orina, en el cual se elevan las concentraciones extra e intracelulares de urea (Beck *et. al.*, 1998), en el capítulo 3 se analizó el efecto de concentraciones bajas de urea sobre los parámetros cinéticos de la BADH.

La urea tiene un efecto menor en la *Vmax* de la enzima cuando las concentraciones de los sustratos son saturantes. Sin embargo, cuando dichas concentraciones son subsaturantes la *Vmax* disminuye más de 60%. El tipo de inhibición provocado por urea fue competitvo con respecto a NAD^+, bajo condiciones saturantes y subsaturantes de BA. Con respecto a BA, la inhibición fue no-competitva bajo condiciones saturantes y subsaturantes de NAD^+. Los datos de inhibición por urea concuerdan con el

mecanismo ordenado seguido por la BADH, donde NAD^+ es el primer sustrato que se une a la enzima.

El disulfiram (DSF) es un fármaco utilizado desde hace más de 50 años en el tratamiento del alcoholismo. El efecto farmacológico se debe a la inhibición irreversible de la acetaldehído deshidrogenasa hepática (ALDH), provocando la elevación de los niveles de acetaldehído al ingerir alcohol, que da lugar a una seria de respuestas físicas caracterizadas por nausea, vómito, taquicardia e hipertensión (Huffman *et al.*, 2003; Mirsal *et al.*, 2005).

Existen reportes en la literatura que proponen que el mecanismo de inhibición por DSF sobre las aldehído deshidrogenasas (ALDH's) es a través de la formación de un puente disulfuro intermolecular entre la cisteína y el DSF (Kitson, 1983; Kitson, 1987; Veverka *et al.*, 1997). En contraste, otros autores proponen la formación de un puente disulfuro intramolecular entre cisteínas vecinales (Shen *et al.*, 2000; Lipsky *et al.*, 2001). Se ha encontrado que el DSF inactiva a la BADH de *P. aeruginosa* y de *A. hypochondriacus* L. (Velasco-García et al., 2003). El disulfiram provoca que la BADH de *P. aeruginosa* se disocie o forme agregados cuando los ensayos de inactivación se realizan a 23°C o a 37°C, respectivamente (Velasco-García *et al.*, 2006).

En el capítulo 4, se estudió el efecto del DSF sobre la BADH de riñón. Los estudios fueron realizados a pH 7.0 y fuerza iónica fisiológica. La enzima se inactivó totalmente en presencia de 30 μM DSF. La inactivación por DSF sigue un proceso bifásico, lo cual puede apuntar a la comunicación entre dos

sitios de la enzima, en donde la cisteína catalítica perdería la reactividad. Esta inactivación fue revertida por DDT y 2-mercaptoetanol; por su parte, el glutatión reducido (GSH) no fue capaz de revertir dicha inactivación, lo que resulta importante ya que GSH es el agente reductor fisiológico. Esta falta de efecto se puede deber a la incapacidad o inhabilidad para alcanzar el sitio activo debido a las dimensiones de la molécula de DSF, lo cual provoca que GSH no pueda reducir el puente disulfuro formado en el sitio activo de la enzima. Al estudiar el efecto de los ligandos en la inactivación provocada por disulfiram, se encontró que el NAD^+ y NADH son los que mayor protección ofrecen a la enzima. Dicha protección fue dependiente de la concentración de ligando. La protección ejercida a bajas concentraciones de NAD^+ tiene importancia fisiológica, ya que el mecanismo cinético de la enzima es Iso Bi Bi Ordenado en el cual el NAD^+ es el primer substrato que se une a la enzima. Estos resultados indican que la BADH puede estar protegida por NAD^+ contra la inactivación por DSF *in vivo*.

CONCLUSIONES

Las condiciones ambientales presentes en las células de la médula renal tienen influencia en la actividad y estabilidad de BADH. La fuerza iónica dada por K^+ aumenta la termoestabilidad y regula el efecto de altas concentraciones de urea en la actividad y estabilidad de la enzima. Las concentraciones de urea en el riñón en condiciones de antidiuresis inhibien a la enzima, atacando directamente el sitio de unión de la coenzima, sin embargo, en condiciones fisiológicas este sitio esta saturado por NAD^+, por lo tanto se podrá sintetizar glicina betaina y llevarse a cabo la osmorregulación.

La cisteína del sitio activo es esencial para la oxidación de betaína aldehído a glicina betaína, esta puede ser modificada por fármacos que tienen especificidad por grupos sulfhidrilos causando la inactivación de la enzima. La presencia de NAD^+ y fuerza iónica fisiológica provoca cambios conformacionales en el sitio activo protegiéndolo contra dicha inactivación. Bajo estas condiciones reactivos específicos de grupos sulfhidrilos del tipo de disulfiram no impiden que se lleve a cabo la síntesis de glicina betaína.

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CAPÍTULO 1

Effects of monovalent and divalent cations on the thermostability of porcine kidney betaine aldehyde dehydrogenase

Articulo de Investigación Original

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Effects of Monovalent and Divalent Cations on the Thermostability of Porcine Kidney Betaine Aldehyde Dehydrogenase

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Abstract

In renal cells, the synthesis of glycine betaine, in a reaction catalyzed by betaine aldehyde dehydrogenase (BADH), plays an important role in osmotic regulation. In this work we have studied the effect of monovalent, K^+, and divalent, Mg^{2+}, cations as chloride salts of equal ionic strength, on the thermal stability of porcine kidney BADH. The extent of enzyme inactivation at 40°C was much less in the presence of 150 mM KCl than it was either in the presence of 50 mM $MgCl_2$ or in the absence of salts. Also, heat inactivation at 45°C was fully reversible only if K^+ ions were present. Activity-temperature curves, obtained by assaying the remaining BADH activity after 15-min incubations of the enzyme in temperatures ranging from 30 to 60°C, gave the highest apparent T_m value for the enzyme in the presence of K^+ ions, while in the presence of Mg^{2+} ion this value was even lower than that of the enzyme at low ionic strength. The enzyme at low ionic strength formed aggregates of very high molecular weight at temperatures above 39°C. At both low and near physiological ionic strength given by 150 mM KCl, changes in tryptophan fluorescence intensity indicated two thermal-induced transitions in the enzyme. K^+ cations significantly stabilized the enzyme during the first transition but not during the second. The pure enzyme preparation has NADH bound, which dissociated by dialysis against the low ionic strength buffer devoid of K^+ but remained tightly bound in the presence of this cation, even at 60°C. In summary, our results support the requirement of physiological ionic strength, given by a monovalent cation, for maximum thermostability of kidney BADH and suggest the participation of K^+ in the binding of the coenzyme.

Keywords: betaine aldehyde dehydrogenase; glycine betaine; renal osmoregulation; monovalent cations; enzyme thermostability

Introduction

Kidney medulla cells are exposed to wide changes in the ionic and osmotic composition of their environment during diuresis and antidiuresis. When concentrated urine is excreted, the extracellular NaCl concentrations may reach molal levels, causing reduction of cell volume, as a consequence of water loss from the cells, and increased intracellular concentrations of Na^+, K^+ and Cl^- ions. Recovery of normal cell volume involves in a first step the import of Na^+ and Cl^- ions via specific transport systems, which leads to a high intracellular Na^+ concentration. But this increase is transitory as most of Na^+ ions entering the renal medulla cells are replaced by K^+ ions due to the activation of the Na^+/K^+-ATPase. Therefore, during water deprivation the intracellular concentrations of Na^+ ions moderately increase while those of K^+ ions rise significantly (Beck et al., 1998). But although this mechanism leads to complete cell volume restoration, the resulting high intracellular ionic strength would have deleterious effects on cell function. Because of that, in the second and much slower stage, the renal cells adjust osmotically by accumulating large amounts of small organic osmolytes, such as glycine betaine, glycerophosphorylcholine, sorbitol, inositol, and taurine (Burg, 1995), which not only allow normal

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cell volume but also normal electrolyte concentrations (Csonka and Hanson, 1991). The accumulation of compatible osmolytes is thus crucial for the viability of renal medulla cells.

Glycine betaine is synthesized from betaine aldehyde in an oxidation reaction catalyzed by betaine aldehyde dehydrogenase (BADH, EC 1.2.1.8). Porcine kidney BADH has been purified (Guzmán-Partida and Valenzuela-Soto, 1998) and kinetically characterized (Figueroa-Soto and Valenzuela-Soto, 2000). This enzyme, as other known animal BADHs (Chern and Pietruzko, 1999; Johansson et al., 1998), is tetrameric (Valenzuela-Soto et al., 2003) and shows a marked preference for NAD^+ over $NADP^+$ (Guzmán-Partida and Valenzuela-Soto, 1998). Previous works showed that porcine kidney BADH retains 50% or more of its maximum activity at concentrations of 1.0 M Na^+ or K^+ ions in the assay medium (Guzmán-Partida and Valenzuela-Soto, 1998). Additional studies indicated that it requires monovalent cations at near physiological concentrations to maintain its tetrameric native structure (Valenzuela-Soto et al., 2003). These results correlate with the physiology of renal tissue, where BADH must be active under high ionic strength conditions.

As glycine betaine synthesis is crucial to renal cell osmotic adjustment, we were interested in characterizing the ion requirements of kidney BADH for its stability. Also, we wanted to know if divalent cations have the same effects as monovalent cations on the enzyme. With this aim, in this work we pursued the characterization of the mammal kidney BADH by analyzing its thermal stability in the absence and presence of either monovalent, K^+, or divalent, Mg^{2+}, cations, both as chloride salts of near physiological ionic strength.

Methods

Enzyme purification and assay

BADH was purified from porcine kidney and its activity assayed at 30° C by earlier reported procedures (Guzmán-Partida and Valenzuela-Soto, 1998). The pure enzyme was stored at −20° C in a 10 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, 10 mM β-mercaptoethanol, 200 mM KCl, and 10% (v:v) glycerol. Previous to the thermal treatments, BADH (0.15 mg/mL) was dialyzed overnight at 4° C against 10 mM HEPES-Na buffer, pH 7.0, 1 mM EDTA, 10 mM β-mercaptoethanol (buffer A), against buffer A with the addition of 150 mM KCl (buffer B), or against buffer A with the addition of 50 mM $MgCl_2$ (buffer C).

Kinetics of inactivation by temperature

Immediately after dialysis, BADH (0.15 mg/mL) was incubated at 40° C and its remaining activity determined by the standard assay at indicated times. The kinetics of reactivation were studied in enzyme samples which were partially inactivated by a 15-min incubation at 45° C and then cooled to 30° C. The recovery of activity was followed for an additional 90 min period. The time-courses of enzyme inactivation and reactivation were analyzed by nonlinear fit of the experimental data to single exponen-

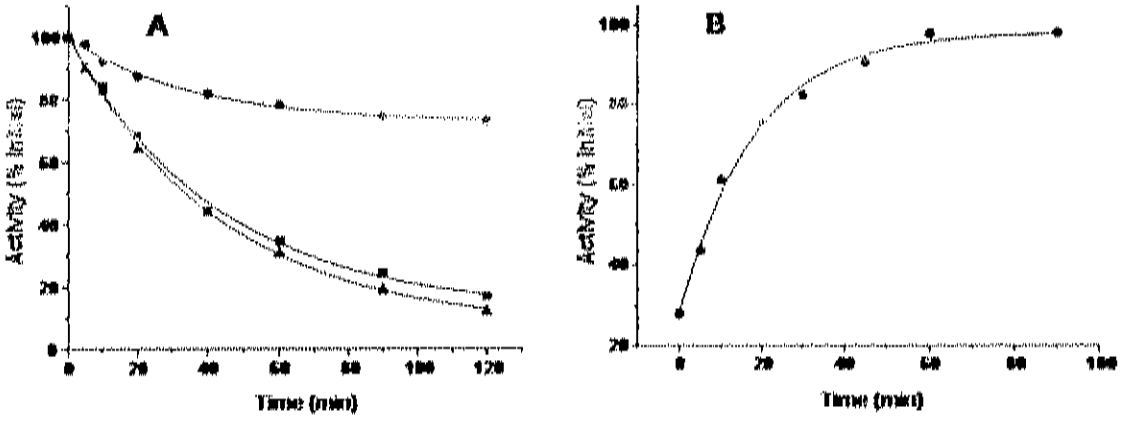

Figure 1. Effect of low and physiological ionic strength on thermal inactivation and reactivation kinetics of porcine kidney BADH. (A) Inactivation at 40° C in buffer A (■), buffer B (●) or buffer C (▲). (B) Recovery of activity at 30° C of enzyme in buffer B previously inactivated at 45° C for 15 min.

Table 1. Effect of cations on the kinetics of inactivation at 40° C of kidney BADH.

Condition	Activity lost[1] (% initial)	Activity at equilibrium[1] (% initial)	k_{obs} ($\times 10^{-2}$)[1] (min^{-1})
Low ionic strength	88.9 ± 2.4	12.0 ± 2.5	2.34 ± 0.18
150 mM KCl	27.7 ± 1.0	72.4 ± 1.0	2.81 ± 0.31
50 mM $MgCl_2$	93.9 ± 1.6	6.4 ± 1.8	2.30 ± 0.11

1. The kinetic parameters are the estimates resulting from a fit of the data in Fig. 1 to a single exponential decay equation ± the S.D. of the estimates.

tial decay and growth equations, respectively. The Microcal Origin program for non-linear regression fitting was used in all analysis of these and the rest of the experimental data.

Thermal stability

In these experiments, samples of BADH (0.15 mg/mL) in the three buffers were incubated in a series of 15 min periods at indicated temperatures in a range from 25 to 61° C. The residual activity, the turbidimetry, indicated by absorbance at 600 nm, and the intrinsic protein fluorescence spectrum of the samples were measured after incubation at each temperature. Fluorescence spectra were recorded using an excitation wavelength of 296 nm and emission wavelength of 300–450 nm (5-nm bandwidth). The spectra were corrected by subtraction of the solvent spectrum obtained under identical conditions. Changes in fluorescence emission intensity at a single wavelength were used to calculate the fraction of unfolded protein at increasing temperatures (F_U) according to the following equation:

$$F_U = (f_N - f)/(f_N - f_U)$$

where f is the observed fluorescence intensity at a given temperature, f_U the fluorescence intensity when the protein is completely unfolded, and f_N the fluorescence intensity of the native protein. The resulting data were plotted *versus* temperature and fitted to a double sigmoidal equation. Apparent T_m is defined as the temperature at the midpoint of the inactivation or unfolding transition.

Results and Discussion

Kinetics of thermal-induced inactivation and reactivation

BADH from porcine kidney was inactivated when incubated at 40° C at low or at physiological ionic strength given by either KCl or $MgCl_2$ (Fig. 1A). Enzyme inactivation wax a first-order, monophasic process, and reached a plateau indicative of the establishment of an equilibrium between inactive and active forms of the enzyme. The kinetic parameters of inactivation estimated under the three conditions are given in Table 1. The rate of inactivation was slightly slowed down by K^+ but not by Mg^{2+} ions. The main effect of K^+ ions was to significantly increase the amount of enzyme that remained active at 40° C. Also, heat inactivation at 45° C in the presence of K^+ ions was fully reversible by cooling the samples to 30° C (Fig. 1B), whereas the enzyme at low ionic strength only recovered a small percentage (7%) of its original activity.

Thermal inactivation

The K^+ ions stabilizing effects were also observed when porcine kidney BADH was incubated for 15 minutes in a temperature range between 25 to 61° C. The thermal inactivation profiles of the enzyme samples in the three conditions are shown in Fig. 2A and the apparent T_m values given in Table 2. In the presence of KCl, the apparent T_m was 4° C higher than that at low ionic strength, but in the presence of $MgCl_2$ the estimated value was even below the latter, which confirmed that divalent cations cannot replace monovalent cations for enzyme stabilization.

The turbidity of the samples indicated enzyme aggregation starting after a 15 min incubation at 39, 42, and 43° C in the low ionic strength, KCl, and $MgCl_2$ samples, respectively (Fig. 2B). At low ionic strength the increase in absorbance at 600 nm was transitory, most likely due to precipitation of protein aggregates of very high molecular weight. In fact, the soluble protein concentration of the heated samples, determined after a brief centrifugation, progressively and significantly decreased in the low ionic strength samples, while remaining constant at high ionic strength (Fig. 2C).

Thermal denaturation

The BADH thermal-induced unfolding transitions were monitored both at low ionic strength (buffer A)

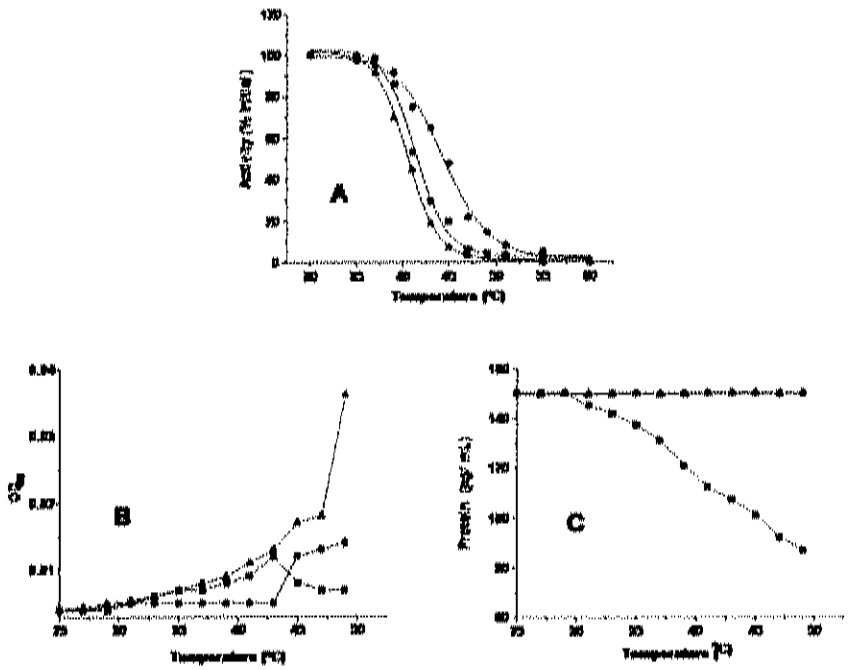

Figure 2. Thermal inactivation of porcine kidney BADH. (A) Activity *versus* temperature profiles at low ionic strength (buffer A) (■) or at physiological ionic strength given by KCl (buffer B) (●) or by $MgCl_2$ (buffer C) (▲). (B) Turbidity and (C) soluble protein concentration of enzyme samples in buffer A (■), B (●), or C (▲).

and at physiological ionic strength in the presence of 150 mM KCl (buffer B) by following the changes in fluorescence emission intensity at 334 nm after an excitation at 296 nm. Intrinsic protein fluorescence spectra are shown in Fig. 3. The enzyme native state at 25° C was characterized by maximum emission at 335.2 and 336.7 nm for the enzyme in the absence and the presence of 150 mM KCl, respectively. The denaturation process was accompanied by both a decrease in fluorescence intensity and a red shift of the maximum emission wavelength, which indicates an increased exposure of the tryptophan residues to the aqueous solvent.

At temperatures above 40° C a second emission peak at 430–440 nm was observed in the enzyme in the presence of 150 mM KCl but not in the enzyme at low ionic strength (Fig. 4). This peak clearly arose from fluorescence resonance energy transfer from the excited tryptophan residue(s) to protein bound NADH as the emission wavelength of tryptophan overlap with the absorption spectrum of the coenzyme. In the enzyme in the presence of

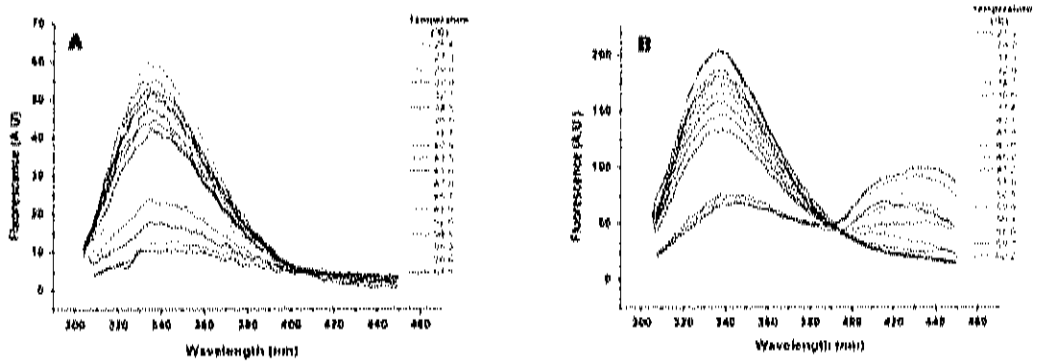

Figure 3. Temperature-induced changes in intrinsic protein fluorescence of porcine kidney BADH. (A) Tryptophan emission spectra of enzyme at low ionic strength and (B) at physiological ionic strength in the presence of 150 mM KCl were recorded at the indicated temperatures after a 15-min incubation period.

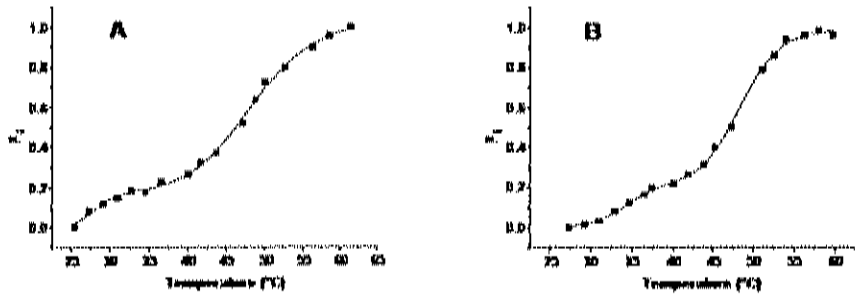

Figure 4. Temperature-induced unfolding transitions of porcine kidney BADH. (A) Enzyme at low ionic strength and (B) at high ionic strength in the presence of 150 mM KCl. Changes in tryptophan fluorescence emission at 434 nm were used to calculate the fraction of unfolded protein, as described in Methods.

KCl, no fluorescence due to NADH was observed in the whole temperature range studied (Fig. 4B), even when using an excitation wavelength of 340 nm (not shown). These findings indicate that the pure enzyme contained bound NADH, which dissociated by dialysis against the low ionic strength buffer devoid of K^+ ions but remained tightly bound in the presence of this cation even at temperatures above 60° C. In addition to its stabilizing effects, K^+ ions appears to play a role in the binding of the coenzyme to kidney BADH consistent with previously observed synergy between K^+ ions and oxidized coenzyme in preventing enzyme inactivation by dilution (Valenzuela-Soto et al., 2003). Porcine kidney BADH might bind a monovalent cation in the vicinity of the nucleotide binding site similarly to human liver ALDH2 (Perez-Miller et al., 2003). It remains to be determined if other monovalent cations, such as Na^+ which was the cation observed in the crystal structure of ALDH2, also increase the affinity of the enzyme for the coenzyme.

Table 2. Effects of K^+ and Mg^{2+} ions on apparent T_m of kidney BADH.

Condition		$appT_m^1$
Low ionic strength		
By activity[2]	41.3	± 0.2
By fluorescence[3]	1st transition	28.1 ± 0.6
	2nd transition	48.3 ± 0.4
150 mM KCl		
By activity[2]	44.3	± 0.3
By fluorescence[3]	1st transition	34.5 ± 0.7
	2nd transition	48.4 ± 0.2
50 mM MgCl		
By activity[2]	40.4	± 0.1

1. Apparent T_m values are the estimates from a fit of data in Fig. 2 or in Fig. 4 to a single or double sigmoidal equation, respectively, ± the SD of the estimates.
2. The thermal-induced transition was monitored by determining enzyme activities after 15 min of incubation at the temperatures indicated in Fig. 2 .
3. The thermal-induced transitions were monitored by recording intrinsic fluorescence spectra after 15 min of incubation at the temperatures indicated in Fig. 4.

The observed increases in quantum yield of NADH as the temperature increased (Fig. 4B) might be due to higher efficiency of energy transfer as a consequence of greater protein flexibility at high temperatures which permits tryptophan residue(s) to approach the bound NADH molecule, and/or to the presence of a great proportion of NADH molecules bound in an elongated fashion, which prevents quenching by contact of the adenine and the fluorescent nicotinamide ring (Lakowicz, 1999).

The energy transfer between protein tryptophan residues and protein bound NADH precluded the correct estimation of the spectral center of mass in the case of the BADH sample in the presence of 150 mM KCl. Therefore, we choose to use the fluorescence at 334 nm, a wavelength at which changes are well observed, to monitor the thermal-induced transition. However, it has to be kept in mind that the energy transfer phenomenon may produce important alterations in the fluorescence emission at this wavelength and therefore, the conclusions drawn from the fluorescence experiments reported here, particularly those of the enzyme in the presence of KCl, need future confirmation in studies in which an enzyme devoid of NADH is used.

The thermal-induced unfolding of BADH at both low and near physiological ionic strength in

the presence of K^+ ions clearly showed two transitions (Fig. 4), with apparent T_m values given in Table 2. The first transition of the enzyme at low ionic strength ended at 32.6° C and that of the enzyme at physiological ionic strength at 40.7° C. Also, the first transition midpoint, apparent T_m, was 6.5° C lower in the absence of K^+ than in its presence. In the second transition, we did not find significant differences between samples at low and high ionic strength in enzyme stability as assessed by apparent T_m values. The molecular events underlying the first thermal induced transition are not yet clear to us since the enzyme activity was conserved in the temperature range involved in this transition.

Under our experimental conditions, thermal denaturation of porcine kidney BADH was not reversible once the second transition is complete. Therefore, the unfolding curves and parameters presented in Figs. 2A and 4, and Table 2, respectively, represent non-equilibrium conditions, and we did not attempt to obtain thermodynamic parameters from these data.

In summary, in this work we have confirmed that monovalent cations are important for maintenance of the active conformation of porcine kidney BADH, and found that their stabilizing effects are important at moderate temperatures (below 40° C) but not at high temperatures. The effects of K^+, or of monovalent cations, are specific as these are not observed at a similar ionic strength given by a divalent cation such as Mg^{2+} ions. [illegible] increase the affinity of the enzyme for the coenzyme. Finding that K^+ ions [illegible] bound more tightly leads one to wonder if in vivo the enzyme is partially inhibited by being in the E-NADH complex. It is possible that [illegible] compared with that in the [illegible] cytosolic BADH from [illegible] does not occur.

Acknowledgments

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CAPÍTULO 2

Ionic Strength Dependency of Urea Effect on Renal Betaine Aldehyde Dehydrogenase Stability

Articulo de Investigación Original

Hector G. Ayala-Castro and Elisa M. Valenzuela-Soto

Ionic Strength Dependency of Urea Effect on Renal Betaine Aldehyde Dehydrogenase Stability

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Abstract

As part of the urinary concentrating mechanism, renal medulla cells are exposed to a hyperosmotic environment containing high concentrations of inorganic ions and urea. Medullary cells synthesize and accumulate organic osmolytes such as glycine betaine, glycerophosphorylcholine, sorbitol, and inositol to counteract these stressful conditions. Glycine betaine is synthesized from betaine aldehyde in a reaction catalyzed by betaine aldehyde dehydrogenase (BADH). Previous work with swine kidney BADH has shown that this enzyme requires physiological ionic strength to maintain its native tetrameric structure and, specifically, the ionic strength given by a monovalent cation for maximum thermostability. In this study, we examined the effect of urea at physiological and high ionic strength on renal betaine aldehyde dehydrogenase. Concentrations [illegible] to obtain physiological and high ionic strength; urea was tested in a 0 to 7 M range; the assays were carried out at 30 and 37°C. Physiological concentrations of urea (0.3–1 M) had no effect on BADH activity at 30 or 37°C under physiological ionic strength and pH 7.0. BADH activity is inhibited 50% at 3.6 M urea and 30°C. On the other hand, the enzyme incubated with urea 1 M was totally inactivated at 37°C, whereas tetramer it was partially inactivated at 30 °C. Inactivation was prevented by 0.15 or 0.3 M K^+ ions at two temperatures. Native-PAGE showed that higher ionic strength preserves the tetrameric conformation of BADH at 1 M urea at 30 or 37°C. At physiological temperature and pH, the BADH inactivation effect of urea is regulated by ionic strength.

Keywords: betaine aldehyde dehydrogenase; glycine betaine; ionic strength; kidney; renal osmoprotection; urea

Introduction

In response to exposure to a hyperosmotic environment, prokaryotic, plant, and animal cells accumulate organic osmolytes [illegible] conditions owing to the urine concentrating mechanism (Beck et al. 1998). The passive accumulation of urea in the medullary interstitial fluid increases the maximum urine concentrations which can occur in the kidney, therefore urea has a unique role in the urine concentrating mechanism (Levinsky and Berlinder 1959). During antidiuresis urea concentrations reach levels greater than 1000 mmol.l^{-1} (Yancey 1988).

It is well known that urea has an unfolding effect on proteins, and that it does so by both direct and indirect mechanisms. Direct urea interactions [illegible] of the protein, particularly [illegible] and facilitates the exposure of the hydrophobic core residues (Bennion and Daggett 2003).

Glycine betaine (GB) is one of the major non-perturbing osmolytes that accumulates in many cells exposed to hypertonicity (Yancey et al. 1982). Kidney medulla cells accumulate glycine betaine, glycerophosphorylcholine, sorbitol, and inositol (Burg 1995). GB is synthesized from betaine aldehyde oxidation in a reaction catalyzed by betaine aldehyde dehydrogenase (BADH EC 1.2.1.8.).

In renal cells GB is synthesized from choline (Moeckel and Lien 1997). Porcine kidney BADH

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has been purified (Guzman-Partida and Valenzuela-Soto 1998) and kinetically characterized (Figueroa-Soto and Valenzuela-Soto 2000). Its molecular and kinetic properties closely resemble those of other ALDH9s (Guzman-Partida and Valenzuela-Soto 1998; Chern and Pietruszko 1999; Figueroa-Soto and Valenzuela-Soto 2000; Vaz et al. 2000). The quaternary structure of renal BADH is a homotetramer with a molecular mass of 232 kDa (Valenzuela-Soto et al. 2003).

The enzyme requires ionic strength to maintain its tetrameric conformation; at low ionic strength the enzyme dissociates, forming dimers that are inactive and very stable (Valenzuela-Soto et al. 2003). Furthermore, renal BADH under low ionic strength is inactivated by temperature with an *app*Tm = 41.7°C while under physiological ionic strength given by 0.15 M KCl the enzyme shows an appTm = 44.3°C (Valenzuela-Soto et al. 2005). Divalent cations tested at the same ionic strength are unable to protect the enzyme activity or stability from thermal inactivation (Valenzuela-Soto et al. 2005).

Studies of urea's effect on the enzymes involved in osmolyte synthesis are scarce. It has been demonstrated that 1 M urea inhibits renal aldose reductase (Burg 1997). Here, we investigate the effect of urea on porcine kidney betaine aldehyde dehydrogenase [illegible]

[illegible]

[illegible] mM β-mercaptoethanol.

The effect of urea on enzyme activity and stability

The activity of BADH was assayed at 0, 1, 2, 3, 4, 5, 6 and 7 M urea, in the presence and absence of 0.15 M KCl. The stability assays were carried out by incubating the enzyme at 1 M urea for 120 min at 30 or 37 °C in the presence of 0, 0.15 or 0.30 M KCl. An aliquot was taken at different time intervals and activity assayed. Fluorescence spectra were recorded with a PTI-840 fluorescence spectrophotometer, using an excitation wavelength of 296 nm and emission wavelength of 300–450 nm. Fluorescence spectral centers of mass (intensity-weighted average emission wavelengths, λ_{av}) were calculated according to the following equation:

$$\lambda_{av} = \Sigma \lambda I(\lambda) / \Sigma I(\lambda)$$

where λ is the emission wavelength and $I(\lambda)$ represents the fluorescence intensity at wavelength λ.

Gel electrophoresis

Native-PAGE was carried out in a Phast system in 4-15% polyacrylamide gradient gel, following the manufacturer's instructions. The enzyme was incubated at 1 M Urea, or 1 M urea plus 0.15 M KCl or 1 M urea plus 0.3 M KCl at 30°C or 37°C for 120 min. Protein was identified by silver staining.

Kinetic analysis

Activity data were analyzed by nonlinear regression using the program Microcal Origin (Originlab Corporation).

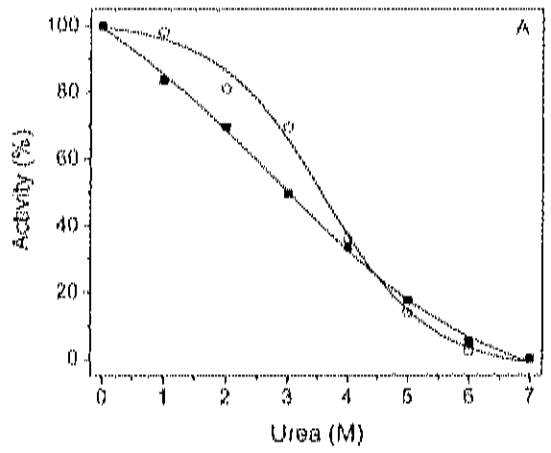
Results and Discussion

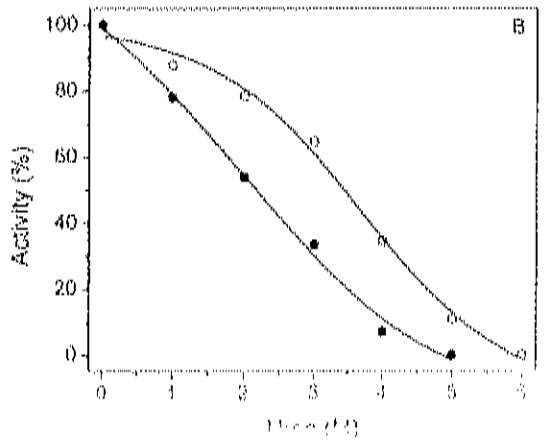
In 1 M urea, at 30°C and low ionic strength the enzyme maintains 98% of its activity, whereas at 37°C the activity decreased 10% (Fig. 1). Higher [illegible] inhibitory [illegible]

[illegible] in BADH activity at 3.7 M [illegible]

[illegible]

[illegible] enzyme. Intrinsic fluorescence emission studies were carried out on BADH to investigate whether urea produces changes in the protein tertiary structure. Urea-induced unfolding curves for 30 °C are shown in Fig. 2. The calculated denaturation midpoints (C_m) for BADH plus 1 M urea, BADH-urea plus 0.15 M KCl and 0.3 M KCl were 4.3 ± 0.2 M, 4.8 ± 0.4 M and 4.4 ± 0.2 M, respectively. This indicates that the decrease in enzyme activity was not caused by protein denaturation; urea inhibited enzyme activity at concentrations above 1.0 M. This effect is currently being studied.





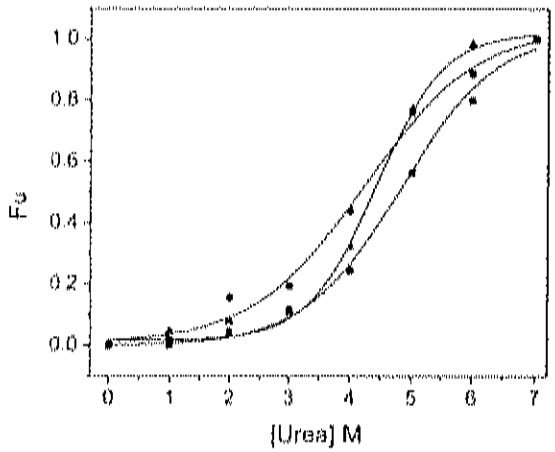


Figure 2. Urea-induced unfolding curve of renal BADH. Enzyme plus 1 M urea under low ionic strength (●) and physiological ionic strength equivalent to 0.15 M KCl (■) or plus 0.30 M KCl (▲).

3A). When incubated at 30°C, 90% of the enzyme was inactivated after 60 min (Fig. 3A). While under physiological and high ionic strength, the enzyme retained 49% and 55%, respectively, of its activity (Fig. 3A).

nal BADH is active without the osmolytes included in the activity assay. The results indicate that renal BADH is fully active and therefore glycine betaine is available to carry out osmoregulation and counteract the harmful effect of urea on other enzymes in the cells of the medulla.

The effect of physiological and ionic strength urea concentrations on BADH stability was analyzed. BADH was inactivated at 1 M urea at 30 °C and 37°C (Fig. 3). Enzyme inactivation was biphasic with a first-order kinetics and reached equilibrium after 120 min of incubation at 30°C (Fig.

with a previous work

process is time dependent. Urea had a strong effect on renal BADH at 37°C; however this effect was reduced by ionic strength. The kinetic parameters of inactivation are given in Table I. Enzyme inactivation could be the result of denaturation caused by urea and to explore this possibility, intrinsic fluorescence emission studies were done.

The intrinsic fluorescence emission analyses of BADH incubated in presence and absence of 1 M urea, or urea 1 M plus 0.15 M KCl or urea 1 M plus 0.30 M KCl, were carried out at different time intervals at 30°C and 37°C. Small changes

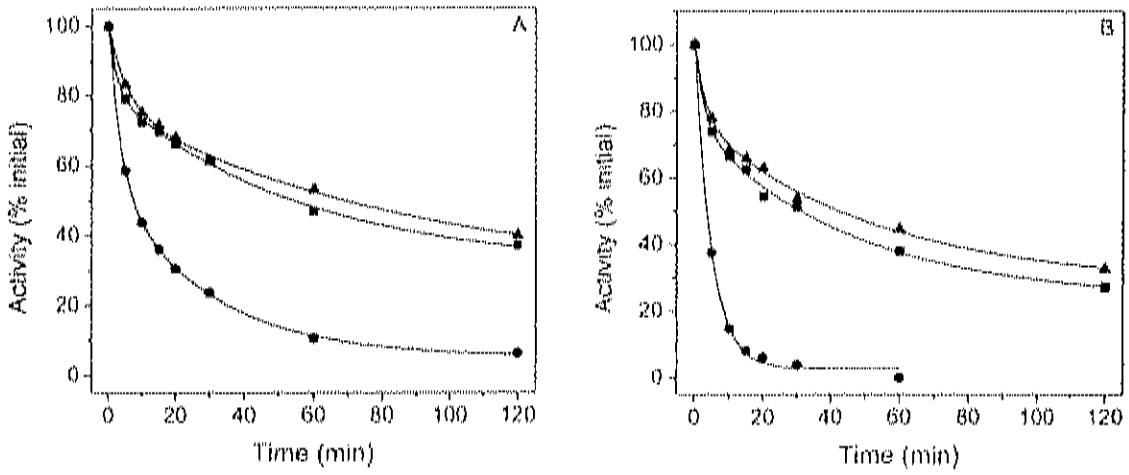


Figure 3. BADH inactivation kinetics at 30 °C (A) and 37 °C (B). Enzyme incubated at 1 M urea (●), 1 M urea plus KCl 0.15 M (■) and 1 M urea plus KCl 0.3 M (▲).

Table 1. Effect of ionic strength on the kinetics of inactivation at 1 M urea of renal BADH.

Condition	Amplitude 1 (% initial)	Amplitude 2 (% initial)	Kobs 1 (min^{-1})	Kobs 2 (min^{-1})
		30 °C		
Low ionic strength	43 ± 2.6[a]	52 ± 2.3[a]	0.30 ± 0.03[a]	0.036 ± 0.004[a]
150 mM KCl	19 ± 1.7[b]	51 ± 1.4[a]	0.15 ± 0.08[a]	0.018 ± 0.002[b]
300 mM KCl	23 ± 1.7[b]	23 ± 1.9[b]	0.2 ± 0.03[a]	0.02 ± 0.001[b]

[illegible]

[illegible] or high ionic strength had a Z_{av} of 251 and 349, respectively (Fig. 4B). We can conclude that no significant changes in the tertiary structure of the protein were provoked by 1 M urea at the temperatures tested in this study.

The decrease in enzyme activity could be a consequence of changes in its tetrameric conformation. To explore this possibility, a native-PAGE was carried out with the enzyme incubated for 60 min. For renal BADH, native-PAGE showed that 1 M urea induced changes in protein aggregation at 30°C (Fig. 5A) and at 37°C [illegible] tetramer molecular mass) (Fig. 5A lane 1), while there were several protein bands at 37 °C (Fig. 5B lane 1). Urea decreases tetramer concentration and increases trimer and dimmer formation at both temperatures (Fig. 5A lane 2 and 5B lane 2). In contrast, ionic strength allows the majority of the enzyme to stay in the tetramer conformation at 30 °C (Fig. 5A lane 3 and 4) however, 0.3 M KCl showed the best effect on BADH aggregation conformation (Fig 5A lane 4). The ionic strength effect was less dramatic at 37°C, but even so a strong protein band was observed for the enzyme

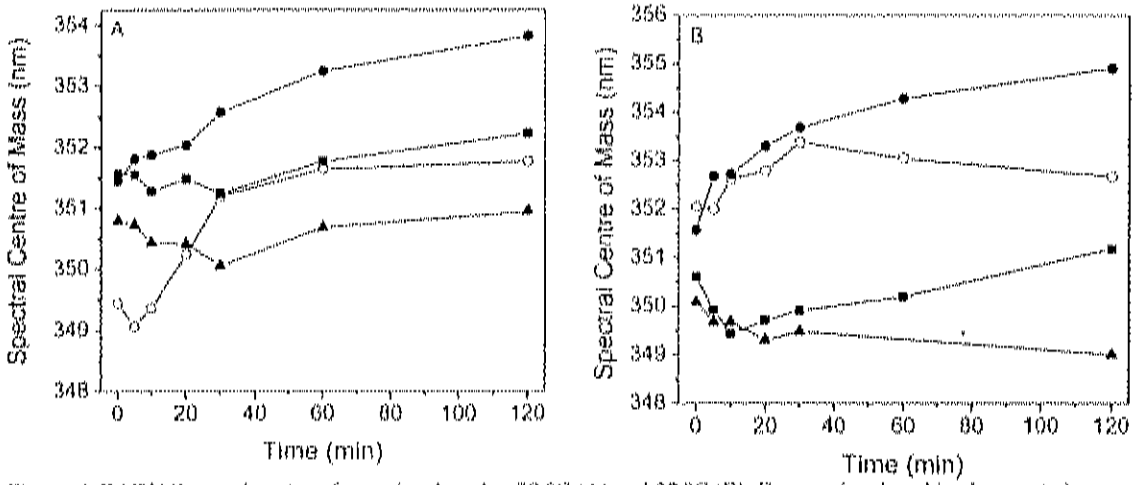
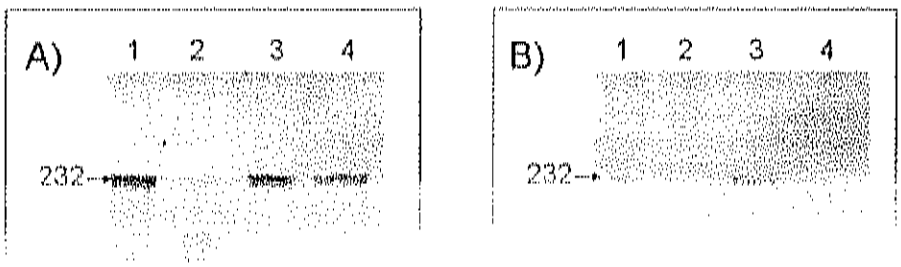


Figure 4. BADH Spectral center of mass incubated at 30 °C (A) and 37 °C (B). Enzyme incubated in absence (●) or presence (■) of 1 M urea, or 1 M urea plus KCl 0.15 M (▲) and 1 M urea plus KCl 0.3 M (▼).



at 232 kDa with 0.15 M and 0.3 M KCl (Fig. 5B

In this study we found that

of urea had an inhibitory effect on BADH activity. On the other hand, urea had an important effect on BADH aggregation at physiological concentration and temperature. However, the aggregation of BADH caused by urea was reverted or halted by ionic strength. In addition, we confirm that renal BADH conformation is stabilized by ionic strength. During antidiuresis urea and NaCl concentrations increase, thus osmolyte synthesis is necessary to achieve osmoregulation (Burg, 1995). Under renal physiological conditions 1 M urea would be ex-

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CAPÍTULO 3

Urea competitively inhibits porcine kidney betaine aldehyde dehydrogenase

Artículo de Investigación Original

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ABSTRACT

Urea concentrations are high in renal medullary cells. Betaine aldehyde dehydrogenase is located in cortex and medulla cells and is therefore exposed to high urea concentrations. Enzyme activity assays at 0-2 M concentrations of urea were carried out at pH 7.0, under saturating and subsaturating substrate concentrations. The enzyme, at both substrate concentrations, was inhibited by urea in a competitive manner with respect to NAD^+, and in a non-competitive way against betaine aldehyde. Ionic strength had no effect on the type of inhibition caused by urea. Under NAD^+ saturating and subsaturating concentrations, values of K_i were 1.9 ± 0.1 M and 1.6 ± 0.1 M. For BA saturating and subsaturating concentrations, values of K_i were 0.33 ± 0.05 M and 0.4 ± 0.06 M. Urea inhibits by preferential binding to the free enzyme, forming an E-urea complex. At high NAD^+ concentrations an E-urea-NAD^+ complex is formed which is able to bind BA. The resulting complex is inactive.

INTRODUCTION

In mammals, the most abundant protein catabolism waste product is nitrogen, which is excreted as urea in the urine. Urea concentration in the urine exceeds 1 M during antidiuresis [1]. Urea plays an important role during the urinary concentrating mechanism because the passive or active accumulation of urea in renal medullary interstitial fluid increases the maximum urine concentrations that can occur in the kidney [2-4]. However, urea can also have deleterious effects because it destabilizes many macromolecular structures [5,6]. In addition, urea inhibits important protein functions such as ligand binding [5].

There is ample literature describing the effect of urea on protein denaturation and structure at high concentrations, however there have been few kinetic analyses of the reversible inhibition of enzyme activity by urea [7-9]. Furthermore, there are few kinetic studies about the effect [illegible] [10-12].

As mentioned, renal medulla cells are exposed to [illegible] as well as levels of hypertonicity so extreme that they would be lethal in general circulation. These cells adapt to their environment through the controlled accumulation of compatible organic osmolytes [13]. Unlike [illegible] osmolytes to contend with the hyperosmotic environment [illegible] renal tubule cells protects against the osmotic force of urine as it becomes concentrated in the distal tubules and collecting ducts [13]. GB is synthesized from betaine aldehyde (BA) in a reaction catalyzed by betaine aldehyde dehydrogenase (BADH, EC 1.2.1.8 betaine aldehyde: NAD^+ oxidoreductase); which is located in the renal cortex and medulla [15].

Porcine kidney BADH belongs to the ALDH superfamily and is classified as class 9 [16]. This enzyme has been purified [17] and kinetically characterized [18]. BADH follows an iso bi bi ordered steady state mechanism: NAD^+ is the first substrate to bind the enzyme and NADH the last product to be released from it [18]. Active BADH is a tetramer with 54 kDa subunits [19].

In a previous study, it was demonstrated that porcine kidney BADH requires ionic strength to maintain its tetrameric conformation; at low ionic strength the enzyme dissociates, forming dimers which are inactive and very stable [19]. Additionally, the enzyme requires the physiological ionic strength provided by a monovalent cation for maximum thermostability [20]. With the aim of studying the possible the relationship between urea and BADH in kidney, earlier studies by our research team demonstrated that urea concentrations above 3 M had the effect of inactivating BADH at pH 7.0 and 20°C; with the [illegible] porcine kidney BADH [illegible]

Materials and metho[illegible]

Enzyme purification [illegible]

[illegible] phosphate buffer, pH 7.0, 1 mM EDTA, 10 mM β-mercaptoethanol, 0.2 M KCl and 10% glycerol. The enzyme (0.065 mg/mL) was dialyzed overnight at 4 °C against 10 mM HEPES-KOH buffer, pH 7.0, 1mM EDTA, 10 mM β-mercaptoethanol (buffer A, to obtain low ionic strength), or against buffer A plus 150 mM KCl (buffer B,

physiological ionic strength). BADH activity was measured spectrophotometrically as reported elsewhere [17].

Inhibition assays

The enzyme was assayed using 100 mM Hepes buffer, at pH 7.0 and 30 °C at low or physiological ionic strength using 10 mM Hepes or 0.15 M KCl respectively. To realize the inhibition patterns, the NAD^+ concentrations were 0.025, 0.05, 0.01, 0.25, and 0.5 mM, and the BA concentrations were 0.05, 0.1, 0.2, 0.4, 0.6 mM. The urea concentrations tested were 0, 0.125, 0.25, 0.375, 0.5, 1.0, 1.5, 2.0 M. All assays were done at least twice and on two different days, with duplicate measurements each time, using an Ultrospec 4000 spectrophotometer.

Data analysis

Kinetic data were analyzed using the non-linear regression program Grafit [illegible] fitting [illegible] following equations [illegible]

Competitive inhibition (Eq. 1)

$$v = \frac{V_{max} * [NAD^+]}{[illegible]}$$

Non-competitive inhibition (Eq. 2)

$$v = \frac{V_{max} * [BA] * \frac{1}{1 + [Urea] / K_{I(Urea)}}}{K_m + [BA]}$$

Dixon competitive inhibition (Eq. 3)

$$v = \frac{K_m + [NAD^+]}{V_{max} * [NAD^+]} + \frac{[Urea] * \left(\frac{K_{m(NAD^+)}}{K_{i(Urea)}}\right)}{V_{max} * [NAD^+]}$$

Dixon non-competitive inhibition (Eq. 4)

$$v = \frac{1}{V_{max}} * \left(1 + \frac{K_{m(BA)}}{[BA]}\right) * \left(1 + \frac{[Urea]}{K_i}\right)$$

Results

Since BADH inactivation by urea was dependent on ionic strength [21], the reversible enzyme inhibition kinetic was analyzed under two ionic strength conditions: low (0.01) and physiological (0.15).

[illegible] velocity *versus* urea concentration are shown in *inset* Fig. 1A.

When the enzyme was assayed under physiological ionic strength the Lineweaver-Burk plots showed that the inhibition type was again competitive (Fig. 1B), with an apparent V_{max} of 0.91 ± 0.04 μmol/min/mg and a K_{mNAD} of 70 ± 9.6 μM. Changes in the initial velocity *versus* urea concentration showed competitive

inhibition (*inset* Fig. 1B). The K_i calculated with the competitive Dixon equation (Eq. 3) did not change under conditions of low ionic strength (Table 1).

Under NAD^+ saturating concentration (0.5 mM) the Lineweaver-Burk plot showed a pattern of intersecting lines indicating non-competitive inhibition with respect to BA (Fig. 2A). The data points fit the non-competitive equation best (Eq. 2). Apparent V_{max} = 1.54 ± 0.07 μmol/min/mg and K_{mBA} = 403 ± 30 μM (Table 1). With the Dixon equation (Eq. 4) a K_i of 1.91 ± 0.11 M was calculated (Table 1). The Dixon plot of the initial velocity *versus* urea concentration data is given in *inset* Fig. 2A.

The presence of ionic strength provided by KCl (0.15 M) maintained non-competitive inhibition (Fig. 2B). Ionic strength provoked an increase in the K_{mBA} (482 ± 71 μM), while the apparent V_{max} value did not change (Table 1). With the Dixon non-competitive equation (Eq. 4) a decrease in the K_i from 1.91 ± 0.11 M to 1.20 ± 0.08 M was revealed.

Lineweaver-Burk plots showed competitive inhibition (Fig. 3A). The data analysis (Eq. 1) showed an apparent V_{max} = 0.33 ± 0.02 μmol/min/mg and a K_{mNAD} = 92 ± 14 μM (Table 1). Data were analyzed using the competitive Dixon equation (Eq. 3) and a K_i of 397 ± 59 mM was determined (Table 1). At physiological ionic strength and fixed BA concentration, the Lineweaver-Burk plot again showed competitive

inhibition with respect to NAD^+ (Fig. 3B). The data points fit the competitive inhibition equation best (Eq. 1), with an apparent V_{max} of 0.37 ± 0.01 μmol/min/mg and a K_{mNAD} of 86 ± 10 μM. The Dixon competitive inhibition equation (Eq. 3) returned a K_i value of 131 ± 16 mM (Table 1). Dixon plots of the initial velocity *versus* urea concentration showed a competitive inhibition pattern (*inset* Fig. 3A and 3B).

At the fixed NAD^+ (0.025 mM) subsaturating concentration, the Lineweaver-Burk plot showed non-competitive inhibition with respect to BA (Fig. 4A). The data points fit the non-competitive equation best (Eq. 2). Urea decreases the V_{max} value to 0.35 ± 0.01 μmol/min/mg and K_{mBA} = 234 ± 19 μM. In addition, a K_i of 1.57 ± 0.09 M was determined by the Dixon non-competitive equation (Eq. 4) (Table 2). *Inset* Fig. 4A shows the initial velocity *versus* urea concentration Dixon plot.

[illegible]

[illegible]

Discussion

Urea plays a key role in kidney function. It may reach concentrations of 500 mM in the human kidney during antidiuresis, and can exceed 2 M in desert rodents [1,22,23]. For rabbit renal medullary cells in tissue culture, GB content decreased in the presence of urea [22-24]. Our results provide a the first explanation of how urea affects GB synthesis.

The competitive inhibition pattern shows that urea is binding to free enzyme (EUrea) and competing with the coenzyme for the active site. Betaine aldehyde binds to the enzyme until the complex BADH-NAD^+ is formed, thus the non-competitive inhibition detected for BA demonstrates that urea preferentially binds to the free enzyme. However, the non-competitive inhibition found in the presence of BA means that urea can also bind to the $ENAD^+BA$ complex. The urea inhibition mechanism is shown in scheme 1, where urea binds to free BADH, but under NAD^+ saturating concentrations urea can also bind to the $ENAD^+$ complex, producing $EUreaNAD^+$ and this resulting complex can still bind BA, forming an inactive complex, $ENAD^+BAUrea$.

Urea decreased the K_{mNAD} 30%, a result similar to that found for aldose reductase [11,12] which was explained as the consequence of conformational changes in the ENADPH complex [illegible]) involved in [illegible] [illegible] [illegible]

[illegible] K_m [illegible] substrate [illegible] formation.

Unlike the previous findings on BADH stability and the protection of enzyme stability against high urea concentrations [21], in this study the data show that ionic strength was not playing a key role in the inhibition of BADH by urea. When all the data are considered, an important question that arises regarding how BADH can

function adequately under the high urea conditions present in the renal inner medulla cells. One explanation could be the saturating NAD^+ concentrations that can be found in the medulla cells since the concentration of NAD^+ in rat renal cells has been estimated to be 1 mM [28]. At that coenzyme concentration, the enzyme will be saturated with NAD^+ and the impact of urea inhibition will be reduced.

Acknowledgments

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Tables

Table 1. Apparent kinetic constants for porcine kidney BADH at saturating substrate concentrations

Fixed saturating substrate	Variable substrate	V_{max} (μmol/min/mg)	K_m (μM)	K_i (M)	Kinetic pattern
BA	NAD^+	0.95 ± 0.02	22 ± 2.7	0.33 ± 0.04	Competitive
BA	$NAD^+ + K^+$	0.91 ± 0.03	70 ± 9.6	0.33 ± 0.04	Competitive
NAD^+	BA	1.5 ± 0.07	403 ± 32	1.91 ± 0.10	Non-competitive
NAD^+	$BA + K^+$	1.5 ± 0.12	482 ± 71	1.12 ± 0.08	Non-competitive

substrate concentration:

fixed	Variable	V_{max}	K_m	K_i	kinetic
BA	$NAD^+ + K^+$	0.364 ± 0.01	1[illegible] ± 16	0.1[illegible] ± 0.03	[illegible]
NAD^+	BA	0.350 ± 0.01	223 ± 19	1.57 ± 0.09	Non-competitive
NAD^+	$BA + K^+$	0.346 ± 0.02	515 ± 54	0.56 ± 0.04	Non-competitive

Figure Legends

Figure 1. Competitive inhibition of BADH by urea at fixed saturating BA concentrations. BA concentration was 0.6 mM. Enzyme activity was assayed at low (A) and physiological (B) ionic strength. Lineweaver-Burk plots of initial velocity *versus* variable NAD^+ concentration (0.5, 0.25, 0.1, 0.05, 0.025 mM) and fixed urea concentrations of 0 (○), 0.5 (●), 1.0 (□), 1.5 (■) and 2.0 (△) M. *Inset*: Dixon plots, $1/v$ against [urea], at NAD^+ 0.025 (7), 0.05(◇), 0.1 (◆), 0.25 (⌖), 0.5 (⌖) mM.

Figure 2. Non-competitive inhibition of BADH by urea at saturating NAD^+ concentrations. NAD^+ concentration was 0.5 mM. Enzyme activity was assayed at low ionic strength (A) and physiological (B) ionic strength. Lineweaver-Burk plots of initial velocity *versus* variable BA concentration (0.6, 0.4, 0.2, 0.1 0.05 mM) and fixed urea concentrations of 0 (○), 0.5 (●), 1.0 (□), 1.5 (■) and 2.0 (△) M. *Inset*: Dixon plots, $1/v$ against [urea], at NAD^+ 0.025 (▲), 0.05(◇), 0.1 (◆), 0.25 (⌖), 0.5 (⌖) mM.

Figure 3. Competitive inhibition of BADH by urea at subsaturating NAD^+ concentrations. NAD^+ concentration was 0.025 mM. Enzyme activity was assayed at low ionic strength (A) and physiological (B) ionic strength. Lineweaver-Burk plots of initial velocity *versus* variable NAD^+ concentration (0.5, 0.25, 0.1, 0.05, 0.025 mM), and at fixed urea concentrations of 0 (○), 0.5 (●), 1.0 (□), 1.5 (■) and 2.0 (△) M. *Inset*: Dixon plots, at NAD^+ 0.025 (▲), 0.05(◇), 0.1 (◆), 0.25 (⌖), 0.5 (⌖) mM.

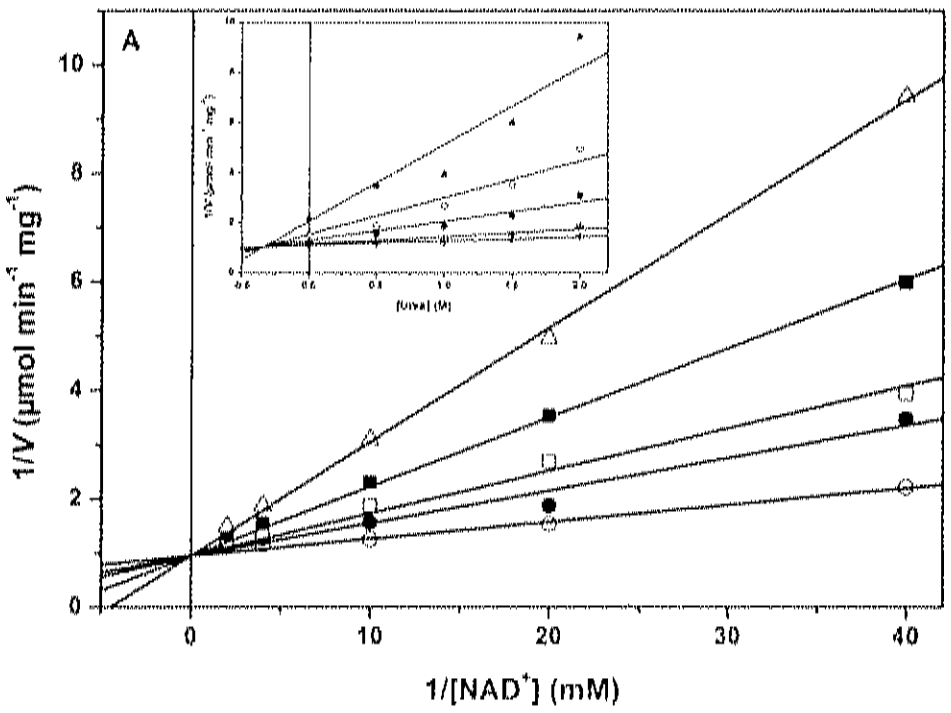
Figure 4. Non-competitive inhibition of BADH by urea at subsaturating NAD^+ concentrations. NAD^+ concentration was 0.025 mM. Enzyme activity was assayed at

low ionic strength (A) and physiological (B) ionic strength. Lineweaver-Burk plot of initial velocity *versus* BA (0.6, 0.4, 0.2, 0.1 and 0.05 mM) and at fixed urea concentrations of 0 (○), 0.5 (●), 1.0 (□), 1.5 (■) and 2.0 (△) M. *Inset*: Dixon plot, $1/v$ against [urea], at BA concentrations of 0.05 (⊠), 0.1 (⊠), 0.2 (⊠), 0.4 (⊠) and 0.6 (⊠) mM.

Scheme 1. Proposed mode of urea binding to betaine aldehyde dehydrogenase

E is the free enzyme, K_{ic} is the [illegible] competitive inhibition constant, K_{mc} is the [illegible] competitive inhibition constant, and K_{cat} is the catalytic constant.

Figure 1.


A
1/V (µmol min-1 mg-1)
1/[NAD+] (mM)
0
2
4
6
8
10
0
10
20
30
40

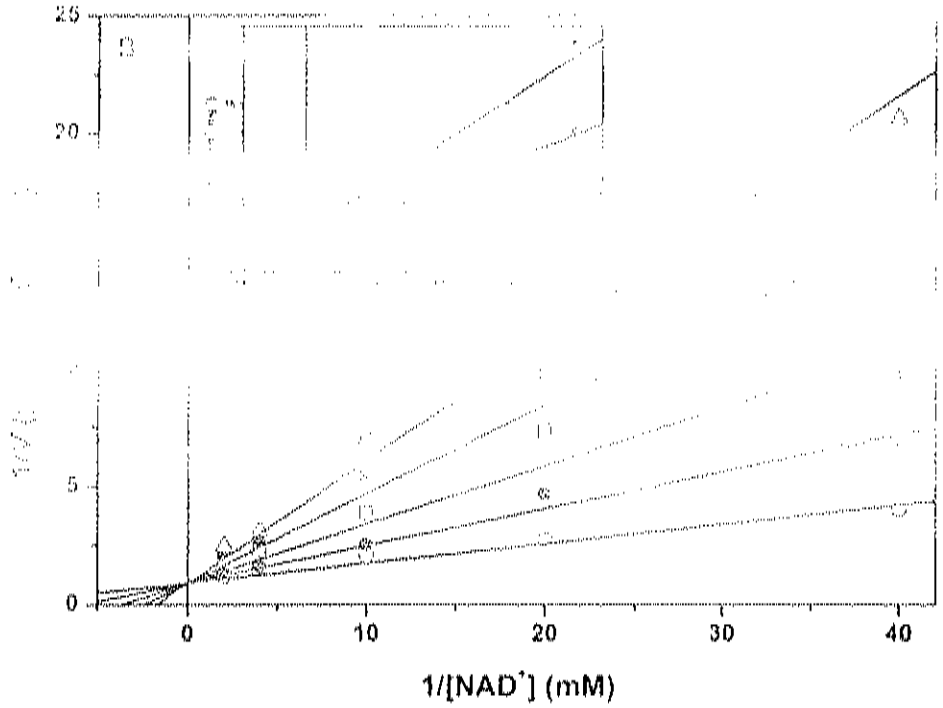

1/[NAD+] (mM)
0
5
20
25
0
10
20
30
40

Figure 2.

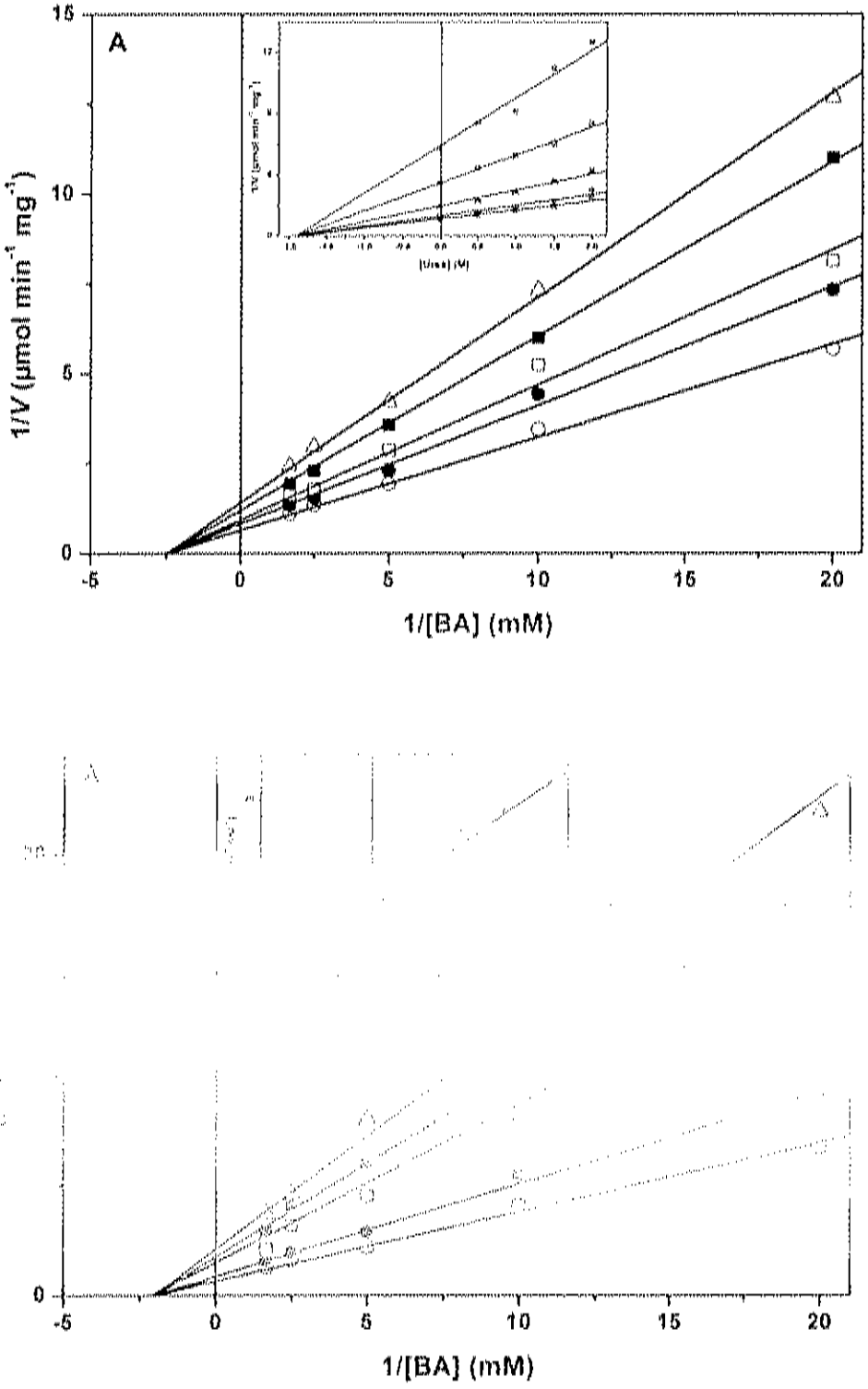


Figure 3.

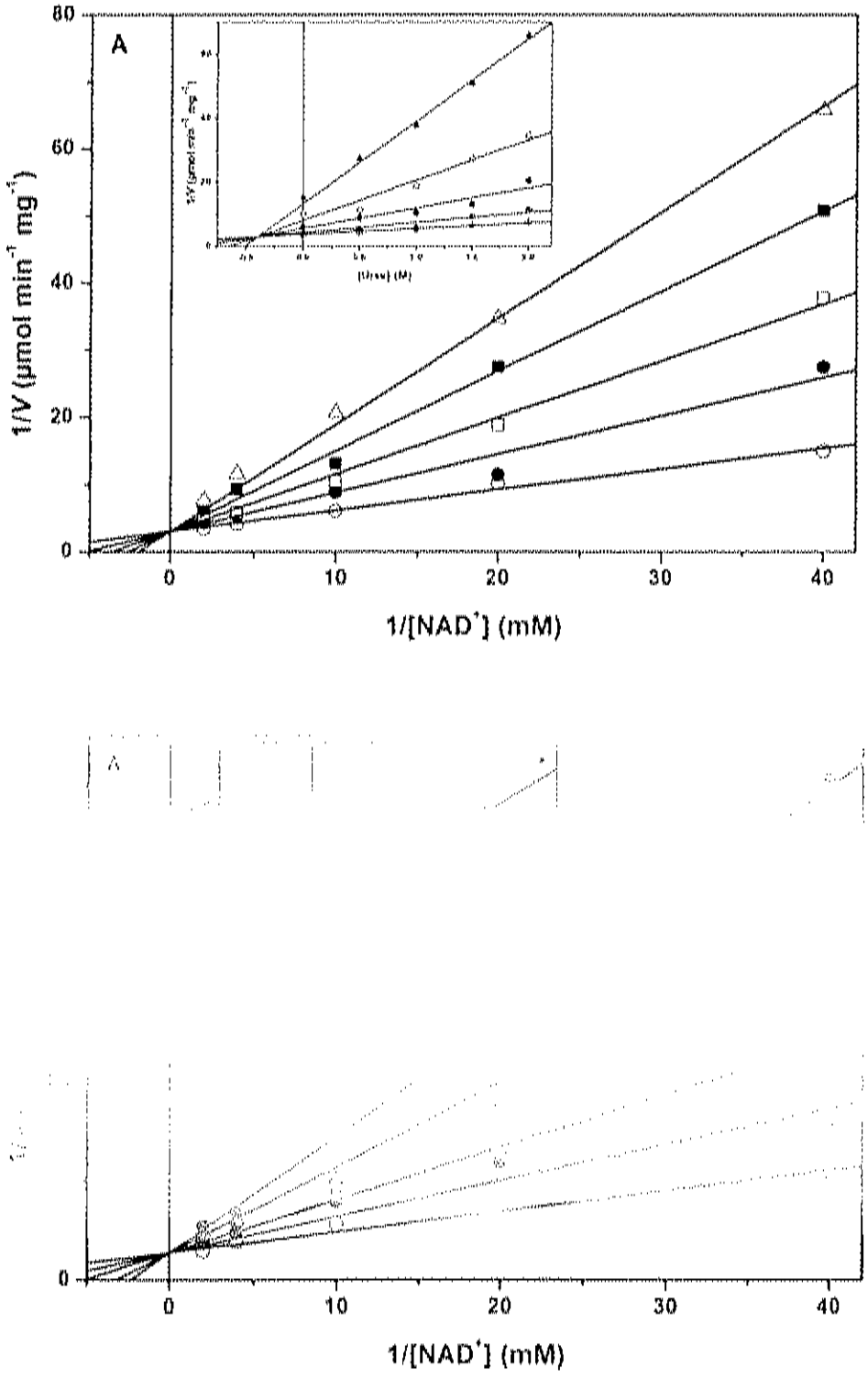
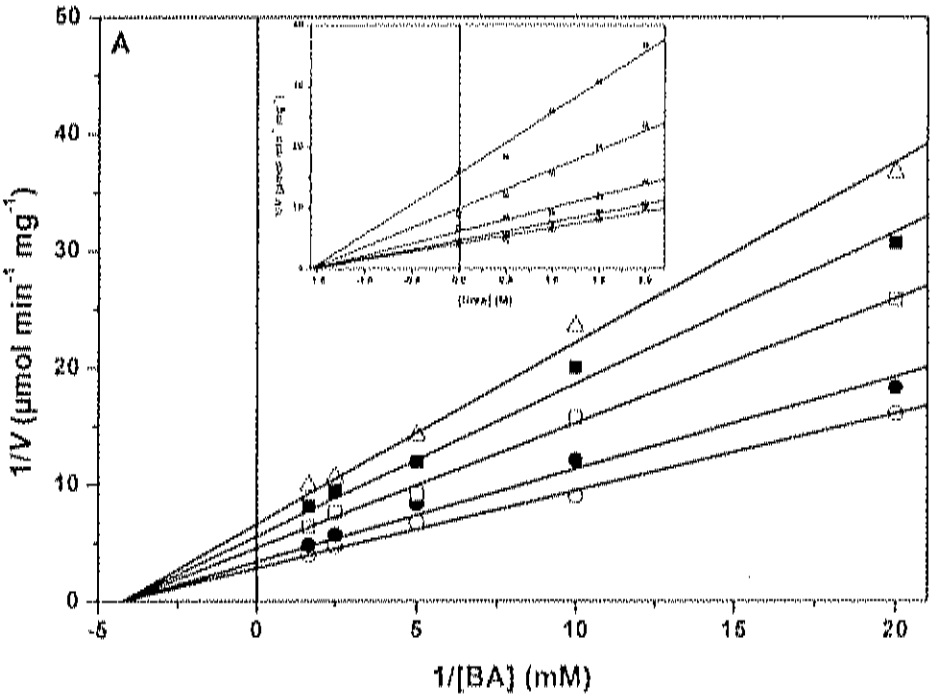
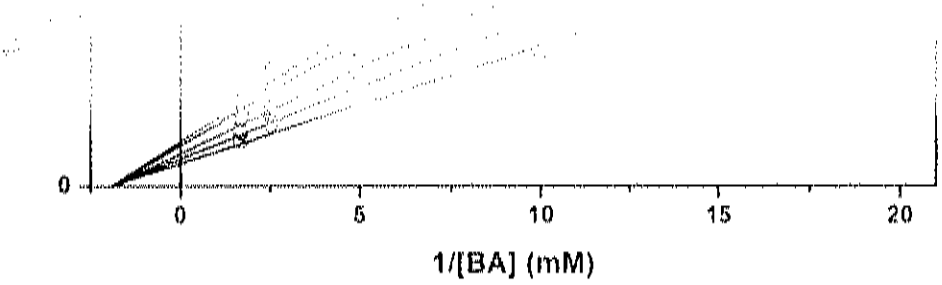


Figure 4.


A
1/V (μmol min-1 mg-1)
1/[BA] (mM)
50
40
30
20
10
0
-5
0
5
10
15
20


0
0
5
10
15
20
1/[BA] (mM)

CAPÍTULO 4

Complex, unusual conformational changes in kidney betaine aldehyde dehydrogenase revealed by chemical modification with disulfiram

Articulo de Investigación Original

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Complex, unusual conformational changes in kidney betaine aldehyde dehydrogenase revealed by chemical modification with disulfiram

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*Corresponding author. [illegible]

[illegible]

Abbreviations used: DSF, [illegible]

HEPES, (N-(2-hydroxyethyl)piperazine-[illegible]-ethanesulfonic acid), pBADH, betaine aldehyde dehydrogenase from porcine kidney.

Abstract

The NAD^+-dependent animal betaine aldehyde dehydrogenases participate in the biosynthesis of glycine betaine and carnitine, as well as in polyamines catabolism. We studied the kinetics of inactivation of the porcine kidney enzyme (pkBADH) by the drug disulfiram, a thiol-reagent, with the double aim of exploring the enzyme dynamics and investigating whether it could be an *in vivo* target of disulfiram. Both inactivation by disulfiram and reactivation by reductants were biphasic processes with equal limiting amplitudes. Under certain conditions half of the enzyme activity became resistant to disulfiram inactivation. NAD^+ protected almost 100% at 10 μM but only 50% at 5 mM, and vice versa if the enzyme was pre-incubated with NAD^+ before the chemical modification. NADH, betaine aldehyde, and glycine betaine also afforded greater protection after pre-incubation with the enzyme than without pre-incubation. Together, these findings suggest two kinds of active sites in the seemingly homotetrameric [illegible]

Introduction

Betaine aldehyde dehydrogenase (BADH, betaine aldehyde: NAD^+ oxidoreductase, EC 1.2.1.8) catalyzes the last, irreversible step in the synthesis of the osmoprotector glycine betaine: the $NAD(P)^+$-dependent oxidation of betaine aldehyde. Glycine betaine plays an important role in keeping the osmotic balance in renal cells [1,2] and is also a methyl donor for methionine synthesis [3]. Animal BADHs—which are part of the ALDH9 family of the aldehyde dehydrogenase (ALDH) superfamily [4,5]—are also involved in polyamines catabolism and synthesis of γ-aminobutyric acid and carnitine [4,6]. *In vivo* inhibition of animal BADHs might thus have severe adverse effects because of the importance of the metabolic processes in which these enzyme are involved.

One of the best-known ALDH inhibitors is the drug disulfiram (DSF), long and widely used in the aversion treatment of alcoholism. DSF irreversibly inhibits the [illegible] [13] (Scheme 1). Several undesirable side effects of the DSF treatment have been [illegible] even though thiols are the most reactive of the protein functional groups, and cysteine residues are present in most proteins. In spite of its relevance, given the present and

possibly future use of DSF as a drug, an extensive study of the DSF-inhibition of ALDH9s—which have a reactive catalytic cysteine residue—is still lacking. We choose the porcine kidney BADH (pkBADH) to investigate the possibility of ALDH9s being targets of this drug, as well as to explore factors that could affect this inhibition.

PkBADH is localized both in cortex and medulla cells [17]. This enzyme is a homotetramer [18] with a marked preference for NAD^+ over $NADP^+$ [19]. Its molecular and kinetic properties [20] closely resemble those of other animal BADHs [6,21,22], consistent with the finding that animal genomes have just one gene for this enzyme [4,5]. Its kinetic mechanism is steady state, bi bi ordered, where NAD^+ binds to the enzyme prior to the aldehyde [20]. The observed mixed inhibition of NADH against NAD^+ was taken as an indication of the existence of a rate-limiting isomerization step of the free enzyme. On the basis of this kinetic mechanism and previously

Betaine aldehyde, DTT, EDTA, glycine betaine, GSH, HEPES, 2-mercaptoethanol, NAD$^+$ (sodium salt), NADH, and DSF were obtained from Sigma-Aldrich SA de CV, México. All other chemicals and solvents used in this study were of analytical grade.

Purification and activity assay of pkBADH

BADH was purified from porcine kidneys and its activity assayed at 30 °C following earlier reported procedures [19]. The molar concentration of pkBADH was estimated by determining the protein concentration according to Bradford [24], and assuming a molecular mass of 230 kDa for the tetramer. Although the protein had undergone several chromatographic and dialysis steps during its purification, a $[redacted]$ ratio of [redacted]

with nitrogen just before use to prevent air oxidation of the enzyme cysteines. DSF [redacted] dissolved in nitrogen-saturated water. Samples of [redacted] were incubated with DSF at indicated concentrations, and aliquots removed after

several incubation periods to determine the residual enzyme activity. Activity data were analyzed by nonlinear regression fitting to single or double exponential decay equations (eqs. 1 and 2, respectively), using the Origin software (OriginLab, Northampton, MA, USA).

$$A_t = A_\infty + A_0 e^{-k_{obs}\cdot t} \quad (1),$$

$$A_t = A_\infty + A_1 e^{-k_{obs1}\cdot t} + A_2 e^{-k_{obs2}\cdot t} \quad (2),$$

where A_t, A_0 and A_∞ are the enzyme activities at time t, zero, and infinite, respectively, expressed as percentage of the initial activity; k_{obs} is the observed pseudo-first order rate constant in monophasic inactivation; k_{obs1} and k_{obs2}, and A_1 and A_2 are the observed pseudo-first order rate constants and the amplitudes of each of the two phases, respectively, in biphasic inactivation.

Reactivation of the DSF-inactivated enzyme was attempted with 5 mM DTT,

[illegible]

') + A·(1 [illegible] (3)

[illegible]

Inactivation of pkBADH by DSF under pseudo-first order conditions occurred in a time- and dose-dependent manner (Fig. [illegible]). The inactivation occurred in the presence of 2% (v/v) methanol in the DSF solution, as no significant differences were observed (data not shown). Using a DSF concentration range 10 to 30 μM,

inactivation kinetics were biphasic with rate constants differing in one order of magnitude, and inactivation partial (Table 1). The residual activity at infinite time, A_∞, decreased as the DSF concentrations increased—reaching a value near zero at 30 μM DSF—whereas the amplitude of the two inactivation phases, A_1 and A_2, increased, each one reaching about 50% of initial activity at 30 μM DSF (Table 1). The two pseudo-first order rate constants of inactivation, k_{obs1} and k_{obs2}, increased with the inhibitor concentration (Table 1).

Two inactivation phases in an oligomeric enzyme, as pkBADH, could result from pre-existing active sites heterogeneity in regard to the reactivity of the catalytic thiol, or from communication between active sites so the modification of the thiol of one site decreases the reactivity of the thiol in another site. In both cases the amplitude of the two phases should be equal and 50% of the initial enzyme activity [illegible]

can be [illegible]

[illegible]

this enzyme is a dimer of [illegible]

there can be differences [illegible]

or between the active sites of the two dimers. It is not yet possible for us to explain this likely heterogeneity in structural terms, as the only three-dimensional structure

known of an ALDH9, that of the cod liver enzyme [26], does not show any differences between actives sites. It may be that the dynamics of the active site lead to subtle rearrangements in the environment of the catalytic cysteine that do not show up in the crystal structure but have functional consequences.

One interesting feature of the inactivation of pkBADH by DSF is the partial inactivation, which arises from part of the sites becoming resistant to inactivation during the course of the incubation of the enzyme with DSF. This was proven by adding fresh DSF—at 15 μM, the same concentration initially added—after 60 min of incubation, when inactivation had apparently stopped. No further inactivation was observed even after an additional 60-min incubation period (data not shown). This result rules out the possibilities of either DSF instability or protection afforded by the tightly bound nucleotide as possible causes of the observed partial inactivation. It

thiol groups by DSF. This group could be that of the

slower than the rate of

slow inactivation phase

concentration tested, since the amplitude of the phases would depend on how fast is

rate of the appearance of active sites resistant to DSF modification compared to the rate of modification of the essential cysteine, and both rates may differ from one dimer to the other or from one subunit to the other.

DSF inactivation did not produce any changes in the enzyme tetrameric structure at room temperature, nor at 37 °C, as assessed by size-exclusion chromatography (data not shown). This result differs from that obtained for the *P. aeruginosa* BADH, which partially dissociates into monomers after being modified by DSF at room temperature, and forms aggregates of very high molecular weight at 37 °C [11].

Enzyme reactivation by mono- and di-thiols

[illegible] or 5 mM DTT resulted in [illegible] mM GSH could not, however, [illegible]

[illegible] of reactivation by both DTT and [illegible] were [illegible]

[illegible]

again indicate a very different environment, and therefore reactivity, of the disulfide in the two kinds of active sites.

Effects of ligands on BADH inactivation by DSF

The inability of GSH to reverse the DSF-mediated inactivation of pkBADH was an important finding pointing out the potential danger to renal cells—or to other cells in which BADH plays important physiological roles—of *in vivo* modification of this enzyme by DSF. We therefore explored possible mechanisms of protection against this inactivation. Protection by ligands would seem to be the most plausible.

In these studies we used 30 μM DSF, a concentration causing almost total inactivation of the enzyme (Fig. 1A). The level of protection afforded by NAD^+, from [illegible]

[illegible]

[illegible]

structural evidence in any ALDH—the latter results indicate that the enzyme was indeed not saturated by 10 μM NAD^+, for otherwise no change would have been

observed on increasing NAD^+ concentration. Again, the most probable reason behind this finding is the existence of two kinds of active sites, differing in both their essential thiol reactivity and in their affinity for the nucleotide. It would appear that binding of NAD^+ to one of them—that with the higher affinity for the nucleotide—protects the catalytic cysteine against inactivation, whereas binding to the other—that with the lowest affinity—has the opposite effect, i.e. favors the modification of this cysteine.

As previously found in the inactivation experiments carried out in the absence of ligands, the limiting residual activity reached at about 5 mM NAD^+ (inset Fig. 2) did not result from protection by the nucleotide but rather from half of the total sites becoming resistant to DSF inactivation, as proved by adding fresh DSF after the activity plateau was reached (data not shown). This result suggests half of the sites

the enzyme was pre-incubated with the nucleotide for 4 h at pH 9.0 to DSF addition.

and glycine betaine (Fig. 4). No change in reactivity towards DSF was observed in the

enzyme incubated for up to 4 h in the modification buffer in the absence of ligands (data not shown). The changes in reactivity triggered by the binding of ligands are a further indication of slow conformational rearrangements in the active site, significantly decreasing the reactivity of the essential thiol of this or of other active sites.

Decreased reactivity of the catalytic thiol upon incubation with saturating concentration of ligands and biphasic kinetics of inactivation were previously observed in studies of other BADHs [27,30,31], and several other wild-type and mutated ALDHs exhibit inter-subunit communication—showed either as half of the sites reactivity [32-35] or as positive cooperativity in nucleotide binding [36-38]. But to the best of our knowledge, the appearance of catalytic thiols resistant to chemical modification, as well as the contrasting behavior of low and high concentrations of NAD^+ in affording protection against chemical modification, as well as the contrasting behavior of low and high concentrations of

be rendered specific by the ability of proteins to modulate the reactivity of their important residues through changes in their environment.

Acknowledgements

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Figure Legends

Scheme 1. Reaction of DSF with protein thiol groups. A) Formation of a mixed disulfide. B) Formation of an intra-molecular disulfide.

Fig. 1. (A) Kinetics of inactivation of pkBADH by DSF. The enzyme (0.65 μM as tetramers) was incubated in buffer A at 30°C in the presence of 10 (circles), 15 (squares), 20 (up triangles), 25 (down triangles), or 30 μM DSF (rhombs). At the indicated times, aliquots were withdrawn and assayed for remaining activity. (B) Kinetics of reactivation of the DSF-inactivated pkBADH. Inactivation (closed circles) was carried out by incubation with 30 μM DSF under the same conditions than in panel A. After 30 min of reaction, 5 mM DTT (closed squares), 10 mM 2-mercaptoethanol (open squares), or 10 mM GSH (down triangles) were added to the inactivated enzyme, and recovery of activity was

double exponential decay

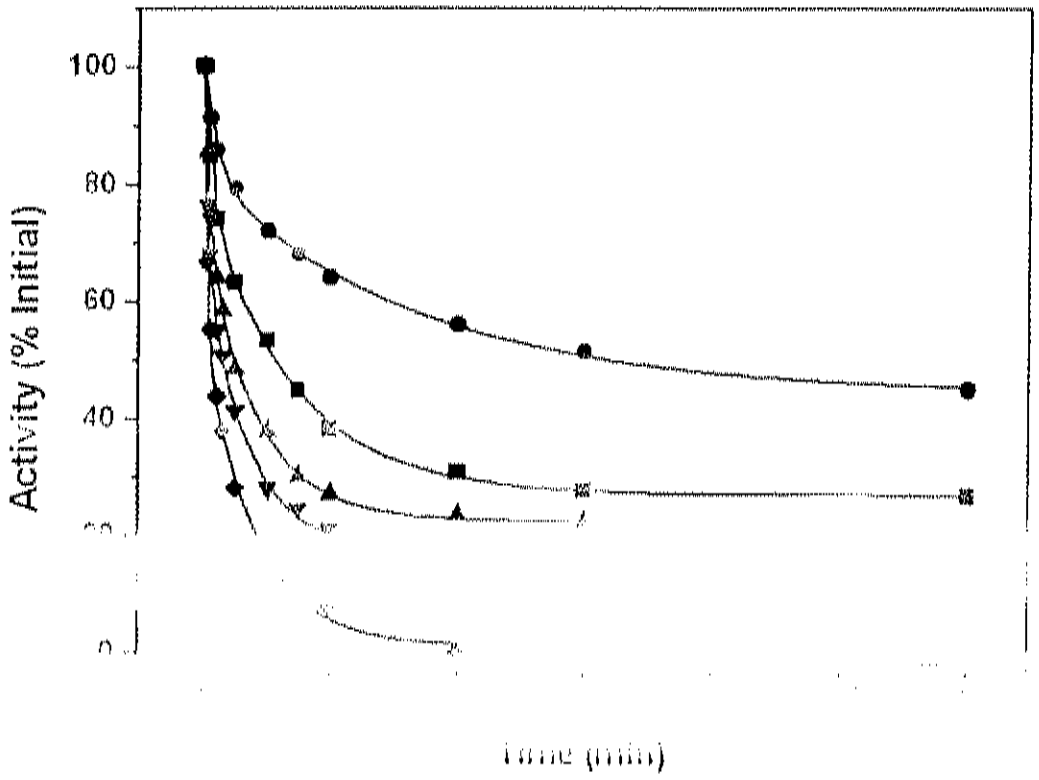
the result of the fit of the data to a single exponential decay equation (eqn. 1).

Inset: Dependence of the residual activity at infinite time, A_∞, on NAD$^+$ concentration.

Fig. 3. Effect of time of pre-incubation with 10 μM NAD$^+$ on the kinetics of inactivation of pkBADH by DSF. The nucleotide was added to the enzyme at 0 (open circles), 1 (closed circles), 4 (open squares) or 24 h (closed squares) prior to incubation with 30 μM DSF. Other conditions were as in Fig. 2. The points are the experimental data; the lines are the result of the fit of the data to a single exponential decay equation (eq. 1).

Fig. 4. Effect of ligands on the kinetics of inactivation of pkBADH by DSF. The enzyme was incubated with 20 μM DSF in the presence of 0.2 mM NAD$^+$ (A), 0.5 mM NADH (B), 0.5 mM betaine aldehyde (C) or 100 mM glycine betaine

Figure 1.



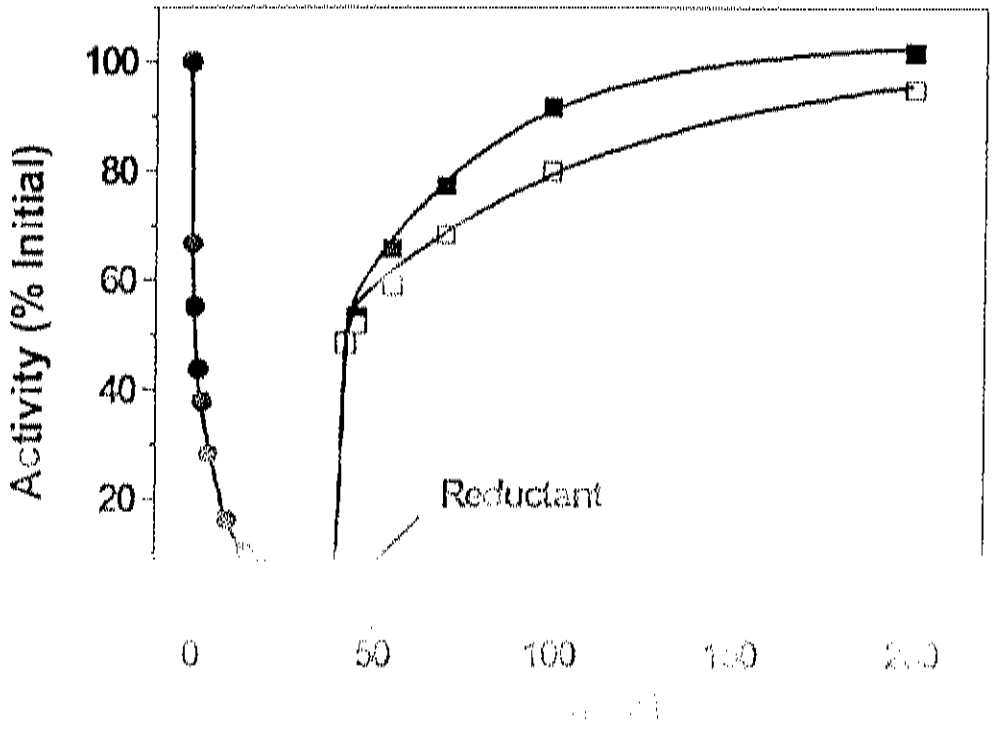

Activity (% Initial)
100
80
60
40
20
Reductant
0
50
100

Figure 3.

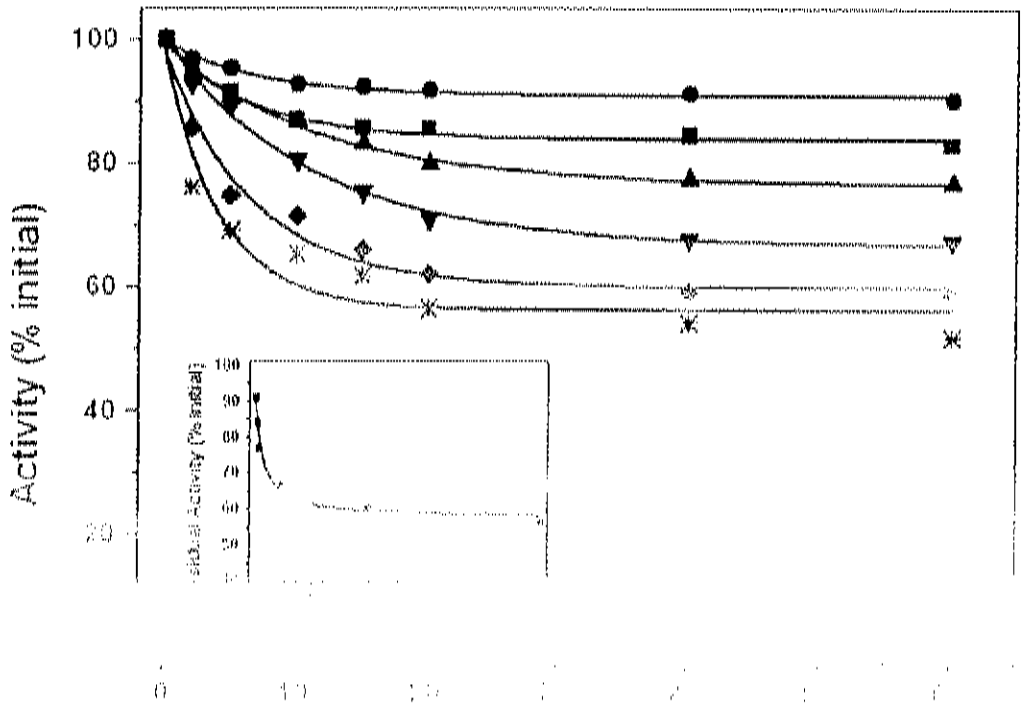


Figure 4.

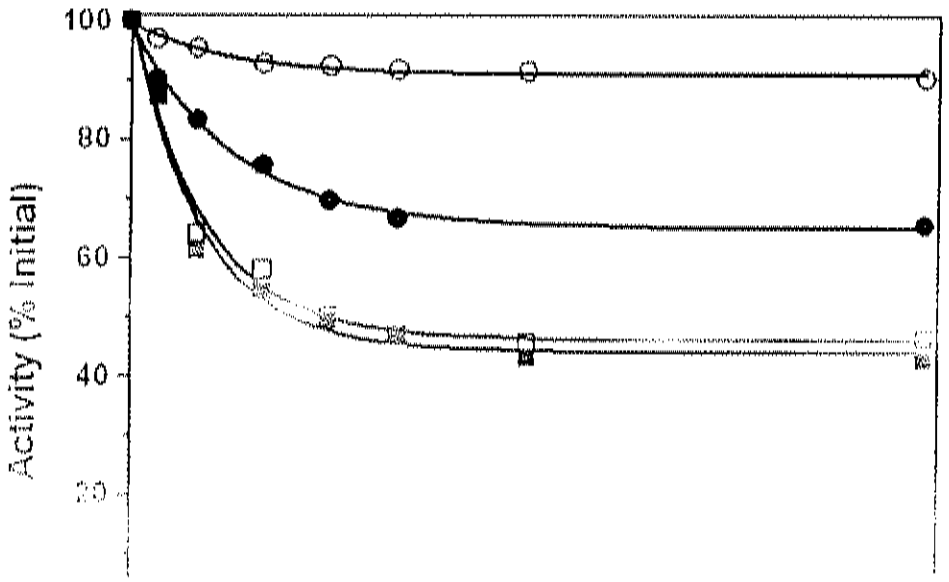
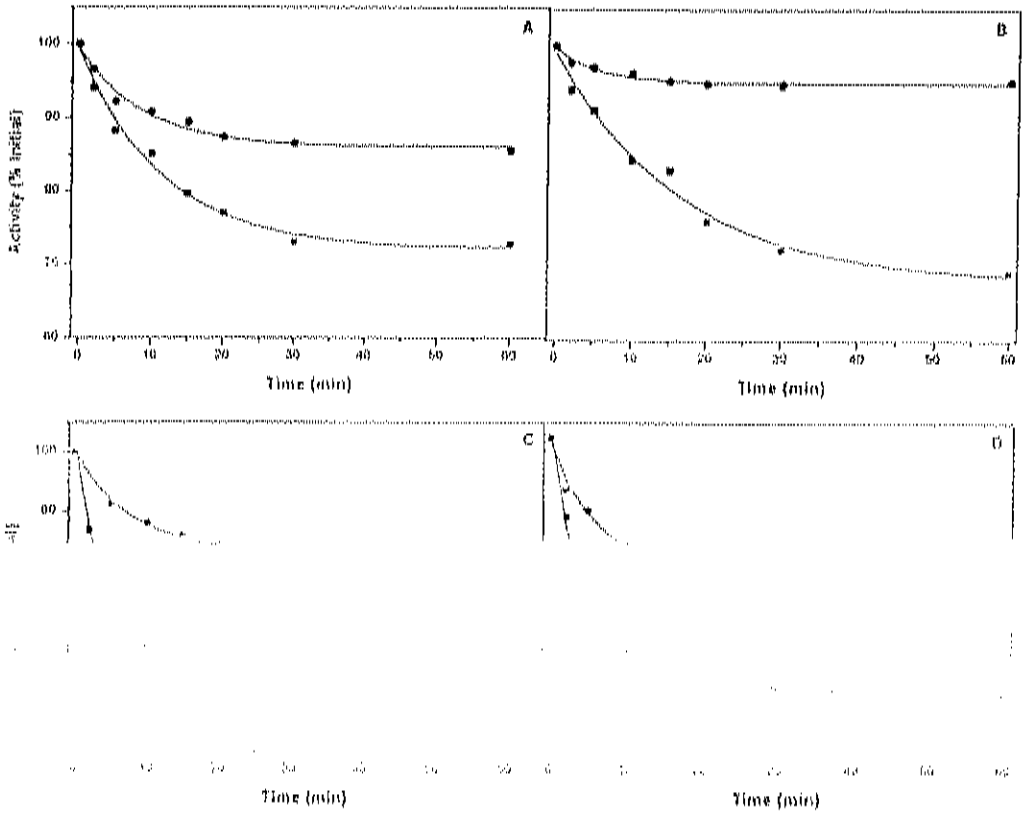


Figure 3.



Scheme 1

A)


Enzyme
Cys-SH
Disulfiram
(DSF)
Diethyldithiocarbamate

B)

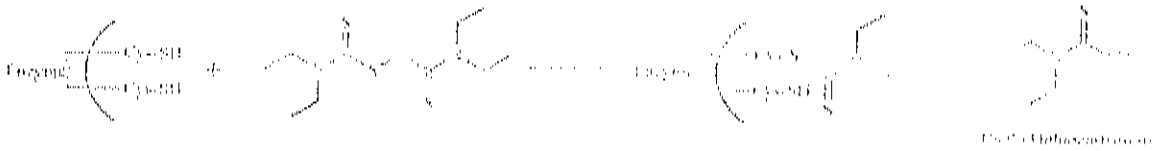

Enzyme
Cys-SH
Cys-SH

Table 1

Kinetic parameters[a] of the inactivation of pkBADH by DSF.

[DSF] (μM)	k_{obs1} (min^{-1})	k_{obs2} (min^{-1})	A_1 (%)	A_2 (%)	A_{ss} (%)
10	0.497	0.030	18	37	44
15	0.678	0.074	35	47	27
20	1.076	0.121	39	49	22
25	1.745	0.145	32	50	17
30	2.406	0.175	31	53	6