The effect of metabolic acidosis on the synthesis and turnover of rat renal phosphate-dependent glutaminase

Jennifer TONG, Gina HARRISON and Norman P. CURTHOYS

Department of Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, U.S.A.

Regulation of the mitochondrial phosphate-dependent glutaminase activity is an essential component in the control of renal ammoniogenesis. Alterations in acid–base balance significantly affect the amount of the glutaminase that is present in rat kidney, but not in brain or small intestine. The relative rates of glutaminase synthesis were determined by comparing the amount of [³⁵S]methionine incorporated into specific immunoprecipitates with that incorporated into total protein. In a normal animal, the rate of glutaminase synthesis constitutes 0.04% of the total protein synthesis. After 7 days of metabolic acidosis, the renal glutaminase activity is increased to a value that is 5-fold greater than normal. During onset of acidosis, the relative rate of synthesis increases more rapidly than the appearance of increased glutaminase activity. The increased rate of synthesis reaches a plateau within 5 days at a value that is 5.3-fold greater than normal. Recovery from chronic acidosis causes a rapid decrease in the relative rate of glutaminase synthesis, but a gradual decrease in glutaminase activity. The former returns to normal within 2 days, whereas the latter requires 11 days. The apparent half-time for glutaminase degradation was found to be 5.1 days and 4.7 days for normal and acidotic rats respectively. These results indicate that the increase in renal glutaminase activity associated with metabolic acidosis is due primarily to an increase in its rate of synthesis. From the decrease in activity that occurs upon recovery from acidosis, the true half-life for the glutaminase was estimated to be 3 days.

INTRODUCTION

During normal acid–base balance, the rat kidney extracts very little glutamine from the plasma (Squires *et al.*, 1976). Renal extraction and catabolism of glutamine is increased rapidly after onset of metabolic acidosis (Hughey *et al.*, 1980; Schrock & Goldstein, 1981). The increased catabolism is due to an increased flux through the mitochondrial phosphate-dependent glutaminase and glutamate dehydrogenase (Schoolwerth *et al.*, 1978; Schoolwerth & LaNoue, 1983). The increased ammoniogenesis provides an expendable cation that is readily excreted in the acidified urine (Tannen, 1978). The resulting α -oxoglutarate provides the carbon skeleton necessary to sustain the increase in renal gluconeogenesis that is associated with an increase in renal ammoniogenesis (Goodman *et al.*, 1966).

The initial increase in renal metabolism of glutamine occurs primarily through changes in the concentrations of metabolites that regulate the glutaminase and glutamate dehydrogenase activities (Boyd & Goldstein, 1979; Vinay *et al.*, 1980). Subsequent adaptations include the induction of phosphoenolpyruvate carboxykinase (Alleyne & Scullard, 1969), phosphate-dependent glutaminase (Leonard & Orloff, 1955) and glutamate dehydrogenase (Seyama *et al.*, 1973). The activity of each enzyme is increased 3–5-fold in the kidney of a chronically acidotic rat. The maximal increase in carboxykinase activity is achieved within 1–2 days (Alleyne & Scullard, 1969). The induction is due to an increased rate of synthesis (Iynedjian *et al.*, 1975) that correlates with an increase in the amount of specific mRNA (Iynedjian & Hanson, 1977; Cimbala *et al.*, 1982). In contrast, the increases in glutaminase and glutamate dehydrogenase activities occur more slowly, requiring 7 days to achieve a maximal response.

Immunological experiments have established that the increased glutaminase activity is due to the presence of an increased amount of the enzyme (Curthoys *et al.*, 1976a). Previous experiments using actinomycin (Goldstein, 1965) suggested that the slight increase in renal glutaminase activity that occurs after 1–2 days of acidosis is due to an increased rate of synthesis. However, in similar experiments, ethionine did not block the increase in glutaminase activity (O'Donovan & Lotspeich, 1968). The greater sensitivity of acidotic animals to inhibitors of protein synthesis prevented the continuation of either experiment for a period of time sufficient to compare the effect of inhibition on a more significant change in glutaminase activity. Thus the mechanism responsible for the adaptation in glutaminase activity is unclear. In the present study, specific immunoprecipitation procedures were used to compare the relative rates of synthesis and apparent rates of degradation of the glutaminase in normal and acidotic rats.

EXPERIMENTAL

Materials

White male Sprague–Dawley rats (200–250 g) were obtained from Zivic Miller and were maintained on Purina rat chow. Acute acidosis was induced by stomach-loading of ether-anaesthetized rats with 20 mmol of NH₄Cl/kg body wt. Chronic acidosis was induced by providing 1.5% (w/v) NH₄Cl as the sole source of drinking water. After drinking the NH₄Cl solution for 7 days, a set of acidotic rats were given water and allowed to recover. [³⁵S]Methionine (1 Ci/ μ mol) was purchased from New England Nuclear. DEAE-Affi-Gel Blue and goat anti-(rabbit IgG)–horseradish peroxidase

conjugate were obtained from Bio-Rad. All other biochemicals were purchased from Sigma.

Solubilization of the glutaminase

Rats were decapitated, and the kidneys, brain and intestine were immediately excised. The tissues were homogenized in a Potter-Elvehjem homogenizer in 3 vol. (v/w) of a buffer containing 330 mM-sucrose, 0.2 mM-EDTA and 20 mM-Tris/HCl, pH 7.5. The crude homogenate was diluted with $\frac{1}{4}$ vol. of 5% (v/v) Triton X-100, incubated for 15 min at 4 °C and then centrifuged for 30 min at 210 000 *g* in a 50Ti rotor. All of the glutaminase activity was recovered in the resulting supernatant. Phosphate-dependent glutaminase activity was determined by using glutamate dehydrogenase to quantify the amount of glutamate formed (Curthoys & Weiss, 1974). The assay conditions were 20 mM-glutamine, 150 mM-phosphate, 0.2 mM-EDTA and 50 mM-Tris/HCl, pH 8.6, at 37 °C. Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Immunoprecipitation of the glutaminase

The phosphate-dependent glutaminase was purified from rat kidney (Curthoys *et al.*, 1976b) and used as antigen to prepare a rabbit immune serum (Curthoys *et al.*, 1976a). Approx. 15 ml of serum was dialysed against 20 mM-Tris/HCl/28 mM-NaCl/0.02% NaN₃, pH 8.0, and applied to a DEAE-Affi-Gel Blue column (5 cm × 15 cm) pre-equilibrated with the same buffer. The eluate fractions were analysed by SDS/polyacrylamide-gel electrophoresis, and the fractions containing IgG molecules were concentrated and dialysed against 10 mM-Hepes/150 mM-NaCl buffer, pH 7.4; 1 ml of the purified IgG fraction precipitated 20 units of glutaminase (i.e. 60 µg).

The specificity of the antibodies was established by using them for immunoblot analysis of the glutaminase. A sample of a freshly prepared homogenate of kidney tissue containing 100 µg of protein was subjected to SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) and then transferred to nitrocellulose for immunostaining (Towbin *et al.*, 1979). After blocking with 3% (w/v) gelatin, the nitrocellulose was incubated with a 1:200 dilution of the purified antibodies. It was then incubated with a goat anti-(rabbit IgG)-horseradish peroxidase conjugate and stained with 4-chloro-1-naphthol.

Normal and acidotic rats were injected intravenously with 0.8 mCi of [³⁵S]methionine and killed 2 h later in order to measure the relative rates of synthesis. The apparent rates of degradation were determined with rats killed 1, 3, 5, 7 and 9 days after injection. A sample of the Triton-solubilized renal homogenate containing 1.5 units of glutaminase and 200 µl of purified antibodies was diluted to 1 ml that contained 10 mM-Hepes and 150 mM-NaCl, pH 7.4. Control samples were prepared by omitting the specific antibody. The samples were incubated at 4 °C for 90 min and then underlayered with 0.5 ml of 0.5 M-sucrose. The immunoprecipitates were pelleted by centrifugation at 10 000 *g* for 10 min. The resulting supernatants lacked glutaminase activity. The pellets were washed twice with 10 mM-Tris/acetate, pH 7.4, containing 1% Triton X-100, and twice with buffer without detergent. The immunoprecipitates were incubated for 10–20 min at 37 °C in 0.6 ml of NCS

Fig. 1. Effect of metabolic acidosis on the activity of rat renal phosphate-dependent glutaminase

The activity of the glutaminase per mg of protein was determined in crude homogenates of renal tissue obtained from rats made acidotic by providing 1.5% NH₄Cl as their sole source of drinking water (●). After 7 days, a set of chronic acidotic rats were given water and allowed to recover (○). Each point is the mean ± S.E.M. of the values determined for four rats.

solubilizer (Amersham) and then diluted with 2.4 ml of ACS scintillation solution (Amersham). The samples were counted for radioactivity and corrected for background by subtracting the radioactivity contained in the control samples. If a second portion of antibodies was added to the supernatant of the initial immunoprecipitate, the radioactivity contained in the second precipitate was similar to that observed in the control samples. Total protein in the Triton-solubilized homogenate was precipitated by adding 10 vol. of 5% (w/v) trichloroacetic acid/1% HClO₄, incubating for 5 min at 4 °C and then centrifuging at 2000 *g* for 5 min. The pellet was washed once, solubilized, and counted for radioactivity. The relative rate of glutaminase synthesis was determined as the ratio of [³⁵S]methionine specifically incorporated into glutaminase to the [³⁵S]methionine incorporated into total protein.

Specific immunoprecipitates and control samples were subjected to 10%-polyacrylamide-slab-gel electrophoresis in the presence of SDS (Laemmli, 1970). One set of lanes was cut into 5 mm slices that were dissolved by heating to 60 °C in 0.2 ml of 60% (w/v) HClO₄ and 0.4 ml of 30% (w/v) H₂O₂. The radioactivity in each gel slice was determined by scintillation counting. Identical samples were also analysed by fluorography (Chamberlain, 1979) or by transfer to nitrocellulose followed by immunostaining (Towbin *et al.*, 1979).

RESULTS

Changes in glutaminase activity

Chronic acidosis was induced by giving rats 1.5% NH₄Cl in their drinking water for a period of up to 14 days (Fig. 1). The specific activity of the renal phosphate-dependent glutaminase was unchanged after 1 day on NH₄Cl. The activity then increased slowly. After 7 days of acidosis, the glutaminase activity reached a plateau at a value that is approx. 5-fold greater than normal. A second set of rats was given NH₄Cl for 7 days

Fig. 2. Effect of metabolic acidosis on the activity of phosphate-dependent glutaminase in brain and small intestine

The activities of the glutaminase per mg of protein were determined in crude homogenates of brain (●) and intestine (○) obtained from rats made acidotic by drinking 1.5% NH₄Cl for various lengths of time. Each point is the mean $\pm$ S.E.M. of the values determined for four rats.

and then allowed to drink water. Within 1 day, blood pH and HCO₃⁻ concentration returned to normal (Parry & Brosnan, 1978). However, the specific activity of the glutaminase decreased very slowly, requiring 11 days to reach a normal value.

The phosphate-dependent glutaminase contained in either rat brain (Curthoys *et al.*, 1976a) or intestine (Pinkus & Windmueller, 1977) is immunologically identical with the enzyme isolated from kidney. As shown in Fig. 2, the specific activity of glutaminase in brain was almost 2-fold greater than that observed in a normal kidney, whereas that in intestine was about one-third of that in kidney. In contrast with the adaptive response observed in renal tissue, the specific activities of the glutaminase in brain and intestine were not altered during chronic acidosis.

Immunoprecipitation of the glutaminase

The specificity of the anti-glutaminase antibodies was established by immunoblot analysis of a freshly prepared homogenate of rat kidney tissue (Fig. 3 inset, lane 1). Only two peptides, one of M_r 65000 and one of M_r 68000, were reactive in the immunostaining procedure. The two peptides were identical in size with the two peptides that are contained in the undegraded form of the phosphate-dependent glutaminase purified from rat brain (Haser *et al.*, 1985).

A rat was injected with 0.8 mCi of [³⁵S]methionine and killed 2 h later. Samples of the Triton-solubilized renal homogenate were incubated with specific anti-glutaminase antibodies. The radioactivity contained in the resulting immunoprecipitate was at least 20 times that present in a sample prepared in the absence of specific antibodies. When analysed by SDS/polyacrylamide-gel electrophoresis, the radioactivity contained in the specific immunoprecipitate migrated as a single peak with a mobility identical with that of the glutaminase (Fig. 3). The width of the peak and the small shoulder on the leading edge were due to the fact that, upon solubilization, the renal

glutaminase undergoes a limited and non-inactivating proteolysis (Clark & Curthoys, 1979). The M_r -68000 peptide is degraded during the incubation required for immunoprecipitation. As a result, an immunoblot of the glutaminase immunoprecipitated from a Triton-solubilized homogenate retained very little of the M_r -68000 peptide, but instead exhibited a series of peptides of M_r slightly lower than 65000 (lane 2). A very similar pattern of peptides was observed when an immunoprecipitate of ³⁵S-labelled glutaminase was subjected to electrophoresis and fluorography (lane 3). Therefore, the radioactivity associated with the specific precipitate is predominantly incorporated into peptides that are derived from the native glutaminase.

Relative rates of synthesis

The effect of altered acid-base status on the relative rates of synthesis of the glutaminase is shown in Fig. 4. In a normal animal, 0.04% of the incorporated [³⁵S]methionine was recovered in the specific immunoprecipitate of the glutaminase. During onset of acidosis, the associated increase in the relative rate of synthesis occurred more rapidly than the change in glutaminase specific activity. The slight increase in the relative rate of synthesis observed after 3 h of acute acidosis was not statistically significant. However, after the rats had drunk NH₄Cl for 24 h, the relative rate of glutaminase synthesis had increased 2.1-fold. The increase in the relative rate

Fig. 3. Specificity of immunoprecipitation of the glutaminase labelled *in vivo* with [³⁵S]methionine

The kidneys of an acidotic rat that had been injected with 0.8 mCi of [³⁵S]methionine were homogenized and solubilized with Triton X-100. Samples of the supernatant containing 1.5 units of glutaminase were incubated in the presence (●) or absence (X) of anti-glutaminase antibodies, and the resulting precipitates were subjected to SDS/polyacrylamide-gel electrophoresis. The gels were cut into 5 mm slices that were solubilized and counted for radioactivity. The insert shows: lane 1, an immunoblot of the glutaminase contained in a crude renal homogenate that was not treated with Triton X-100; lane 2, an immunoblot of the glutaminase immunoprecipitated from a Triton-solubilized crude homogenate; lane 3, a fluorogram of ³⁵S-labelled glutaminase immunoprecipitated from a Triton-solubilized crude homogenate. The '65' marks the mobility of the M_r -65000 peptide.

Fig. 4. Effect of metabolic acidosis on the relative rate of glutaminase synthesis

Rats were injected with 0.8 mCi of [35 S]methionine and killed 2 h later. Samples of Triton-solubilized renal homogenate containing 1.5 units of glutaminase were incubated in the presence and absence of specific antibodies. The resulting immunoprecipitates were solubilized and counted for radioactivity. The amount of [35 S]methionine incorporated into total protein was determined by precipitation with trichloroacetic acid. The relative rate of synthesis is equal to the ratio of [35 S]methionine specifically incorporated into the glutaminase divided by the [35 S]methionine incorporated into total protein. Each bar represents the mean $\pm$ S.E.M. of the values determined for three to five rats.

Fig. 5. Determination of the apparent rates of degradation of glutaminase in normal and acidotic rats

Chronic acidosis was induced by providing rats with 1.5% NH_4Cl for 5 days. Sets of acidotic ($\circ$) and normal (Δ) rats were then injected with 0.8 mCi of [35 S]methionine and killed on various days after injection. Samples of Triton-solubilized renal homogenates containing 1.5 units of glutaminase were incubated in the presence and absence of specific antibodies. The resulting precipitates were solubilized and counted to determine the amount of radioactivity specifically contained in the glutaminase. Each point is the mean of values determined from two rats. Linear regression analysis was used to determine the line that best fit each set of data.

Fig. 6. Estimation of the half-life of the renal glutaminase

The decrease in glutaminase specific activity (E) after recovery from acidosis (data from Fig. 1) was plotted according to the equation which describes the approach to steady-state conditions. E_A and E_N are the mean specific activities of the glutaminase in the fully induced chronic acidotic state and in the normal state, respectively.

of synthesis reached a plateau before the increase in enzyme activity reached a maximum. After 5 days of chronic acidosis, the rate was 5.3-fold greater than normal. When rats were allowed to recover from acidosis, the relative rate of synthesis decreased rapidly and returned to normal within 2 days. The fact that it required an additional 9 days for the glutaminase activity to return to a normal steady-state value suggests that the enzyme must turn over slowly.

Rates of degradation

The apparent rates of degradation of the glutaminase were measured in normal and chronic-acidotic rats injected with [35 S]methionine (Fig. 5). The apparent half-lives for the glutaminase were estimated to be 5.1 days and 4.7 days for normal and acidotic rats respectively. The difference in apparent rates of degradation was not significant. Therefore the changes in glutaminase activity were due primarily to an altered rate of synthesis.

The relative rate of glutaminase synthesis returned to normal after 2 days of recovery from acidosis (Fig. 4). Thus K_S , the zero-order rate constant for synthesis, and K_D , the first-order rate constant for degradation, during the period 2–11 days after acidosis are likely to be equal to the rate constants that determine the normal steady-state amount of the glutaminase. Under these conditions, the decrease in the specific activity of glutaminase (E) is described by the equation:

$$dE/dt = K_S - K_D E$$

Integration of this equation gives:

$$E = \frac{K_S}{K_D} + \left(E_A - \frac{K_S}{K_D} \right) e^{-K_D t}$$

where E_A is the specific activity of glutaminase in the fully induced acidotic state. Under normal steady-state

conditions, the specific activity of the glutaminase (E_N) is constant, i.e. $dE/dt = 0$, and thus $E_N = K_S/K_D$.

Substituting this into the equation yields:

$$E = E_N + (E_A - E_N)e^{-K_D t}$$

which can be rearranged to:

$$-\ln[(E - E_N)/(E_A - E_N)] = K_D t$$

A plot of the data that describes the decrease in glutaminase activity after recovery from acidosis according to this equation generates a straight line (Fig. 6). The slope determined by linear-regression analysis yields an estimated rate constant for degradation of 0.23 day^{-1} . This value corresponds to a half-life for the glutaminase of 3.0 days.

DISCUSSION

The phosphate-dependent glutaminase purified from rat kidney contains a series of peptides in the M_r range 59 000–45 000. Previous studies have established that the various peptides are generated by a partial, but non-inactivating, proteolysis of the native glutaminase (Clark & Curthoys, 1979). All of the peptides associated with the purified glutaminase are structurally related. Furthermore, when the purified glutaminase was stoichiometrically inactivated with either L-2-amino-5-chloro-4-oxo[5- ^{14}C]pentanoic acid (Shapiro *et al.*, 1978) or 6-diazo-5-oxo-L-[6- ^{14}C]norleucine (Clark *et al.*, 1982), all of the peptides bound the two affinity labels. $F(ab)_2$ fragments of antibodies prepared against the purified glutaminase can be used to immunoprecipitate this activity quantitatively from a freshly solubilized homogenate of kidney. The resulting precipitate is devoid of any peptides in the 58 000–45 000 M_r range (Haser *et al.*, 1985). Instead, the predominant peptide recovered in the immunoprecipitate has M_r 65 000.

The phosphate-dependent glutaminase purified from rat brain contains two peptides, one of M_r 68 000 and one of M_r 65 000 (Haser *et al.*, 1985). Partial digestion of two peptides associated with the brain glutaminase and the M_r -65 000 peptide recovered from the immunoprecipitate of the renal glutaminase gives an identical pattern of proteolytic fragments. The immunoblot of the non-solubilized crude homogenate of kidney (Fig. 3) indicates that the renal glutaminase also contains the same two peptides observed in the purified brain glutaminase. The M_r -68 000 peptide associated with the renal glutaminase is degraded to lower- M_r forms within 90 min after solubilization with Triton X-100 (R. A. Shapiro, W. G. Haser & N. P. Curthoys, unpublished work). This observation explains the lack of the M_r -68 000 peptide in the immunoprecipitates of the renal glutaminase. Since the partial proteolysis is non-inactivating, the complete removal of enzyme activity ensures that immunoprecipitation of the proteolysed glutaminase is quantitative. Therefore, the pattern of bands observed in the immunoblot and the fluorograph of the immunoprecipitate of the ^{35}S -labelled enzyme is consistent with the well-characterized sensitivity of the glutaminase to a renal proteinase. The identity of the two patterns provides additional evidence that the immunoprecipitation procedure is highly specific.

The relative rates of glutaminase degradation measured in normal and chronically acidotic rats were equivalent. This observation suggests that the degradation process is

unaltered during metabolic acidosis. The observed increase in the relative rate of glutaminase synthesis is sufficient to account for the greater steady-state glutaminase activity that is achieved during chronic acidosis. Owing to the relatively slow turnover of the glutaminase, an adaptation to a new steady-state activity occurs more slowly than the changes in the rate of glutaminase synthesis. This fact is particularly evident during recovery from the maximally induced acidotic state. The gradual decrease in glutaminase activity was used to estimate the first-order rate constant for glutaminase degradation. This value corresponds to a half-time for glutaminase turnover of approx. 3 days. In contrast, the apparent half-time for glutaminase turnover calculated from the decrease in incorporated [^{35}S]methionine was approx. 5 days. The difference between the two measured values is probably due to reutilization of the label. The relative difference in values is consistent with the report that the absolute and apparent half-times for the turnover of rat renal γ -glutamyl transpeptidase are 1.5 and 3.0 days respectively (Capraro & Hughey, 1985).

A significant increase in the relative rate of synthesis of phosphoenolpyruvate carboxykinase is observed within 2 h after administration of an acute acid load (Iynedjian *et al.*, 1975). In contrast, the slight increase in the relative rate of glutaminase synthesis observed 3 h after onset of acidosis was not significantly different from the rate measured in a normal animal. Thus onset of metabolic acidosis may cause a more rapid increase in the rate of transcription of the phosphoenolpyruvate carboxykinase gene. Alternatively, the observed difference in response could merely reflect a difference in the half-lives of the two mRNAs. The approach of the rate of enzyme synthesis to a new steady-state value is also a function of the degradation constant of the mRNA. In rat liver, the phosphoenolpyruvate carboxykinase mRNA turns over with a half-life of only 40 min (Cimbala *et al.*, 1982). Thus the carboxykinase mRNA in kidney could be degraded more rapidly than the glutaminase mRNA. Under these conditions, the same increase in the rate of synthesis of the two mRNAs would result in a more rapid approach of the rate of synthesis of the carboxykinase to a new steady-state value.

The difference in the distribution of the two enzymes within the various cells of the renal nephron could cause an apparent difference in their response to acute acidosis. Rat renal phosphoenolpyruvate carboxykinase is found only in the proximal-tubular cells of the renal nephron (Burch *et al.*, 1978). During metabolic acidosis, the increase in this activity occurs predominantly in the early or convoluted segment of the proximal tubule. Since the carboxykinase occurs only in a single portion of the nephron, the increase in activity that occurs within these cells is reflected as a similar fold increase in homogenates of whole tissue. In contrast, the glutaminase activity in a normal rat is greatest in the distal portions of the nephron, intermediate in the proximal convoluted tubule and very low in the glomeruli and proximal straight tubule (Curthoys & Lowry, 1973). During chronic acidosis, the glutaminase activity is increased 20-fold in the proximal convoluted tubule, but is unchanged in other segments of the renal nephron. As a result of this distribution, the cell specific increase in glutaminase activity results in only a 4–5-fold increase in the whole tissue.

Previous studies (Leonard & Orloff, 1955; Goldstein, 1965) have reported that the total renal glutaminase activity increases by 10–30% within 1 day after onset of acidosis. In the present study, glutaminase activity was unchanged within 1 day. However, quantitative microanalysis has shown that, within this time period, the glutaminase activity in the proximal convoluted tubule has increased 2.5-fold (Curthoys & Lowry, 1973). Therefore the normal enzyme distribution is likely to mask the initial induction of glutaminase synthesis that occurs within the proximal convoluted tubules. These considerations also suggest that the 5-fold increase in the relative rate of glutaminase synthesis observed in crude homogenates of kidney tissue is due to a much greater increase in the rate of synthesis that occurs in this specific type of cell. Therefore the increase in both the renal phosphoenolpyruvate carboxykinase and phosphate-dependent glutaminase during acidosis may occur in response to the same stimulus and through an identical mechanism.

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