Annu. Rev. Nutr. 1995. 15:133–59

REGULATION OF GLUTAMINASE ACTIVITY AND GLUTAMINE METABOLISM

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KEY WORDS: mitochondria, urea, ammonia, liver, kidney

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0199-9885/95/0715-0133$05.00

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ABSTRACT

Glutamine is synthesized primarily in skeletal muscle, lungs, and adipose tissue. Plasma glutamine plays an important role as a carrier of nitrogen, carbon, and energy between organs and is used for hepatic urea synthesis, for renal ammoniagenesis, for gluconeogenesis in both liver and kidney, and as a major respiratory fuel for many cells. The catabolism of glutamine is initiated by either of two isoforms of the mitochondrial glutaminase. Liver-type glutaminase is expressed only in periportal hepatocytes of the postnatal liver, where it effectively couples ammonia production with urea synthesis. Kidney-type glutaminase is abundant in kidney, brain, intestine, fetal liver, lymphocytes, and transformed cells, where the resulting ammonia is released without further metabolism. The two isoenzymes have different structural and kinetic properties that contribute to their function and short-term regulation. Although there is a high degree of identity in amino acid sequences, the two glutaminases are the products of different but related genes. The two isoenzymes are also subject to long-term regulation. Hepatic glutaminase is increased during starvation, diabetes, and feeding a high-protein diet, whereas kidney-type glutaminase is increased only in kidney in response to metabolic acidosis. The adaptations in hepatic glutaminase are mediated by changes in the rate of transcription, whereas kidney-type glutaminase is regulated at a posttranscriptional level.

INTERORGAN METABOLISM OF GLUTAMINE

Sites of Synthesis

Glutamine, a nonessential amino acid, plays an important role in the interorgan transport of both nitrogen and carbon. Within the body, glutamine comprises more than 60% of the free α–amino acids. It is present in concentrations of 0.5–0.8 mM in plasma and of up to 20 μmol/ml in the intracellular water of some tissues, such as skeletal muscle (104). The plasma glutamine pool turns over very rapidly at a rate of 900–1300 μmol/kg per h in the rat (108) and 318–373 μmol/kg per h in humans (23). ^{13}C-labeled tracer studies have shown that some dietary glutamine (consumption is approximately 4–8 g/day) is absorbed intact into the portal blood stream (25, 71). However, portal-drained viscera invariably exhibit a net uptake of arterial glutamine (135, 136), indicating that most of the large glutamine pool within the body is synthesized de novo.

This review begins with a description of glutamine synthesis and of the various enzymes that have been implicated in glutamine catabolism. We then focus on the biosynthesis, mitochondrial localization, and structural and kinetic properties of the liver- and kidney-type glutaminases. We conclude with a discussion of the two glutaminase cDNAs and the regulation of the expression of their respective genes.

The synthesis and hydrolysis of glutamine in mammalian tissues was first described by Krebs in 1935 (59). Although the exact pathway of precursor formation is not fully understood, the only known reaction yielding glutamine is that catabolized by glutamine synthetase (EC 6.3.1.2, L-glutamine: ammonia ligase ATP). This reaction converts glutamate and ammonia to glutamine with the hydrolysis of ATP (Equation 1).

$$\text{L-glutamate} + NH_3 + ATP \rightarrow \text{L-glutamine} + ADP + P_i \qquad 1.$$

Glutamine synthetase is found in most tissues, and although skeletal muscle possesses relatively low activity when expressed per gram of tissue, by virtue of its large mass this tissue is a major site of glutamine synthesis within the body (133). During stress and hypercatabolic states (metabolic acidosis, cancer, sepsis, trauma, or the injection of endotoxin), glutamine is released from muscle, and intracellular glutamine levels fall (51, 64). If the condition is prolonged, a new steady state is reached with a lower intracellular glutamine concentration and an elevated rate of release of glutamine maintained by increased glutamine synthesis.

Detailed analysis of arteriovenous difference measurements in the rat showed that glutamine release from skeletal muscle could not account for the high rates of glutamine utilization (94, 133). This finding led to the identification of the lungs as a major organ of net glutamine synthesis (134). Most studies in the rat indicate that the lungs release as much, if not more, glutamine than the total skeletal musculature. However, such estimates are subject to large errors because a very small arteriovenous difference is multiplied by a very large blood flow. Although the lungs release glutamine in times of surgical trauma or sepsis, they show glutamine balance in healthy human subjects (106). Thus, the role of the lungs may vary with organism and physiological state (106, 134).

A recently identified site of net glutamine synthesis and release is adipose tissue. Arteriovenous difference measurements in humans (30) and microdialysis experiments in rats (57, 58) indicate that the total release of glutamine by adipose tissue may be similar in magnitude to the amount released by skeletal muscle.

Most organs that possess glutamine synthetase activity also have some glutaminase activity. The net glutamine balance across an organ is the difference between these two activities. This is true for the lungs and adipose tissue

and particularly for organs such the brain, liver, and kidney, all of which show net glutamine release under appropriate physiological or pathological conditions (105). Net release of glutamine by the brain is seen in both control and diabetic subjects, but the magnitude of release is relatively small compared with that from other organs (37). Renal glutamine synthetase, and hence renal glutamine release, is organism specific in that it is present in rats and sheep but not in humans or dogs (65).

In liver, glutamine synthetase is expressed only in a discrete 1–3 cell layer of hepatocytes surrounding the venous exit of the acinus (32). Hepatic glutamine synthesis is thus physically separated from glutamine hydrolysis since liver glutaminase is located predominantly in periportal cells (81, 129). A complex intercellular glutamine cycle has been proposed (42) and may play a key role in the maintenance of ammonia homeostasis, with perivenous glutamine synthesis effectively detoxifying any ammonia that escapes urea synthesis in the periportal cells. However, such zonation is probably equally important in the maintenance of glutamine homeostasis since the liver shows net release of glutamine when other organs exhibit increased rates of glutamine utilization. A similar compartmentalization occurs in rat kidney, where glutamine synthetase is expressed only in the proximal straight tubules (7). Increased flux through renal glutamine synthetase and net release of glutamine are observed only during metabolic alkalosis (22).

Sites of Catabolism

Glutamine is probably utilized by all cells for the synthesis of purines, pyrimidines, glucosamine, and other amino acids. However, such biosynthetic pathways account for only a small fraction of total body glutamine turnover. A number of cell types, including the absorptive epithelial cells of the small intestine (enterocytes), cells of the immune system (e.g. thymocytes, lymphocytes, and macrophages), the fetus, hair follicles, and many tumors, exhibit very high rates of glutamine utilization (52, 53, 56, 84, 85, 125, 127, 135, 136). Although these cells require large amounts of glutamine for biosynthetic purposes, a limited amount (<5%) of the extracted glutamine is used for such pathways. The vast majority is used for energy production because glutamine is the major respiratory fuel in such cells. This dependence on high rates of glutamine catabolism suggests that the mucosa of the intestine is the major site of glutamine utilization in the body, although it has been argued that the cells of the immune system constitute an equally large "organ" of glutamine catabolism (85). Glutamine is only partially oxidized in the intestinal cells, and the carbon end products, i.e. lactate, alanine, and aspartate, are utilized by other tissues (125). The production of large amounts of alanine and ammonia from glutamine catabolized by the small intestine contributes to the synthesis

of urea in the liver. Thus, the contribution of plasma glutamine to urea synthesis is greater than that arising solely from glutamine extracted by the liver (70).

The liver uses glutamine to synthesize glucose and urea. In liver, the glutaminase enzyme is expressed predominantly in the periportal cells (42, 81); these cells express high levels of activity of other enzymes of the glutamine pathway, such as those of the urea cycle and gluconeogenesis. Evidence from tracer experiments in vivo indicates that the ammonia released from glutamine may be preferentially channeled into the urea cycle (77, 78). Interestingly, liver-type glutaminase has been detected only in cells with a functional urea cycle. However, because the liver also exhibits net glutamine output under some conditions, it can be considered the major site of glutamine homeostasis.

In normal acid-base balance, the kidney extracts and metabolizes very little glutamine (107). However, the onset of metabolic acidosis results in increased renal extraction of plasma glutamine (29, 46), which is catabolized largely within the S1 and S2 segments of the proximal convoluted tubule (35). Increased flux through glutaminase is accompanied by an increased flux through the mitochondrial glutamate dehydrogenase (92). The resulting ammonium ions are largely excreted in the urine, a process that facilitates the excretion of metabolic acids or anions while conserving sodium and potassium ions. The resulting α-ketoglutarate is initially metabolized to phosphoenolpyruvate and, depending on the organism, is either converted to glucose (36) or oxidized to CO_2 (118, 119). The two decarboxylation reactions associated with the conversion of α-ketoglutarate to phosphoenolpyruvate result in the net synthesis of bicarbonate ions, which are added to the plasma and which partially compensate the systemic acidosis.

In the brain, glutamine also serves as an important precursor for the synthesis of neurotransmitter substances (61). Glutaminase activity is high in the nerve terminals and neurons and much lower in glial cells (4). However, the glial cells have a high level of glutamine synthetase (132). Thus, glutamine is taken up by neurons, where it is either catabolized to glutamate, one of the most abundant neuroexcitatory substances, or further metabolized to γ-aminobutyric acid, an important neuroinhibitory substance. When released by acidnergic neurons, the γ-aminobutyric acid or glutamate is taken up by the glial cells and used to resynthesize glutamine. The release of glutamine and its uptake by neurons complete an intercellular cycle of glutamine utilization.

GLUTAMINASES

Definition and Reaction

Many enzymes can utilize glutamine as a substrate, but only one gives rise to stoichiometric amounts of glutamate and ammonia (79) and is therefore a true

glutaminase (Equation 2). This activity is termed phosphate-dependent glutaminase (EC 3.5.1.2, L-glutamine amidohydrolase) and is also known as phosphate-activated glutaminase, glutaminase I, or mitochondrial glutaminase.

$$\text{L-glutamine} + H_2O \rightarrow \text{L-glutamate}^- + NH_4^+ \qquad 2.$$

History of Related Enzymes

The activity known as glutaminase II is in fact a combination of a number of isozymes of glutamine aminotransferase and ω-deamidase (14). The preferred keto acids for the transaminases are phenylpyruvate and the keto analogues of methionine, glycine, serine, cysteine, and asparagine. Because of the low abundance of the preferred keto acid substrates, these reactions are not believed to be of quantitative importance in glutamine metabolism. They may conserve essential amino acid carbon skeletons by reamination to the amino acids.

Another activity, phosphate-independent glutaminase (also called maleate-activated glutaminase), is a reaction of γ-glutamyl transpeptidase (16, 113). Although it was once thought to play a major role in renal ammonia production, this activity is no longer considered a physiological function of γ-glutamyl transpeptidase.

Glutamine is also a substrate for a number of other reactions, most notably the family of eight amidotransferases involved in the synthesis of purines, pyrimidines, glucosamines, nicotinamide adenine dinucleotide (NAD^+), and asparagine. These enzymes possess a conserved active site sequence, and some evolved from the fusion of a glutaminase gene with a synthetase gene (139). Many of these enzymes can utilize ammonia as the substrate, albeit at very high nonphysiological concentrations.

Other reactions utilizing glutamine include phenyacyl CoA *N*-acyltransferase (80), which conjugates glutamine to phenylacetic acid, and transglutaminases, which only react with glutamine incorporated into protein and catalyze an acyl transfer in which the γ-carboxamide groups of glutamine residues are the acyl donors. Such reactions are of minor quantitative importance in glutamine metabolism.

Many prokaryotic asparaginases exhibit high glutaminase activity. These enzymes have been tested as anticancer agents because they deplete plasma glutamine levels. Although high levels of asparaginase are found in some mammalian tissues, notably liver and kidney, the mammalian enzymes show little or no reactivity toward glutamine (138).

Isoenzymes

In the first paper to report the enzymatic hydrolysis of glutamine in mammalian tissues, Krebs (59) noted that the glutaminase activity of liver differed from that of other tissues, such as kidney and brain. Specifically, he found differ-

ences in the pH optima and sensitivity to inhibition by the end product glutamate. These two isozymes have been extensively studied over the past 60 years and are now known to differ not only in kinetic properties, but also in protein structure. They are the products of different but related genes.

KINETIC DIFFERENCES Kidney-type glutaminase is expressed in kidney, brain, intestine, and fetal liver (15). The enzyme is localized within the mitochondria. Its kinetic properties have been characterized using permeabilized mitochondrial extracts or highly purified enzyme. Kidney-type glutaminase requires a polyvalent anion for activity. The most commonly used activator is phosphate, which produces half-maximal activation at concentrations of 20–30 mM. The kidney isoenzyme exhibits a broad specificity with respect to activators, and the physiological effector is unknown. It also exhibits a relatively low K_m for glutamine (2–5 mM) and is strongly inhibited by glutamate. As discussed below, the kinetic properties of this isoenzyme are affected by the state of polymerization of the protein.

The liver-type enzyme is found only in postnatal liver (67, 103) and, like the kidney-type enzyme, is found within the mitochondrial matrix in loose association with the inner membrane (55, 72, 76). Compared with the kidney-type enzyme, purified liver-type glutaminase exhibits a relatively high K_m (17 mM) for glutamine, a lower $K_{0.5}$ for phosphate activation, and a flat pH curve with an optimum between 7.8 and 8.2. The enzyme is neither inhibited by glutamate (up to 50 mM) nor activated by maleate but is inhibited by a number of glutamine analogues, such as *N*-acetylglutamine and 6-diazo-5-oxo-L-norleucine (55, 72, 102).

In the late 1960s, Charles (9) showed that liver-type glutaminase was subject to activation by ammonia (one of the products of the reaction), and McGivan (74) demonstrated that enzymatic activity is dependent on ammonia, with NH_3 as the activating species. The kinetic characteristics of glutaminase are different depending on whether the enzyme is membrane associated, or solubilized by sonication or phospholipase A2 treatment (75, 76). When membrane associated, hepatic glutaminase has a K_m for glutamine of 6 mM and exhibits hyperbolic behavior with respect to glutamine concentration. The solubilized enzyme, however, shows a higher $K_{0.5}$ for glutamine and exhibits sigmoidal kinetics.

SHORT-TERM REGULATION The renal metabolism of glutamine is rapidly activated following the acute onset of metabolic acidosis. Within 1–3 h, the arterial plasma glutamine concentration is increased twofold (46, 93), primarily because of an increased release of glutamine from muscle tissue (93). Renal extraction of glutamine becomes significant as the arterial plasma concentra-

tion is increased. Additional responses include prompt acidification of the urine (112), which facilitates the removal of ammonium ions, and pH-induced activation of α–ketoglutarate dehydrogenase (69), which reduces the intracellular concentration of α-ketoglutarate and glutamate. Thus, increased metabolism initially results from an increased availability of glutamine and a decreased concentration of the products of the glutaminase and glutamate dehydrogenase reactions. However, during chronic metabolic acidosis, all of the acute adaptations are partially compensated (6) and the arterial plasma glutamine concentration is decreased to 65% of normal (107).

The short-term regulation of flux through hepatic glutaminase has been studied in isolated hepatocytes, in the perfused liver, and in isolated liver mitochondria (55, 72). At high concentrations (>5 mM), glutamine is readily utilized by liver cells, resulting in the production of glucose and urea. However, at lower (more physiological) levels of glutamine (<1 mM), there is very little evidence of net glutamine utilization (70), although evidence of flux through both glutaminase and glutamine synthetase is apparent (120). Net glutamine utilization by liver in vitro can be achieved by the addition of hormones such as glucagon, vasopressin, thyroxine, angiotensin II, and catecholamines; of analogues of cyclic adenosine monophosphate (cAMP); or of metabolites such as ammonia, bicarbonate, cysteine, and leucine (55, 72). Ochs (86) has proposed that hormones that mediate changes in calcium act at the level of α–ketoglutarate dehydrogenase, whereas glucagon and cAMP act directly at the level of glutaminase. These agents may act by changing the interaction of glutaminase with the inner mitochondrial membrane, since the effects are not seen in disrupted mitochondria. It has been suggested that hormonal regulation of cell and/or mitochondrial volume may play a role in glutaminase regulation. More recently, Ewart & Brosnan (28) proposed that mitochondrial uptake of spermine may be involved in this process, since this polyamine can activate hepatic glutaminase in both intact and disrupted mitochondria. The activation of hepatic glutaminase by its product ammonia may seem somewhat paradoxical. Welbourne (133) proposed that a "feed-forward" activation of the enzyme by ammonia produced from intestinal glutamine catabolism would ensure that the liver maintains glutamine homeostasis at times of increased glutamine absorption from the diet.

Pogson and colleagues (68) have attempted to quantify the regulatory roles of glutaminase and other steps in hepatic glutamine catabolism in isolated hepatocytes. They determined a flux control coefficient of close to 1.0 for glutaminase activity, which indicates that a small change in glutaminase activity would result in a large change in flux through the catabolic pathway. Other points of significant control were identified as the transport of glutamine into the cell (system N) and the transport of glutamine out of the cell (system L), with coefficients of 0.31 and –0.4, respectively.

LONG-TERM REGULATION The level of glutaminase activity is increased in rat kidney during progressive development of chronic acidosis (24, 66). In normal rat kidney, mitochondrial glutaminase activity is greatest in the distal portions of the nephron, intermediate in the proximal convoluted tubule, and very low in glomeruli and proximal straight tubules (19, 137). Within 24 h of the onset of acidosis, glutaminase activity is increased twofold within the proximal convoluted tubule. However, because of the cell specificity of the increase and the greater level of activity associated with distal tubules, a significant increase in glutaminase activity measured in a crude homogenate of whole kidney can only be observed after 2–3 days. The total glutaminase activity increases gradually and eventually plateaus after 7 days of acidosis at a value 4- to 5-fold greater than normal (19). The maximal induction results from a 7- to 20-fold increase of glutaminase activity within the proximal convoluted tubule.

Immunological experiments have established that the increase in renal glutaminase activity results from the presence of an increased amount of protein and not from an activation of preexisting enzyme (18). The relative rate of renal synthesis of glutaminase was determined by pulse labeling experiments (115). In a normal rat, the rate of glutaminase synthesis constitutes 0.04% of the total protein synthesis. Following onset of acidosis, the relative rate of glutaminase synthesis increased gradually and then plateaued within 5 days at a value 5.3-fold greater than normal. The apparent half-life for glutaminase degradation was nearly identical in normal and acidotic rats. Thus, the stimulation of glutaminase synthesis accounts for the 5-fold increase in glutaminase activity. Recovery from chronic acidosis causes a rapid decrease in the relative rate of glutaminase synthesis but only a gradual decrease in glutaminase activity (87). From the kinetics of the decrease in activity, the half-life of the glutaminase protein was estimated to be 3 days.

The long-term regulation of hepatic glutaminase was recognized within the past 10 years (123, 124). A number of earlier reports (31, 101) indicated that hepatic glutaminase activity changed adaptively over time, but these reports were based on assay systems that failed to consider the unique kinetic properties of the liver enzyme. Using more appropriate assay conditions, investigators found that hepatic glutaminase activity increased upon feeding high-protein diets, during starvation, and during uncontrolled streptozotocin diabetes and decreased upon feeding low-protein diets (122, 126, 130). Changes in acid-base status, a major determinant of renal glutaminase activity, had no effect on hepatic activity. Changes in hepatic glutaminase activity were similar to changes seen in the activities of key enzymes of hepatic glutamine metabolism, such as phosphoenolpyruvate carboxykinase and the enzymes of the urea cycle. Although the apparent half-life of hepatic glutaminase is not known, this enzyme does not reach a new steady state until 2–3 days after induction of diabetes. Such changes are therefore not likely of primary regulatory im-

portance. However, as with changes in the activity of the urea cycle enzymes (78), the adaptation of hepatic glutaminase allows conservation of nitrogen at times of limited intake or more rapid catabolism and detoxification of excess nitrogen in response to high intake or during hypercatabolic states.

GLUTAMINASE PROTEINS

Purification

Kidney-type glutaminase has been purified from pig (63) and rat (17) kidney; from pig (111), rat (41), and cow brain (10); and from Ehrlich ascites tumor cells (91). The enzyme can be solubilized from mitochondria by treatment with Triton X–100, by freeze-thaw fractionation, or by lyophilization in the presence of a borate and phosphate buffer. The glutaminase undergoes specific and extensive polymerization in the presence of borate and phosphate (M_r > 10,000,000). Conversely, dialysis into a Tris-phosphate buffer produces an active tetrameric enzyme with an apparent mass of 250,000. This reversible association-dissociation has been used to purify the glutaminase either by repeated precipitation and solubilization or by repeated chromatography on a gel filtration column. The enzyme purified from rat brain or acidotic rat kidney exhibits a specific activity of approximately 300 μmol/min per mg protein, which represents an enrichment of approximately 1000-fold.

Liver glutaminase was somewhat refractory to purification. Because it does not undergo the reversible polymerization observed for the kidney-type enzyme, a distinct purification scheme was developed. In the mid-1980s, three groups (43, 88, 102) obtained liver glutaminase of varying purities, and Smith & Watford (102) raised specific antisera to their preparation. Preparations of hepatic glutaminase that are purified 600-fold and that have specific activities of 30–60 μmol/min per mg protein are comprised predominantly of a 58-kDa peptide. Although hepatic glutaminase has been reported to copurify with glutamate dehydrogenase (88), suggesting a physical interaction of the two enzymes, the two activities can be easily separated either by precipitation or by chromatographic methods (102).

Subunit Structure and Oligomerization

In the presence of a saturating concentration of phosphate, the purified renal glutaminase exists as a fully active tetrameric enzyme (33). Removal of phosphate and other polyvalent anions causes the glutaminase to dissociate to an inactive dimer. Phosphate-induced activation closely correlates with the formation of the tetrameric enzyme. Moreover, activation and tetramer formation can be reversed to a similar extent by increasing the concentrations of the inhibitor glutamate. A characterization of the properties of the dimeric

and tetrameric forms of glutaminase covalently bound to sepharose established that polymerization is an essential step in the phosphate-induced activation of the glutaminase (82). The dimeric and tetrameric forms of the glutaminase also exhibit different sensitivities toward inactivation by two glutamine affinity-labeling reagents. Glutaminase is rapidly inactivated by the stoichiometric binding of L-2-amino-4-oxo-5-chloropentanoic acid (95). However, concentrations of phosphate that increase glutaminase activity decrease the rate of inactivation by the chloroketone. Furthermore, inactivation is blocked by glutamate but not by glutamine. These data suggest that the chloroketone reacts preferentially with the dimeric form of the glutaminase and that the enzyme may have a specific binding site for glutamate. In contrast, inactivation of renal glutaminase by 6-diazo-5-L-oxo-norleucine occurs only when the enzyme is catalytically active (13, 96). Glutaminase activity and the rate of inactivation by the diazoketone exhibit very similar phosphate concentration–dependent activation profiles. The ability of glutamate to protect glutaminase against inactivation is also reversed by increasing concentrations of phosphate. These results indicate that the diazoketone interacts preferentially with the tetrameric form of the glutaminase at a site that is specific for binding glutamine.

Shapiro et al (100) developed a sensitive radioactive activity assay to investigate further the hypothesis that glutamine and glutamate interact at separate sites on kidney-type glutaminase. The K_m for glutamine decreased from 36 to 4 mM when the phosphate concentration was increased from 5 to 100 mM. Glutamate was found to be a competitive inhibitor with respect to glutamine at both high and low concentrations of phosphate. However, the K_i for glutamate was increased from 5 to 52 mM by increasing phosphate concentrations. These results indicate that glutamine and glutamate interact at the same site, but the conformation and specificity of this site are determined by the phosphate concentration and the extent of tetramer formation.

Hepatic glutaminase has a subunit size of 58 kDa (102), but the size of the native enzyme has not been determined accurately. Estimates from high-performance liquid chromography (HPLC) gel filtration indicate a native M_r >300,000, but using conventional gel filtration, Patel & McGivan (88) reported values of 270,000–310,000, whereas Heine et al (43) and Huang & Knox (45) estimated an M_r of 170,000 using sucrose gradient centrifugation. These discrepencies could result from the different methods used, since Smith & Watford (102) reported an M_r >300,000 with HPLC gel filtration but obtained a value of 162,000 using sucrose density gradient centrifugation. Although the native molecular weights are identical when determined in the presence of 5 or 100 mM phosphate, the purified enzyme rapidly loses activity when phosphate is removed. Therefore, it has not been possible to determine its M_r in the absence of this effector.

Antibodies

When subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, purified rat renal glutaminase contains a series of peptides ranging in apparent M_r from 48 to 59 kDa (12). However, the individually isolated peptides exhibit identical peptide maps and are covalently labeled by the two glutamine affinity reagents. Thus, the mixture of peptides is apparently produced by partial proteolysis; the generated forms retain full glutaminase activity. The purified renal glutaminase was used to prepare rabbit polyclonal antibodies.

The glutaminase purified from rat brain consists of 66-[1] and 68-kDa peptides present in a 3:1 ratio (99). Antibodies prepared against the isolated rat renal glutaminase were affinity purified vs the 66-kDa peptide obtained from purified rat brain glutaminase. The affinity-purified IgGs react with both 66- and 68-kDa peptides contained in purified brain glutaminase and in isolated brain mitochondria. Partial proteolysis of the isolated 68- and 66- kDa brain glutaminase peptides with *Staphylococcus aureus* V8 protease produced an identical pattern of immunoreactive proteolytic fragments. The pattern of immunostaining of the brain glutaminase was unaltered, even when brain mitochondria were solubilized with Triton X–100 and stored at 4° C for 2 days. An identical pattern was observed when intact rat renal mitochondria were subjected to immunoblot analysis. However, when renal mitochondria were solubilized, the 68-kDa peptide was rapidly degraded to the 66-kDa form. At 4° C, this reaction occurs with apparent first-order kinetics and a half-life of 35 min. Degradation of the 66-kDa peptide of the renal glutaminase occurs with much slower kinetics but is nearly complete within 24 h. Thus, the renal cortical glutaminase also consists of two immunologically and structurally related peptides.

To characterize further the mechanism responsible for the adaptive increase in renal glutaminase activity, Tong et al (116) determined the changes in the relative levels of translatable glutaminase mRNA. The relative levels of translatable glutaminase mRNA increased gradually following onset of acidosis, reaching a 4.2-fold maximal induction after 7 days. At all times, the changes in relative level of translatable glutaminase mRNA correlated well with the previously observed changes in the relative rate of glutaminase synthesis. Therefore, induction of the mitochondrial glutaminase does not result from stimulation of the translocation and processing of its precursor or from the regulation of a required posttranslational modification.

Antibodies to preparations of hepatic glutaminase reacted to a specific

[1]The relative molecular masses of the glutaminase subunits cited here differ slightly from previously published values. The estimates used in this review are based on the amino acid sequence deduced from the cDNA sequence (97).

58-kDa protein present only in liver mitochondria, not in cytosol or in preparations of other tissues. Western blot analysis indicated that the abundance of this protein is increased in the liver of diabetic rats. The IgG fraction isolated from the antisera and linked to protein A–Sepharose beads precipitated hepatic glutaminase activity but exhibited very weak reactivity to the glutaminase in brain, kidney, and intestine. Antibodies to the 58-kDa peptide excised from the gels exhibited similar reactivity. Immunoprecipitant titrations demonstrated a similar equivalence point in extracts of liver from control and diabetic rats, whereas the total activity differed fourfold. Thus, the increased activity in diabetes resulted from an increase in the amount of enzyme protein, with no change in the specific activity of the glutaminase (102).

Using a differential assay based on the kinetic properties of the two glutaminase isozymes, Linder-Horowitz (67) reported that liver-type glutaminase was absent in nonhepatic tissues and appeared in liver close to the time of birth, and that the high glutaminase activity in fetal liver was kidney type. Antibodies to hepatic glutaminase show no reactivity on Western blots to extracts from fetal liver but do detect a 58-kDa band in extracts of neonatal liver, thus confirming the developmental pattern (EM Smith & M Watford, unpublished observations).

LOCALIZATION

Submitochondrial Localization

Association of glutaminase with mitochondria was first proposed by Errera & Greenstein for liver (27) and was later shown experimentally (39). Other reports have noted a mitochondrial localization of glutaminase in kidney, brain, and intestine. The submitochondrial localization of glutaminase has not been determined conclusively and may differ from organism to organism. Digitonin and Lubrol fractionation and swell-shrink sonication techniques were used to demonstrate that rat renal glutaminase is associated with the inner surface of the inner mitochondrial membrane (21). This localization is also suggested by an observed discontinuity of an Arrhenius plot of enzyme activity, by the relative concentration of detergent required to release the glutaminase, and by the inability of investigators to inhibit enzyme activity with membrane-impermeable reagents that rapidly inactivate the solubilized glutaminase (98). However, other studies suggest that rat renal glutaminase is contained primarily within the mitochondrial matrix (50). In contrast, pig renal glutaminase may be primarily associated with the outer surface of the inner mitochondrial membrane (62).

Mitochondrial Transport of Glutamine

In rat or dog renal mitochondria, the catabolism of glutamine requires its initial translocation across the inner mitochondrial membrane. Mitochondrial trans-

port of glutamine occurs through a uniport mechanism (5) that uses a specific carrier (1, 34). The transport of glutamine into rat renal mitochondria causes both glutamine and glutamate to appear within the matrix space (20). Therefore, glutamine transport and glutaminase activity are distinct processes. The inability to measure initial rates of transport in the absence of metabolism has made it impossible to determine whether adaptive changes in the ability of mitochondria to transport glutamine occur during acidosis. However, at 4° C, the rate of efflux from mitochondria preloaded with [^{14}C]glutamine is five times faster than the rate of the glutaminase reaction (54). These results suggest that glutamine transport is not a rate-limiting step in renal metabolism of glutamine. However, the mitochondrial glutamine transporter has not been purified, nor has it been cloned. Thus, this important step in glutamine metabolism remains poorly characterized.

Biosynthesis of Glutaminase

When primary cultures of epithelial cells from rat renal proximal tubules (89) or of HTC hepatoma cells (90) were labeled with [^{35}S]methionine in the presence of 20 μM carbonylcyanide m-chlorophenylhydrazone, only a 74-kDa peptide was precipitated with glutaminase-specific antibodies. Pulse-chase experiments indicated that the 74-kDa cytosolic precursor could be chased quantitatively into the mature mitochondrial subunits of glutaminase. The 74-kDa precursor was processed via a 72-kDa intermediate. In addition, synthesis of the 66-kDa subunit occurred more rapidly and was initiated before synthesis of the 68-kDA subunit. The observed kinetics account for the final 3:1 ratio of the two peptides. These data were initially interpreted to suggest that the 68-kDa peptide may be produced by covalent modification of the 66-kDa subunit. In vitro transcription and translation of a full-length glutaminase cDNA (see following section) also yield a single 74-kDa precursor (97). Incubation of the precursor with isolated rat liver mitochondria results in the synthesis of the mature subunits of glutaminase. Thus, the mature forms of glutaminase arise from a single precursor protein generated from a single mRNA.

An in vitro system was used to characterize further this unique processing pathway and to investigate the potential function of the 68-kDa subunit (109). The processing pathway and the kinetics of the in vitro processing reaction closely approximated those observed in cultured cells. The in vitro processing reaction required a membrane potential, the presence of external ATP, and the involvement of proteinacous receptors. Mitochondrial processing was also blocked by o-phenanthroline, an inhibitor of the matrix processing peptidase. The presequence of glutaminase contains a large proportion of basic amino acids. Two-dimensional gel electrophoresis of mature glutaminase established that the 68-kDa subunit is slightly more basic than the 66-kDa subunit. In

addition, incubation of the 74-kDa precursor with purified matrix processing peptidase yields equal amounts of the two mature peptides. A cDNA construct, pΔGA, was created that lacks the nucleotides encoding amino acid residues 32 through 72 of glutaminase. When transcribed and translated in vitro, pΔGA yields a 70-kDa precursor that is processed by mitochondria to a single mature subunit with an M_r of 66 kDa. Thus, the 68-kDa subunit is not produced by covalent modification; rather, the two mature subunits of glutaminase are produced by alternative processing reactions that can be catalyzed by the matrix processing peptidase (MPP). Submitochondrial fractionation of imported glutaminase and Δ-glutaminase precursors suggests that the 68-kDa subunit may retain the mature glutaminase within the mitochondrial matrix.

The structural determinants necessary for translocation and proteolytic processing of the glutaminase were delineated further by characterizing the processing of different chimeric constructs formed by fusing segments of the N-terminal sequence of the glutaminase precursor to cholramphenicol acetyl transferase (110). A chimeric precursor containing the N-terminal 118 amino acids of glutaminase is translocated and processed in isolated rat liver mitochondria or cleaved by purified mitochondrial processing peptidase to yield one intermediate and two mature subunits analogous to the products of processing of the glutaminase precursor. Thus, all the information required for translocation and synthesis of the mature subunits resides in the N-terminal 118 amino acids of the glutaminase precursor. The characterization of chimeric constructs containing shorter segments of glutaminase sequence suggests that the mitochondrial processing peptidase cleavage reactions that yield the glutaminase intermediate and the 66-kDa subunit depend primarily on information present C-terminal to the respective sites of cleavage.

GLUTAMINASE cDNAs

Isolation and Characterization

The first rat kidney-type glutaminase cDNA was isolated using glutaminase-specific antibodies to screen a rat brain λgt11 cDNA library (3). The glutaminase cDNA selectively hybridized to an mRNA which, when translated, yielded a 74-kDa protein that could be immunoprecipitated with the glutaminase-specific antibodies. An RNA probe transcribed from the glutaminase cDNA hybridized to an mRNA approximately 5 kb in length. This mRNA is expressed in rat brain and normal rat kidney and is increased sixfold in acidotic rat kidney but is not expressed in rat liver. The first cDNA contained 1040 base pairs that encode 326 amino acids from the C-terminus of the glutaminase and a short segment of 3′ nontranslated nucleotides. This glutaminase cDNA was used to screen oligo(dT)-primed rat kidney or random-primed rat brain

cDNA libraries. This procedure yielded three additional cDNAs that correspond to the entire sequence of the glutaminase mRNA (97). The full-length glutaminase cDNA contains 60 base pairs of 5′ nontranslated sequence, 2022 base pairs of an open reading frame, and 2445 base pairs of 3′ nontranslated sequence. It encodes a 74-kDa precursor protein composed of 674 amino acids, including an N-terminal sequence of 16 residues that has a calculated propensity to form an amphipathic α-helix typical of a mitochondrial targeting sequence. Residues 73 through 90 correspond to the N-terminal sequence of the more abundant 66-kDa glutaminase subunit. Thus, the proteolytic processing required to yield this peptide results in removal of a 72–amino acid presequence. The 3′ nontranslated region of the glutaminase mRNA contains two potential polyadenylation sites. Use of the initial site apparently results in the synthesis of the less abundant 3.4-kb glutaminase mRNA. This segment also contains four potential stem-loop structures, a 48-bp repeat of CA dinucleotides, and numerous AU-rich regions that contain AUUUA sequences. Such domains may regulate stability of glutaminase mRNA.

A rat liver λgt11 cDNA library was screened for expression of hepatic glutaminase using the antibodies raised against the 58-kDa protein. As a result, a cDNA was identified and isolated (103). This 1-kb cDNA hybridized to a single mRNA species present only in liver RNA. Recently, the complete coding sequence for hepatic glutaminase was isolated, indicating a mature mRNA of 2.2 kb (11). There is no detectable hybridization to RNA from organs such as kidney, intestine, or brain, all of which contain very high levels of kidney-type glutaminase. Similarly, no hybridization was seen with RNA from fetal hepatic tissue, but the probe did detect a signal just after birth that reached adult levels within two days.

The measurement of glutamine metabolism in the perfused liver indicated (42) that in contrast to glutamine synthetase, which is expressed exclusively in perivenous cells, glutaminase expression was probably localized to the periportal region of the liver acinus. This hypothesis was confirmed using the cDNA to hepatic glutaminase, which detected a higher abundance of the mRNA in periportal hepatocytes (129). By in situ hybridization, glutaminase mRNA is detected only in a limited population of periportal hepatocytes (81).

Sequence

The amino acid sequence of liver glutaminase protein has not been determined, thus preventing direct comparison of the nucleotide sequence of the cDNA with the amino acid sequence of the protein. However, the amino acid sequence deduced from the cDNA was compared with that of kidney-type glutaminase (M Watford, unpublished data). Comparisons of the full-length cDNAs show >70% nucleotide identity in the coding regions. When the predicted amino acid sequences are compared, an even higher degree of overall identity of

>75% is apparent. An 18–amino acid sequence at the C-terminal end of the liver glutaminase shows no identity with the sequence of the kidney enzyme. The pattern of differences—scattered amino acid substitutions within stretches of identical amino acids—suggests that the two glutaminases are the products of different genes.

Many of the glutamine amidotransferases possess glutaminase activity and exhibit a conserved active site containing a triad of cysteine, histidine, and aspartate (139). No such site exists in the amino acid sequences of either liver-type or kidney-type glutaminases, suggesting a separate line of evolution.

GLUTAMINASE GENE

The nucleotide sequence for an entire mammalian glutaminase gene has not been determined. Thus, the genomic organization, the related gene products, and even the number of glutaminase genes remain unknown. However, initial progress in cloning and identifying the promoter of kidney-type glutaminase has been made (L Farrell, R Shapiro & NP Curthoys, unpublished data). The 5′ EcoRI/PstI fragment from the pGA cDNA (97) was used as a probe to screen a rat EMBL3 genomic library. Three glutaminase genomic clones were isolated and aligned by restriction analysis. The overlapping clones contain 31 kb of the glutaminase gene, including 16 kb of sequence that extends 5′ of the common SacI fragment, which hybridizes to the EcoRI/PstI cDNA probe. RNA protection assays indicate that transcription is initiated at a position 142 bp 5′ of the initiating methionine codon. Approximately 3 kb of the genomic clone was sequenced. This region contains 2281 bp of promoter sequence, the entire 5′ untranslated region, 401 bp of coding sequence, and 218 bp of intron I. Exon I encodes all of the glutaminase presequence (72 amino acids) and the stretch of 9 consecutive glutamine residues (CAG repeats) located near the N-terminus of the 66-kDa subunit of glutaminase. Thus, exon I contains all of the information necessary for the correct mitochondrial processing of the glutaminase precursor (110).

The glutaminase promoter lacks an identifiable TATA box. However, the region immediately 5′ of the transcription start site contains a GC-rich region that includes a CCAAT box and two SP1 binding sites. The upstream promoter sequence contains two potential serum response elements and four potential C/EBP binding sites. Various lengths of the glutaminase promoter were linked 5′ to a chloramphenicol acetyl transferase (CAT) gene. Transfection of LLC-PK_1-FBPase$^+$ cells, a porcine renal proximal tubule cell line (38), with the GA_{-402} to $_{+110}$CAT construct results in a high level of CAT activity. This activity is greatly reduced by deleting either the transcription initiation site (–63 to 110 bp) or the GC-rich region containing the CCAAT box and SP1 sites (–401 to –63 bp). Investigations are under way to determine the potential

function of the putative regulatory elements and to identify tissue-specific elements.

REGULATION OF EXPRESSION

Liver Gene

Liver-type glutaminase is only expressed in postnatal hepatocytes, and as described above, changes in enzyme activity associated with diabetes result from changes in the amount of the protein (102). Using the cDNA to liver-type glutaminase, Watford et al (131) and Zahn et al (14) determined that such changes are accompanied by similar changes in the relative abundance of liver glutaminase mRNA. Similarly, changes in liver glutaminase activity during starvation (increased twofold), upon feeding high-protein diets (increased twofold), or upon feeding low-protein diets (decreased) also result from changes of similar magnitude in the abundance of the mRNA (131). Using the mRNA elongation transcription assay in isolated rat liver nuclei, Watford et al (131) and Zahn et al (140) determined that within experimental error, all changes in hepatic glutaminase activity and mRNA abundance result from similar changes in the rate of transcription of the gene. Other sites of regulation, such as mRNA stability, transcript processing, and other intranuclear events, cannot be ruled out, but the evidence suggests that such mechanisms are of limited significance in the regulation of liver glutaminase. Long-term regulation of the genes of hepatic phosphoenolpyruvate carboxykinase and the urea cycle enzymes occurs by the same mechanisms (8, 83, 114, 117), suggesting coordinated regulation of expression of this group of hepatic genes.

The humoral signals that regulate hepatic glutaminase expression have not been identified definitively. The patterns of expression (absent before birth and localized in the periportal hepatocytes) and regulation (increased during diabetes, high protein feeding, and starvation; decreased during low protein feeding) and the mechanisms of regulation (primarily transcriptional) are similar to those observed for the key regulatory enzymes involved in the further metabolism of hepatic glutamine. The common regulators for these genes are glucagon (positive), glucocorticoids (positive), and insulin (negative) (8, 83, 114, 117). The injection of large amounts of dibutyryl cAMP in vivo increases the relative abundance of hepatic glutaminase mRNA. Dibutyryl cAMP also increases this mRNA in hepatoma cells in culture (121, 128). Similarly, abundance of hepatic glutaminase mRNA is increased in H35 cells after treatment with dexamethasone, a synthetic glucocorticoid. Although not investigated at the mRNA level, hepatic glutaminase activity in primary cultures of rat hepatocytes does increase in response to glucagon (73). Thus, the evidence suggests that the hepatic glutaminase gene will exhibit regulatory sequences for these

hormones similar to those seen in other genes. For example, the upstream sequences for carbamoylphosphate synthetase I, ornithine transcarbamylase, and arginase contain elements recognized by C/EBP and related factors. cAMP and glucocorticoid response elements may also be present. A unique sequence has been identified in the promoter of four of the urea cycle genes and termed UCE (urea cycle element) (26, 60). This sequence is likely to be present in coordinately regulated genes. However, the absence of this sequence in the carbamoylphosphate synthetase I gene and its presence in the ornithine aminotransferase gene (which shows perivenous expression) (60) suggest that its function may not be directly related to the urea cycle. A clear understanding of the regulation of the hormonal expression of hepatic glutaminase awaits detailed analysis of genomic sequences.

In addition to its unique role as a short-term activator of hepatic glutaminase, ammonia may also play a role in the regulation of expression of the glutaminase gene. Rat hepatocytes in primary culture rapidly lose both hepatic glutaminase activity and protein (as detected by Western blots). However, the activity and protein can be maintained in the presence of 1 mM NH_4Cl (73). Similarly, in H35 hepatoma cells the rate of transcription of the hepatic glutaminase gene and the relative abundance of its mRNA are increased in the presence of 1 mM NH_4Cl (121). These findings point to a possible function of ammonia in vivo, since portal ammonia levels range from 0.5 to 0.8 mM. However, experiments using NH_4Cl-induced acidosis (130) suggest that ammonia is of little regulatory significance. The hepatic glutaminase is not increased even though the load of NH_4Cl used to induce acidosis would probably have increased the portal ammonia concentration. Therefore, either the acidosis overrides the effect of ammonia on hepatic glutaminase expression, or the change in ammonia concentration is not sufficient to increase expression. As with short-term regulation of hepatic glutaminase, which requires ammonia for activity but probably not for acute changes in activity, basal expression of the hepatic glutaminase gene may also require a certain level of ammonia.

Kidney Gene

The glutaminase-specific cDNA hybridizes to 5.0- and 3.4-kb mRNAs contained in total or polyA$^+$ RNA isolated from rat kidney. The levels of the two mRNAs are affected coordinately in response to changes in acid-base balance (48). The levels of both mRNAs are increased fivefold within one day of the onset of chronic acidosis and reach a maximum (an eightfold increase) after five days. During recovery from chronic acidosis, the levels of the glutaminase mRNAs are returned to normal within one day. The observed changes in mRNA levels correlate well with the previously observed changes in the relative levels of translatable glutaminase mRNA (116). Nuclear run-on assays indicate that the rate of transcription of the renal glutaminase gene is unaffected

by alterations in acid-base balance. Thus, the increase in glutaminase activity during chronic acidosis results from an increase in the levels of total and translatable glutaminase mRNAs that apparently results from an increased stability of the glutaminase mRNA.

The increase in rat renal glutaminase mRNAs also has been characterized in response to acute onset of acidosis (47). The observed increase in glutaminase mRNAs occurs following a 6–8 h lag and reaches a plateau within 16–18 h at a level eightfold higher than normal. In contrast, acute recovery from chronic acidosis results in a rapid and coordinate decrease in the levels of the two glutaminase mRNAs. The acute decrease occurs with first-order kinetics and an apparent half-life of 4 h.

To characterize further the potential regulation of glutaminase mRNA stability, Hansen & Curthoys (40) ligated a 936-bp fragment containing the 3′ nontranslated sequence of the smaller glutaminase mRNA into an expression vector that encodes a chimeric β-globin mRNA. The parent construct contains the promoter/enhancer of Rous sarcoma virus (RSV), the coding region of β-globin genomic DNA, and the 3′ nontranslated region of bovine growth hormone mRNA. Both the parent and the β-globin-glutaminase chimeric constructs were stably transfected into LLC-PK_1-FBPase$^+$ cells. Stable transfectants of the β-globin-glutaminase construct expressed lower levels of the chimeric mRNA than the parent construct. However, only the level of the β-globin-glutaminase mRNA was increased 2.5- or 5-fold by growth in acidic media (pH = 6.9) or by treatment with cycloheximide, respectively. The apparent half-life of the parent β-globin mRNA (>24 h) measured by inhibiting transcription was unaffected by growth in acidic media. In contrast, the apparent half-life of the β-globin-glutaminase chimeric mRNA was increased from 4.5 to 18 h. When the two constructs were stably transfected into COS monkey kidney cells, the levels of the parent β-globin mRNA and of the chimeric β-globin-glutaminase mRNA were equivalent and were unaffected by changes in extracellular pH. These results further support the hypothesis that changes in rat renal glutaminase mRNA levels are mediated primarily through regulation of the stability of the mRNA.

Previous studies suggested that the adaptations in rat renal glutaminase mRNA levels were reproduced in LLC-PK_1-FBPase$^+$ (49). In these experiments, the acute onset of acidosis was modeled by transferring cells grown in normal media (pH 7.5 and 25 mM $NaHCO_3$) to an acidic media (pH 6.9 and 10 mM $NaHCO_3$). However, these experiments were conducted using a rat glutaminase cDNA probe, which produced only a weak signal during Northern analysis. Therefore, the previous study was largely conducted using slot-blot analysis. The investigators had assumed that the hybridization conditions were specific for glutaminase mRNA and that all potential species of glutaminase mRNAs were coordinately regulated in response to changes in extracellular

pH and HCO_3^- concentration. A recent study using improved analytical procedures showed that the reported pH-responsive adaptations in phosphoenolpyruvate carboxykinase mRNA levels in the LLC-PK_1-FBPase$^+$ cells were qualitatively but not quantitatively reproduced (44). Thus, the basal and adaptive levels of potential glutaminase mRNAs in the LLC-PK_1-FBPase$^+$ cells were reexamined using improved Northern analysis procedures and specific probes derived from the porcine glutaminase cDNA (D Porter & NP Curthoys, unpublished data). The results demonstrate that the LLC-PK_1-FBPase$^+$ cells express at least three different species of glutaminase mRNAs that are differentially expressed as functions of cell growth and differentiation and of media conditions. Similar alterations in glutaminase mRNA levels have been observed in Ehrlich ascites tumor cells (2). Thus, the development of an appropriate cell culture system to characterize the regulation of the kidney-type glutaminase gene will require careful definition and standardization of tissue culture procedures and cell growth conditions.

SUMMARY

Function of Glutaminases

The presence of two distinct glutaminase isozymes that are the products of different but related genes probably explains differences in metabolism of glutamine in different cells. These differences are illustrated by the expression of kidney-type glutaminase in fetal liver but not in postnatal liver, where it is replaced by the unique liver enzyme. The function of glutamine metabolism in the liver cell changes during the same period of development. In fetal liver, glutamine is used for biosynthetic purposes and possibly as a respiratory fuel; in postnatal liver, glutamine is a substrate for gluconeogenesis and urea synthesis and little, if any, undergoes complete oxidation. Therefore, although hepatic glutaminase releases ammonia, this ammonia is immediately incorporated into carbamoylphosphate in the urea cycle by the action of carbamoylphosphate synthetase I. All other organs that utilize large amounts of glutamine possess kidney-type glutaminase and release the ammonia formed into the bloodstream or urine without further modification.

Future Directions

Much is now known about the structure and function of the two types of glutaminase. The recent cloning of glutaminase cDNAs and genomic DNA will enable us to expand our knowledge of this important enzyme even further. A more detailed analysis of the transcriptional regulation of liver-type glutaminase and a better definition of the expression of potential isoforms of renal-type glutaminase as a function of cell growth and differentiation are

likely to be forthcoming. These advances will require a determination of the number of genes that encode and express functional glutaminases, a thorough characterization of their promoters, and a more detailed definition of their respective transcripts. This information should help explain the tissue specificity of glutaminase expression and its increased expression and function in transformed cells. The development of procedures to express native and mutated forms of glutaminase in vitro will make it possible to analyze association with the mitochondrial inner membrane or with other proteins in the mitochondrial matrix. Such experiments may provide insight into the acute regulation of glutaminase metabolism and the effective channeling of the products of the glutaminase reaction. Other important questions to address are which molecular mechanisms are responsible for the pH-responsive stabilization of the renal glutaminase mRNA and what signal transduction pathway is involved. The latter experiments may require the development of more responsive cell lines or the more thorough definition and standardization of conditions used to culture existing cell lines. Thus, future experiments should greatly increase our understanding of the role of glutaminase in regulating glutamine catabolism, in cell growth and transformation, and in urea synthesis and pH homeostasis.

ACKNOWLEDGMENTS

The experiments performed in the authors' laboratories were supported by grants DK 37124 and DK 43704 from the National Institutes of Health awarded to NPC and by grant DK 37301 from the National Institutes of Health and Hatch Project #14161 from the New Jersey Agricultural Experiment Station awarded to MW.

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