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Immunoblot analysis of glutaminase peptides in intact and solubilized mitochondria isolated from various rat tissues

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Antibodies were prepared against isolated rat renal glutaminase and affinity-purified against the 65 kDa peptide contained in the purified rat brain glutaminase. The affinity-purified IgGs were then used to compare the glutaminase immunoreactive peptides contained in samples that had been subjected to SDS/polyacrylamide-gel electrophoresis and transferred to nitrocellulose. The purified brain glutaminase and isolated brain mitochondria contain 68 and 65 kDa peptides that exhibit nearly equivalent immunostaining. Partial proteolysis of the isolated 68 and 65 kDa peptides with *Staphylococcus aureus* V8 proteinase produced an identical pattern of immunoreactive proteolytic fragments. However, digestion of the two peptides with chymotrypsin resulted in similar, but slightly different, patterns. The pattern of immunostaining was unaltered even when the brain mitochondria were solubilized with Triton X-100 and stored for 2 days at 4 °C. A very similar pattern was observed when intact renal mitochondria were subjected to immunoblot analysis. However, when renal mitochondria were solubilized, the 68 kDa peptide was rapidly degraded to the 65 kDa form. At 4 °C this reaction occurs with apparent first-order kinetics and a $t_{\frac{1}{2}}$ of 35 min. Degradation of the 65 kDa form of the renal glutaminase occurs with much slower kinetics, but is nearly complete after 24 h. Solubilization of mitochondria isolated from various zones of the kidney indicated that the responsible endogenous proteinase was localized primarily in the cortex. Mitochondria isolated from intestinal or renal papillary tissue contain four glutaminase immunoreactive peptides (M_r 68000, 65000, 61000 and 58000). The smallest of these peptides is identical in size with the single immunoreactive peptide observed in liver tissue.

INTRODUCTION

In mammals, glutamine is the most abundant amino acid found in plasma. It is synthesized in skeletal muscle, in the hepatocytes that surround the terminal hepatic venules and in glial cells within the brain. The plasma pool is derived primarily from the glutamine that is synthesized within muscle tissue (Marliss *et al.*, 1971). However, during periods of fasting or of chronic acidosis, the liver also adds glutamine to the venous blood (Phromphetcharat *et al.*, 1981; Schrock & Goldstein, 1981). Plasma glutamine is primarily extracted by the epithelial cells of the small intestine, where it serves as the principal respiratory fuel (Windmueller & Spaeth, 1980). Glutamine is also catabolized in periportal hepatocytes to stimulate urea synthesis (Haussinger, 1983) and in various neurons to generate neurotransmitter substances (Kvamme, 1983). During metabolic acidosis, the kidney also extracts large amounts of plasma glutamine in order to generate NH_4^+, which is excreted in the urine (Tannen & Sastrasinh, 1984).

In each of these organs, the catabolism of glutamine is initiated by a mitochondrial glutaminase (Kovacevic & McGivan, 1983). The glutaminase found in liver tissue is a unique isoenzyme that is dependent on NH_4^+ (Patel & McGivan, 1984). In contrast, the glutaminases contained in brain, kidney and intestine exhibit a lower K_m for glutamine, require the presence of a polyvalent anion and are immunologically related (Curthoys *et al.*, 1984). However, the enzymes purified from the latter tissues exhibit different structural properties. For example, the glutaminase purified from rat kidney contains five to seven structurally related peptides that range in M_r from 59000 to 48000. The peptides are generated during the purification procedure by an endogenous proteinase that causes a partial, but non-inactivating, proteolysis of the native enzyme (Clark & Curthoys, 1979). The glutaminase immunoprecipitated from freshly solubilized renal tissue contains only a 65 kDa peptide (Haser *et al.*, 1985). In contrast, the glutaminase purified from rat brain or immunoprecipitated from freshly solubilized brain tissue contains a predominant peptide of M_r 65000 and a minor peptide of M_r 68000. The purpose of the present study was to utilize immunoblot procedures to characterize further the relationship between the two forms of peptides contained in the brain glutaminase and to identify the native form of the enzyme contained in the various tissues that catabolize glutamine.

EXPERIMENTAL

Materials

White male Sprague–Dawley rats (200–250 g) were obtained from Zivic Miller (Zelienople, PA, U.S.A.) and were maintained on Purina rat chow. Reagents for polyacrylamide-gel electrophoresis, DEAE-Affi-Gel Blue and goat anti-(rabbit IgG)–horseradish peroxidase conjugate were purchased from Bio-Rad. *S. aureus* V8 proteinase was purchased from Miles Laboratories. ^{14}C-labelled protein M_r standards were obtained from

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BRL. Nitrocellulose membranes were a product of Millipore. All other biochemicals were obtained from Sigma.

Preparation of glutaminase

Rats were decapitated and the kidneys, brain, liver and intestine were immediately excised. The tissues were homogenized in 8 vol. (v/w) of a buffer containing 330 mM-sucrose, 0.2 mM-EDTA and 20 mM-Tris/HCl, pH 7.5, and mitochondria were isolated by differential centrifugation (Curthoys & Weiss, 1974). A kidney was cut to yield a cone of tissue in which the base consisted solely of cortex and the apex contained only papillary tissue (Waldman & Burch, 1963). The cone was then dissected into cortex, medulla and papilla and the individual segments were homogenized in 0.5 ml of homogenate buffer. The homogenates were then centrifuged at 10000 *g* for 10 min at 4 °C. The resulting pellet was resuspended with 3 vol. of homogenate buffer (v/w) and centrifuged in a Beckman Microfuge for 10 s to remove nuclei and cell debris. The resulting supernatant was used as the mitochondrial preparation. The isolated mitochondria were incubated at 4 °C in the presence or absence of 0.3% Triton X-100. At various times aliquots containing 100 μg of protein were added to 50 μl of hot sample buffer (Laemmli, 1970) containing 4% SDS and heated to 90 °C for 2 min.

The phosphate-dependent glutaminase was purified from rat kidney (Curthoys *et al.*, 1976*b*) and rat brain (Haser *et al.*, 1985). Glutaminase activity was determined 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 the standard.

Electrophoretic procedures

A sample containing 60 μg of purified brain glutaminase was subjected to electrophoresis on a preparative 10%-(w/v)-polyacrylamide slab gel in the presence of SDS (Laemmli, 1970). The gel was then stained with Coomasssie Brilliant Blue and destained for 2 h. Individual strips containing the 68 and 65 kDa peptides were cut from the gel and incubated with 0.5 ml of sample buffer for 1 h at 25 °C and then overnight at 4 °C. Alternatively, the strips were cut into 3 mm slices that were used for partial proteolytic digestion with *S. aureus* V8 proteinase (150 ng/sample) or with chymotrypsin (150 ng/sample) as described by Cleveland *et al.* (1977).

Immunoblot analysis was carried out essentially as described by Towbin *et al.* (1979). The samples were subjected to SDS/polyacrylamide-gel electrophoresis and then transferred to nitrocellulose. After blocking with 20 mM-Tris/HCl/500 mM-NaCl, pH 7.4 (TBS), containing 3% gelatin (w/v), the nitrocellulose was incubated with affinity-purified anti-glutaminase antibodies. It was then incubated with a goat anti-(rabbit IgG)–horseradish peroxidase conjugate and stained with 4-chloro-1-naphthol. The immunoblots were then photographed with Kodak slide film. The relative intensities of the 68 and 65 kDa bands were quantified by tracing with a Helena Quick Scan R & D densitometer.

The purified rat renal glutaminase was used to prepare a rabbit immune serum (Curthoys *et al.*, 1976*a*). The IgG fraction was isolated by chromatography on DEAE-Affi-Gel Blue (Tong *et al.*, 1986) and affinity-purified against the 65 kDa peptide of the brain glutaminase. Approx. 10 μg of the 65 kDa peptide was electrophoresed on a preparative SDS/polyacrylamide slab gel and then transferred to nitrocellulose. After blocking with gelatin, two 1.5 cm strips were cut from the vertical ends of the nitrocellulose and immunostained to locate the position of the 65 kDa peptide. A horizontal strip containing this peptide was then cut from the unstained nitrocellulose and incubated with 1.5 ml of TBS containing 7.5 mg of rabbit anti-glutaminase IgG and 1% gelatin (w/v) for 2 h at 25 °C. The strip was then washed for 30 min with two 25 ml changes of TBS to remove free and non-specifically bound antibodies. The specific IgGs were eluted by incubating the strip with 0.5 ml of TBS containing 2 M-NaSCN for 2 h at 25 °C. The eluate was then centrifuged (750 *g* for 5 min) through a 5 ml Sephadex G-25 column equilibrated with TBS and then diluted with 0.25 ml of TBS containing 3% gelatin.

RESULTS

When the purified glutaminase or the immunoprecipitate of the freshly solubilized brain tissue was subjected to SDS/polyacrylamide-gel electrophoresis and stained with Coomassie Brilliant Blue, the intensity of the 65 kDa peptide was approx. 4-fold greater than that of the 68 kDa peptide (Haser *et al.*, 1985). In contrast, immunoblot analysis of identical samples indicates that the brain glutaminase may contain a nearly equivalent amount of the two peptides (Fig. 1, lanes 1 and 2). The ratio of the intensities of the two bands was not altered when the amount of the glutaminase analysed was varied from 0.1 to 1.0 μg. The anti-glutaminase IgGs that were affinity-purified against the isolated 65 kDa peptide react with both the 65 and the 68 kDa peptides (Fig. 1, lanes 3 and 4). Thus the two peptides share common immunological determinants. Partial proteolysis of the isolated 68 kDa peptide with *S. aureus* V8 proteinase results in at least 18 different proteolytic fragments that can be identified by immunostaining (Fig. 1, lane 5). An identical pattern of fragments was generated by proteolysis of the 65 kDa peptide (Fig. 1, lane 6). In contrast, treatment of the isolated 68 and 65 kDa peptides with chymotrypsin resulted in slightly different patterns of proteolytic fragments (Fig. 1, lanes 7 and 8). The larger fragments generated from the 68 kDa peptide all appear to be slightly larger than the corresponding peptides derived from the 65 kDa peptides. However, the pattern of small fragments derived from the two peptides are identical.

The affinity-purified antibodies were also used to compare the immunologically reactive peptides that are present in intact mitochondria. The glutaminases solubilized from brain and kidney exhibit an equivalent immunological titre (Curthoys *et al.*, 1976*a*). The isolated brain and renal mitochondria also exhibit an identical pattern of 68 and 65 kDa peptides (Fig. 2). Immunologically reactive peptides of the same size are also present in mitochondria isolated from the small intestine. However, this tissue also contains a significant amount of at least two additional immunologically reactive peptides which have a slightly lower M_r (61000 and 58000). The glutaminase contained in liver cannot be

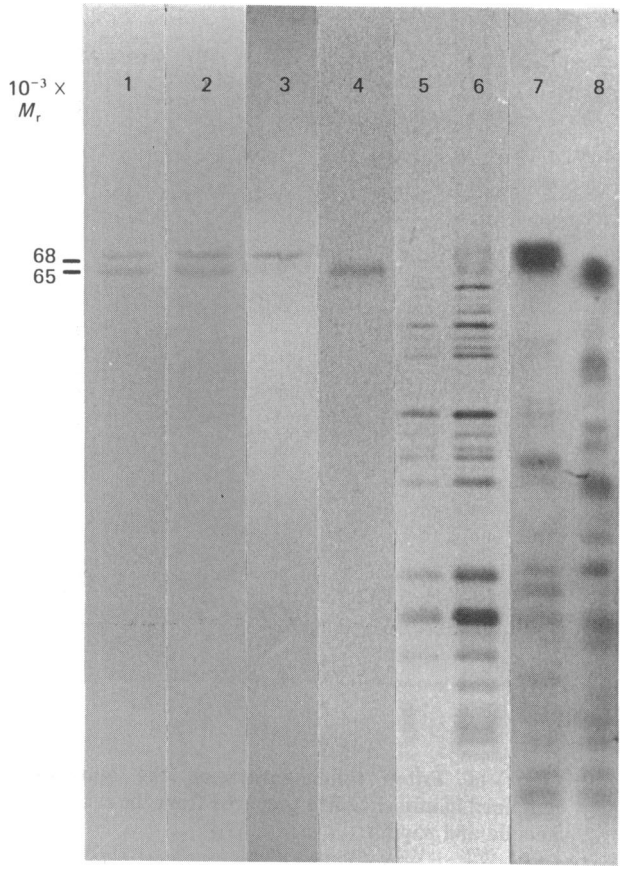

Fig. 1. Immunoblot analysis of the limited proteolytic digest of the peptides associated with the purified rat brain glutaminase

The various samples were subjected to SDS/polyacrylamide-gel electrophoresis, transferred to nitrocellulose and immunostained. The samples contained 200 ng (lane 1) and 600 ng (lane 2) of purified brain glutaminase, the isolated 68 kDa peptide (lanes 3, 5 and 7) and the isolated 65 kDa peptide (lanes 4, 6 and 8). The isolated peptides were incubated in the absence (lanes 3 and 4) or presence of *S. aureus* V8 proteinase (lanes 5 and 6) or chymotrypsin (lanes 7 and 8).

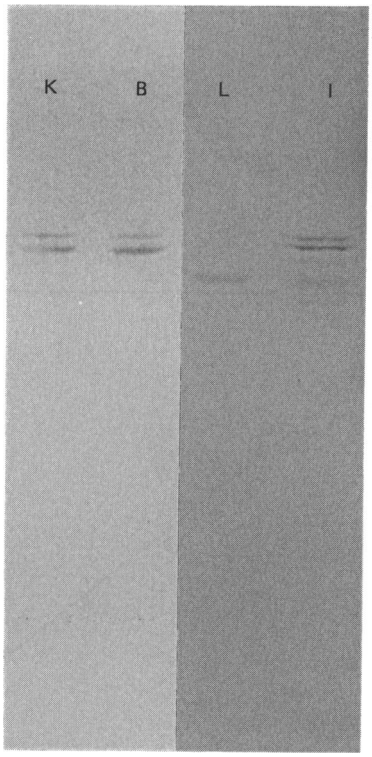

Fig. 2. Immunoblot analysis of the glutaminase contained in mitochondria isolated from various tissues

Samples of mitochondria isolated from kidney (K), brain (B), liver (L) and small intestine (I) were subjected to SDS/polyacrylamide-gel electrophoresis, transferred to nitrocellulose and immunostained.

directly precipitated by the antibodies produced against the isolated renal glutaminase (Curthoys *et al.*, 1976*a*). However, the affinity-purified antibodies do exhibit a slight, but significant, reactivity towards a 58 kDa peptide that is present in isolated hepatic mitochondria.

The ability to purify an undegraded form of the glutaminase from rat brain was attributed to the absence of an endogenous proteinase that is responsible for the cleavage of the purified renal enzyme (Haser *et al.*, 1985). To test further for the presence of a proteinase that might cleave the glutaminase, isolated brain mitochondria were solubilized with 0.3% Triton X-100 and then stored at 4 °C. The relative intensities of the 68 and 65 kDa peptides were unaltered even after 2 days. In addition, no lower-M_r immunoreactive peptides were generated. The pattern of glutaminase peptides was also unaltered when isolated renal mitochondria were incubated for 90 min in the absence of Triton X-100. However, solubilization of the mitochondria results in a rapid disappearance of the 68 kDa peptide and the concomitant increase in the intensity of the 65 kDa peptide (Fig. 3). This process occurs with apparent first-order kinetics and a $t_{\frac{1}{2}}$ of approx. 35 min.

In order to characterize further the proteolysis of the renal glutaminase, mitochondria were isolated from the different zones of the kidney. Solubilization of cortical mitochondria results in a rapid proteolysis of the glutaminase (Fig. 4). The 68 kDa peptide is completely degraded within 90 min, whereas disappearance of the 65 kDa peptide is nearly complete after 24 h. A similar series of reactions is evident when medullary mitochondria were treated with Triton X-100. However, the rate of disappearance of the initial glutaminase peptides is considerably reduced. In contrast, the pattern of immunoreactive peptides associated with papillary mitochondria is unaltered even 48 h after treatment with Triton X-100. However, the papillary mitochondria also contain a greater amount of the two lower-M_r peptides that are present in intestinal mitochondria. The 58 kDa peptide is particularly evident in the papillary mitochondria.

DISCUSSION

The rat renal glutaminase is asymmetrically associated with the internal surface of the mitochondrial inner membrane (Shapiro *et al.*, 1985). Many of the proteins contained in this membrane are initially translated on cytoplasmic ribosomes as precursor proteins (Douglas *et al.*, 1986). The precursors contain an *N*-terminal extension of 20 to 35 amino acids that serve to target the

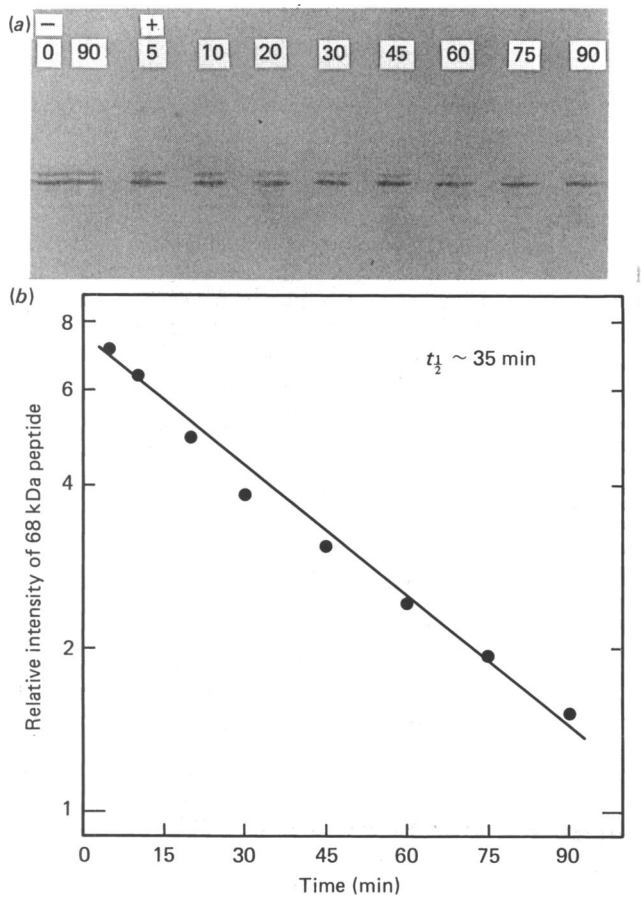

Fig. 3. Effect of Triton solubilization on the glutaminase contained in renal mitochondrial

(*a*) Aliquots of renal mitochondria were incubated at 4 °C for the indicated min in the absence (−) or presence (+) or 0.3% Triton X-100. The samples were then subjected to SDS/polyacrylamide-gel electrophoresis, transferred to nitrocellulose and immunostained. (*b*) The relative intensities of the 68 kDa peptide were determined by densitometric analysis and plotted against time of incubation at 4 °C.

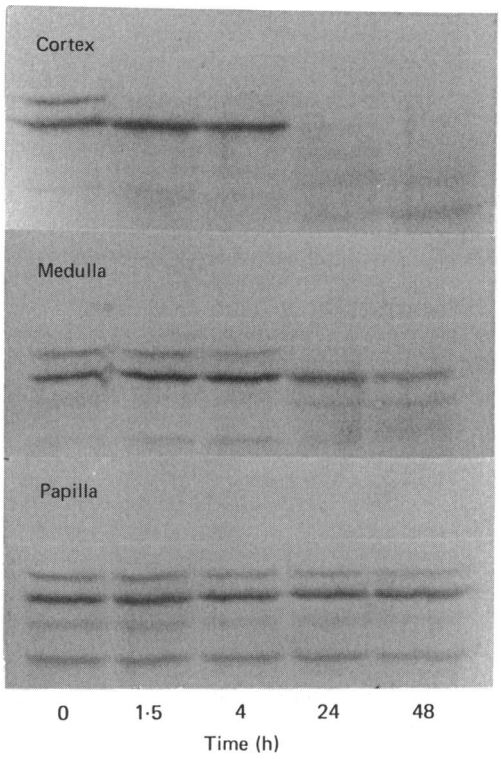

Fig. 4. Effect of Triton solubilization on the glutaminase contained in mitochondria prepared from the real cortex, medulla and papilla

Aliquots of the isolated mitochondria were incubated at 4 °C for the indicated times in the presence of 0.3% Triton X-100. The samples were then subjected to SDS/polyacrylamide-gel electrophoresis, transferred to nitrocellulose and immunostained.

protein for mitochondrial insertion. After translocation across the mitochondrial inner membrane, the *N*-terminal segment is removed by a Zn^{2+}-dependent proteinase. Thus the rat mitochondrial glutaminase is likely to have an M_r that is slightly less than that of its initial translation.

Translation *in vitro* of fractionated rat renal polyadenylated RNA yields a single 72 kDa peptide that is specifically precipitated with anti-glutaminase IgG (J. Tong, R. A. Shapiro & N. P. Curthoys, unpublished work). However, the glutaminase purified from rat brain contains two peptides of M_r 68000 and 65000 (Haser *et al.*, 1985). The apparent lack of stoichiometry observed when the brain glutaminase is stained with Coomassie Brilliant Blue initially suggested that the lower-M_r form may be an artefact caused by proteolysis of the solubilized enzyme. However, the immunoblot analyses presented here indicate that brain and renal mitochondria normally contain two glutaminase peptides that are produced *in vivo* from a common precursor.

In contrast with previous results, immunostaining indicates that the purified brain glutaminase and isolated brain mitochondria contain nearly equivalent amounts of the two glutaminase peptides. Since the antibodies were initially prepared against the purified rat renal glutaminase that lacks peptides of M_r greater than 59000, it is unlikely that the difference in apparent abundance is due to additional determinants that are present only on the 68 kDa peptide. The fact that the relative intensities of the two bands were unaltered when the concentration of protein was varied over a 10-fold range also indicates that neither band was preferentially saturated with the anti-glutaminase IgG. Furthermore, the IgGs were affinity-purified against the 65 kDa peptide and still produced nearly equivalent immunostaining of the two peptides. The use of affinity-purified antibodies greatly reduced the background staining and eliminated a few minor bands that were detectable with unfractionated IgG. These observations suggest that the 68 kDa peptide may result from a post-translational modification that both reduces its electrophoretic mobility and its reactivity towards Coomassie Brilliant Blue.

Previous analysis had indicated that the 68 and 65 kDa peptides were structurally related (Haser *et al.*, 1985). However, the greater sensitivity of the immunostaining procedure made feasible the identification of a greater number of proteolytic fragments. Even with the increased resolution, the patterns of peptide fragments

produced on partial digestion of the 68 and 65 kDa peptides with *S. aureus* V8 proteinase were identical. Thus the initial hydrolytic reaction must remove the terminal segment of the 68 kDa peptide that contains its unique sequence of amino acids or the moiety added by post-translational modification. In contrast, chymotrypsin must preferentially cleave at the opposite end of the glutaminase peptides. As a result, the larger fragments produced from the two peptides retain their apparent difference in M_r, and only the smaller fragments produce an identical pattern. Thus the observed patterns support the conclusion that the 68 and 65 kDa peptides contain large segments of identical amino acid sequence. The observations that the pattern of immunoreactive peptides observed in intact brain mitochondria is unaltered after solubilization and is identical with the pattern observed in the purified glutaminase indicates that the two structurally related peptides are components of the native enzyme.

Intact renal mitochondria also contain nearly equivalent amounts of the 68 and 65 kDa immunoreactive glutaminase peptides. Identical patterns were also observed when intact renal mitochondria were isolated from rats that were made either acutely or chronically acidotic. Thus a shift in the relative abundance of the two peptides apparently does not contribute to the increase in glutaminase activity that occurs during metabolic acidosis. Therefore we have initiated a study of the biosynthesis and processing of the mitochondrial glutaminase in order to characterize further the difference between the two peptides and to determine their functional significance.

Degradation of the glutaminase peptides occurs only after the renal mitochondria are solubilized with Triton X-100. Therefore the responsible endogenous proteinase(s) are probably not contained within the mitochondrial matrix. Renal mitochondria isolated by differential centrifugation are contaminated with lysosomes and brush-border membranes, both of which contain significant amounts of proteinases and aminopeptidases. A rapid proteolysis of the glutaminase occurs only in solubilized mitochondria isolated from the renal cortex and the small intestine (results not shown). This observation suggests that enzyme(s) associated with the brush-border membrane may be responsible for the degradation of the native glutaminase. The conversion of the 68 kDa peptide into the 65 kDa form is complete within 3–4 h after solubilization of the renal mitochondria. This observation explains the inability to detect the larger form of the glutaminase in direct immunoprecipitates of solubilized renal homogenates (Haser *et al.*, 1985). The subsequent degradation of the 65 kDa peptide occurs with slower kinetics. Thus the overall process may involve more than one enzyme, or the sequential cleavage may occur at sites that exhibit different reactivity towards the same proteinase.

The solubilized liver isoenzyme of glutaminase is quite labile and has only been partially purified (Patel & McGivan, 1984). The enrichment of the enzyme correlated with the purification of a 74 kDa peptide. However, in a recent abstract, Heini *et al.* (1986) report the development of an improved procedure for the isolation of the liver glutaminase. The prominent peptide contained in their preparation has an M_r of 59 500. Therefore, the 58 kDa immunoreactive peptide observed in hepatic mitochondria may constitute the liver form of glutaminase. If so, the liver and kidney glutaminases may be evolutionally related and have retained common immunological determinants. Thus the antibodies that were raised against the purified renal enzyme may prove useful in efforts to purify further the hepatic isoenzyme. The degradation of the renal glutaminase results in formation of a 61 kDa intermediate, but it does not generate a 58 kDa peptide. Thus the 61 kDa peptide that is observed in renal papillary and intestinal mitochondria may be derived from the more abundant 68 and 65 kDa peptides. However, the observed 58 kDa peptide may indicate that cells within the two tissues express significant amounts of the hepatic glutaminase.

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