Regulation of Renal Ammoniagenesis

SUBCELLULAR LOCALIZATION OF RAT KIDNEY GLUTAMINASE ISOENZYMES*

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NORMAN P. CURTHOYS‡ and ROBERT F. WEISS

From the Department of Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

SUMMARY

Rat kidney contains two distinct glutaminase isoenzymes; one of which is phosphate-dependent, the other is phosphate-independent but is strongly activated by maleate. The phosphate-dependent glutaminase is contained within mitochondria. Its activity cofractionates with mitochondrial marker activities during differential centrifugation and on sucrose gradients using both isopycnic and sedimentation velocity techniques. Incubation of mitochondria with borate buffer results in increased phosphate-dependent glutaminase, cytochrome oxidase, and malate dehydrogenase activities, indicating that this glutaminase activity may be latent. Sub-mitochondrial fractionation by digitonin-Lubrol treatment in the presence of borate indicated that this glutaminase isoenzyme is contained in the inner mitochondrial membrane. This conclusion was confirmed by a swell-shrink, sonication procedure carried out in the absence of borate. Only 25 to 35% of the phosphate-independent glutaminase was associated with the mitochondrial fraction obtained by differential centrifugation; the remaining activity was pelleted by subsequent centrifugation at 40,000 × g for 30 min (heavy microsomes). The phosphate-independent glutaminase in both fractions banded during isopycnic centrifugation with a mean density (1.166) similar to that of light mitochondria. However, the results of sedimentation velocity centrifugation studies showed that this isoenzyme was contained in an organelle distinct from mitochondria. The phosphate-independent glutaminase did not cofractionate with marker activities for lysosomes, microsomes, or plasma membranes during differential centrifugation or isopycnic gradient analysis and may be contained in some as yet unidentified organelle.

Increased renal synthesis of ammonia in response to metabolic acidosis is an essential component of the kidney's mechanism for maintaining acid-base balance (1, 2). The time course and magnitude of this response has been well characterized but little is known about its regulation (3). The primary source of ammonia synthesized by the kidney is the amide and amine nitrogen of glutamine which is extracted from the plasma (4) and glutaminase catalyzes the first reaction in what is thought to be the primary pathway for renal ammoniagenesis (5). Regulation of this response could largely be attributed to the properties of the enzyme which catalyzes this reaction. The fact that renal glutaminase activity is particulate introduces an additional level of regulation that its enzymatic properties may be altered by its position within the membrane or its accessibility to substrates may be limited by a permeability barrier.

Klingman and Handler (6) suggested that the phosphate-activated glutaminase from hog kidney was contained in mitochondria and showed that the addition of borate stabilized this activity towards extraction with organic solvents. Subsequently, procedures have been developed which make use of the effect of borate on this activity and which yield highly purified preparations of this enzyme from hog kidney (7) and from rat kidney (8). In addition to the phosphate-dependent glutaminase, Katunuma (9) has reported the occurrence and the separation of a second glutaminase isoenzyme in rat kidney which is not affected by phosphate but is strongly activated by maleate. This isoenzyme is also particulate and Katunuma suggested that both isoenzymes were recovered largely within the mitochondrial fraction. Using a highly sensitive assay procedure, Curthoys and Lowry (10) have determined the activities of the two glutaminase isoenzymes in individually dissected populations of the various cell types contained in rat kidney. The ratio of phosphate-dependent to phosphate-independent glutaminase activities varied from 0.05 in proximal straight tubule cells to 50 in proximal convoluted tubule cells. Therefore, if both isoenzymes are mitochondrial, there must be considerable variation in glutaminase composition of mitochondria contained in different tubular structures. We have undertaken a detailed characterization of the subcellular localization of the rat kidney glutaminase isoenzymes in an attempt to gain some knowledge basic to the understanding of the role of these isoenzymes in the regulation of renal ammoniagenesis.

EXPERIMENTAL PROCEDURE

Materials

White male rats which weighed between 300 and 400 g were obtained from Zivic-Miller and were maintained on Purina rat chow.
Glutamic dehydrogenase in 50% glycerol was obtained from Boehringer and Sons. Digitonin and cytochrome c were products of Calbiochem. All other biochemicals were purchased from Sigma Chemical Co.

Methods

Differential Fractionation—Rats were decapitated and kidneys were excised immediately. The tissue was trimmed of fat, weighed, and homogenized with 5 passes of a Potter-Elvehjem homogenizer in 8 volumes of buffer containing 0.25 M sucrose, 20 mM N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid, and 0.2 mM EDTA, pH 7.5. The crude homogenate was then fractionated by differential centrifugation using a modification of the procedure of Hogeboom et al. (11). All steps were performed at 3°C. The crude homogenate was first centrifuged at 60,000 × g for 5 min. The pellet was resuspended with 2 volumes of homogenate buffer and recentrifuged at 80,000 × g for 1 hour to separate the light microsomes (pellet) and matrix proteins (supernatant). All fractions were resuspended with the initial homogenate buffer.

Sucrose Gradients—All sucrose solutions were prepared in 20 mM N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid 0.2 mM EDTA buffer, pH 7.5. Isopycnic centrifugation was performed by layering 0.5 ml of either the mitochondrial or the heavy microsomal fraction on top of a 28-ml linear sucrose gradient (32.5 to 47.5%). The gradients were then centrifuged in an SW 25.1 rotor at 25,000 rpm for 5½ hours. Centrifugation for longer periods of time confirmed that this was sufficient for the organelles to reach equilibrium. For the sedimentation velocity experiments, centrifuge tubes were filled with 23 ml of a linear sucrose gradient (32.5 to 47.5%) layered on top of a 5-ml cushion of 47.5% sucrose. They were then centrifuged at 144,000 × g for 1 hour to separate the light microsomes (pellet) and matrix proteins (supernatant). All fractions were resuspended with the initial homogenate buffer.

RESULTS

Enzyme Assays—Glutaminase isoenzymes were assayed by the procedure of Curthoys and Lowry (10), except that the volume and the concentration of some of the substrates were increased to extend the range of linearity of the spectrophotometric assay. The initial incubation mixture for the phosphate-dependent glutaminase contained 0.15 M potassium phosphate, 20 mM glutamine, 50 mM Tris-Cl, and 0.2 mM EDTA, pH 8.6. The corresponding incubation mixture for the phosphate-independent glutaminase contained 60 mM maleate, 20 mM glutamine, and 0.2 mM EDTA, pH 6.6. For either assay, 100 μl of the appropriate reagent were pipetted onto a citrate-acetate (14) test tube (10 × 75 mm) and incubated at 37°C. The reaction was started by adding 1 to 10 μl of enzyme solution and after a fixed period of time (10 to 30 min) the reaction was stopped with 10 μl of 3 or 1 N HCl (phosphate-dependent or -independent glutaminase assays, respectively). 1 μl of a freshly prepared solution containing 2 mM DPN, 0.25 mM ADP, 0.03% H2O2, 100 μg of glutamic dehydrogenase, and either 80 or 40 mM Tris-Cl, pH 9.4 (dependent or independent, respectively) was added.

For the swell-shrink sonication procedure, the crude mitochondrial preparation was diluted with 5 volumes of 10 mM Tris-phosphate, pH 7.5. After incubating at 3°C for 5 min, 2 volumes of 1.8 M sucrose containing 2 mM ATP and 2 mM MgSO4 were added. An immediately freeze-dried phosphatase (16) were assayed as described previously except that inorganic phosphate was determined by the method of Lowry and Lopez (17). Acid phosphatase was assayed using p-nitrophenylphosphate (18), but because of the large amounts of alkaline phosphate in kidney tissue, the reaction was stopped by 6 N KOH and the absorbance at 410 nm was determined immediately. β-Glucuronidase was assayed by the procedure of Gianetto and de Duve (19). All enzyme activities in this manuscript are reported in terms of micromoles of product min⁻¹ ml⁻¹.

Differential Centrifugation—The various fractions obtained by differential centrifugation of a crude homogenate were assayed for glutaminase activity and for various subcellular marker enzymes (Table I). The phosphate-dependent glutaminase activity is recovered primarily in the mitochondrial fraction. The close correlation between the per cent recoveries for the phosphate-dependent glutaminase and for cytochrome oxidase in the various fractions supports the idea that this isoenzyme is contained within mitochondria. The mitochondrial fraction is also enriched for acid phosphatase and β-glucuronidase activities but to a much lesser extent. In contrast, only one-third of the phosphate-independent glutaminase was recovered in the mitochondrial fraction; the remaining activity was found almost exclusively in the heavy microsomes. None of the marker enzyme activities were sufficiently specific such that less than 5% of the activity of one isoenzyme was detectable in the assay for the second isoenzyme.
**TABLE I**

Enzyme fractionation by differential centrifugation

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Homogenate</th>
<th>Nuclear</th>
<th>Mitochondria</th>
<th>Heavy microsomes</th>
<th>Light microsomes</th>
<th>Cytosol</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDG</td>
<td>44.0</td>
<td>13.4</td>
<td>34.7</td>
<td>2.8</td>
<td>0.1</td>
<td>0.0</td>
<td>51.0</td>
</tr>
<tr>
<td>PIG</td>
<td>34.8</td>
<td>1.6</td>
<td>10.4</td>
<td>19.8</td>
<td>0.0</td>
<td>0.9</td>
<td>32.7</td>
</tr>
<tr>
<td>Cyt Ox</td>
<td>197</td>
<td>42.1</td>
<td>63</td>
<td>8.6</td>
<td>2.0</td>
<td>8.6</td>
<td>163</td>
</tr>
<tr>
<td>Acid P</td>
<td>53.5</td>
<td>12.0</td>
<td>17.5</td>
<td>10.9</td>
<td>4.0</td>
<td>12.7</td>
<td>57.1</td>
</tr>
<tr>
<td>β-GLU</td>
<td>1.18</td>
<td>0.20</td>
<td>0.43</td>
<td>0.12</td>
<td>0.03</td>
<td>0.24</td>
<td>1.02</td>
</tr>
<tr>
<td>γN</td>
<td>62.8</td>
<td>9.8</td>
<td>15.5</td>
<td>18.8</td>
<td>4.1</td>
<td>10.8</td>
<td>59.0</td>
</tr>
<tr>
<td>TCR</td>
<td>15.6</td>
<td>2.0</td>
<td>4.3</td>
<td>2.8</td>
<td>2.0</td>
<td>6.6</td>
<td>17.7</td>
</tr>
<tr>
<td>Glucose 6-phosphatase</td>
<td>18.8</td>
<td>3.9</td>
<td>2.8</td>
<td>8.7</td>
<td>0.5</td>
<td>0.0</td>
<td>15.9</td>
</tr>
</tbody>
</table>

Numbers in parentheses are per cent of total activity recovered.

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>µmoles min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDG P &amp; GLU</td>
<td>53.5</td>
</tr>
<tr>
<td>Acid P</td>
<td>12.0</td>
</tr>
<tr>
<td>β-GLU</td>
<td>17.5</td>
</tr>
<tr>
<td>γN</td>
<td>10.9</td>
</tr>
<tr>
<td>TCR</td>
<td>4.0</td>
</tr>
<tr>
<td>Glucose 6-phosphatase</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Abbreviations are: PDG, phosphate-dependent glutaminase; PIG, phosphate-independent glutaminase; Cyt Ox, cytochrome oxidase (mitochondria); Acid P, acid phosphatase (lysosome); β-GLU, β-glucuronidase (lysosome); γN, γ'-nucleotidase (plasma membrane); TCR, TPNH cytochrome c reductase (microsome); GeFase, glucose 6-phosphatase (microsome).

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**Fig. 1.** Isopycnic centrifugation of mitochondrial fraction obtained by differential centrifugation. Enzyme activities are expressed as micromoles ml⁻¹ min⁻¹. PDG, phosphate-dependent glutaminase; PIG, phosphate-independent glutaminase; Cyt Ox, cytochrome oxidase; RIDCR, rotenone-insensitive DPNH cytochrome c reductase.

**Fig. 2.** Sedimentation velocity centrifugation of mitochondrial fraction. Enzyme activities are expressed as micromoles ml⁻¹ min⁻¹. Abbreviations are as given in Fig. 1.

Activities distributed in a manner similar to the phosphate-independent glutaminase. The only other activities which showed the greatest per cent recovery in the heavy microsomal fraction were γ'-nucleotidase and glucose 6-phosphatase.

**Sucrose Gradients**—The results of isopycnic centrifugation of the mitochondrial fraction obtained by differential centrifugation are shown in Fig. 1. The phosphate-dependent glutaminase activity is found in two distinct peaks which have mean densities of 1.178 and 1.162. These values are in close agreement with the densities reported for heavy and light mitochondria from rat kidney (20). Both outer and inner mitochondrial membrane marker activities (rotenone-insensitive DPNH cytochrome c reductase and cytochrome oxidase, respectively) correlate well with the phosphate-dependent glutaminase activity. This observation provides strong evidence that this glutaminase isoenzyme is contained within mitochondria. Furthermore, the fact that the two cytochrome activities coincide suggests that the mitochondria are intact and that neither form is an artifact generated by damage to the outer membrane during homogenization. Acid phosphatase and β-glucuronidase activities exhibited distinct peaks and were spread over the entire gradient.

The phosphate-independent glutaminase contained in the mitochondrial fraction banded with a density very similar to that of the light mitochondria. Occasionally the phosphate-independent glutaminase activity was superimposable with the phosphate-dependent glutaminase activity contained in the light mitochondria. For this reason, attempts were made to separate the two activities by sedimentation velocity experiments. As shown in Fig. 2 centrifugation for 3 hours at 10,000 rpm was sufficient to band almost all of the mitochondria at the step increase in sucrose density at the bottom of the gradient. This is indicated by the cytochrome oxidase and phosphate-dependent glutaminase activities which showed coincident banding. But under these conditions the phosphate-independent glutaminase has just slightly entered the top of the gradient and is clearly separated from the mitochondrial markers. Therefore, none of the phosphate independent glutaminase is contained within the mitochondria.

The phosphate-independent glutaminase in the heavy microsomal fraction was also characterized by isopycnic centrifugation (Fig. 3). The activity again banded as a single symmetrical peak with a mean density of 1.166. This was the same mean density at which the phosphate-independent glutaminase had banded in mitochondrial gradients. This glutaminase activity did not coincide with the activity of any of the marker enzymes listed in Table I. TPNH cytochrome c reductase and glucose 6-phosphatase activities behaved similarly. Neither produced a symmetrical peak but instead both were skewed towards the top of
the gradient. Of the activities tested, 5'-nucleotidase most closely approximated the positioning of the phosphate-independent glutaminase. But, the two peaks were separated by two fractions and were skewed differently. When examined by sedimentation velocity centrifugation, the phosphate-independent glutaminase in the microsomal fraction entered the gradient at the same rate as it did during analysis of the mitochondrial fraction. It therefore appears that all of the phosphate-independent glutaminase is contained in a single organelle which is distinct from mitochondria, microsomes, lysosomes, or plasma membranes.

Submitochondrial Localization—Initial experiments with the digitonin-Lubrol fractionation procedure of Schnaitman and Greenawalt (12) indicated that the phosphate-dependent glutaminase was almost quantitatively recovered in the low speed pellet (inner membrane and matrix) following fractionation with glutaminase. But, subsequent treatment of the resuspended pellet with Lubrol resulted in loss of 70% of this glutaminase activity and following their separation by centrifugation, the inner membrane and matrix fractions contained less than 2% and 10% of the initial activity, respectively. Previous efforts to purify the phosphate-dependent glutaminase have shown that addition of borate buffer greatly stabilizes this activity towards solubilization with high concentration of detergents (8). Addition of borate to the resuspended low speed pellet from the digitonin treatment and subsequent incubation at room temperature resulted in a 50 to 100% increase in phosphate-dependent glutaminase activity and did not decrease the activities of any of the marker enzymes. Further fractionation with Lubrol in the presence of borate could then be accomplished with excellent recovery of the phosphate-dependent glutaminase.

The results of the digitonin-Lubrol fractionation following the procedure outlined under "Methods" are presented in Table II. Because of the increase in phosphate-dependent glutaminase, cytochrome oxidase, and malate dehydrogenase activities, the per cent activity of the various enzymes in the four submitochondrial fractions were calculated on the basis of total activity recovered. The validity of the fractionation procedure is attested to by the fact that between 50 and 80% of the recovered activities for each of the marker enzymes is found in its appropriate submitochondrial fraction. The distribution of the phosphate-dependent glutaminase in the various fractions supports the conclusion that this isoenzyme is associated with the inner mitochondrial membrane. Recovery of 75% of the phosphate-dependent glutaminase in the inner membrane fraction is in good agreement with the 79% recovery of cytochrome oxidase in the same fraction. The fact that 12% of the phosphate-dependent glutaminase is recovered in the matrix fraction versus less than 2% for the cytochrome oxidase suggests that it may be less firmly associated with the membrane and is partially solubilized by the fractionation procedure.

In order to establish that borate had no effect on the fractionation of the phosphate-dependent glutaminase, the procedure of Sottocassa (14) was also employed to determine its submitochondrial localization. As previously observed, the swell-shrink sonication procedure resulted in increased activity for cytochrome oxidase and malate dehydrogenase. But, in contrast, this procedure caused a 20% decrease in the phosphate-dependent glutaminase activity. As shown in Fig. 4, upon isopycnic centrifugation of the treated mitochondria, the phosphate-dependent glutaminase bands in a position coincident with the

![Graph showing GLUTAMINASE activity over DENSITY](image)

**Table II**  
**Digitonin-Lubrol subfractionation of mitochondria**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total enzyme activity (μmoles min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PDG¹</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>15.3</td>
</tr>
<tr>
<td>Digitonin Supernatant</td>
<td>3.4</td>
</tr>
<tr>
<td>Outer membrane</td>
<td>3.4 (12)</td>
</tr>
<tr>
<td>Intracristate</td>
<td>0.3 (1)</td>
</tr>
<tr>
<td>Digitonin pellet</td>
<td>11.7</td>
</tr>
<tr>
<td>Inner membrane</td>
<td>21.8 (75)</td>
</tr>
<tr>
<td>Matrix</td>
<td>3.5 (12)</td>
</tr>
<tr>
<td>Recovery</td>
<td>29.0 (100)</td>
</tr>
</tbody>
</table>

¹ Abbreviations are: PDG, phosphate-dependent glutaminase; RIDCR, rotenone-insensitive DPNH cytochrome c reductase; Aden K, adenylate kinase; Cyt Ox, cytochrome oxidase; Mal D, malate dehydrogenase.

Adenylate kinase activity appeared to be extremely labile to dilution involved in preparing the intracristate fraction (12). But, the per cent of initial activity recovered in digitonin supernatant versus digitonin pellet (32% versus 2%, respectively) as compared to recoveries obtained with a lower digitonin to protein ratio (9% versus 82%, respectively) suggests that the intracristate proteins are effectively released by this procedure.
cytochrome oxidase activity. The mean density of the peak fraction (1.19) was greater than that of intact mitochondria. The bulk of the malate dehydrogenase and rotenone-insensitive DPNH cytochrome c reductase activities were found at the top of the gradient. This confirms the finding that the phosphate-dependent glutaminase is contained within the inner membrane of the rat kidney mitochondria.

**Discussion**

The fact that the phosphate-independent glutaminase in both the mitochondrial and the heavy microsomal fractions showed identical behavior when subjected to both isopycnic and sedimentation velocity analysis supports the conclusion that this isoenzyme is contained within a single organelle which is distinct from mitochondria. The phosphate-independent glutaminase did not correlate with any of the marker enzyme activities tested in either the differential fractionation or isopycnic centrifugation experiments. This suggests that this glutaminase is not contained within the lysosomes, plasma membrane, or microsomes. Its behavior to differential centrifugation and its localization in the proximal straight tubule cells (10) indicate that it is not contained in kidney brush border membrane. It is possible that the phosphate-independent glutaminase is contained within some as yet uncharacterized organelle. Alternatively, its independent behavior may be due to an artifact introduced by our homogenization procedure. Although the isolated mitochondria appear to be intact, a considerable proportion of the total activity for some of the other organelle markers is recovered in the cytosol. Solubilization of the phosphate-independent glutaminase requires use of either high concentrations of detergents or proteolytic digestion (21). It is possible that this glutaminase is firmly associated with a large membrane fragment released by homogenization from one of the organelles considered. (Further characterization of the localization of the phosphate-independent glutaminase may require development of a gentler method for the disruption of kidney tissue.)

The experiments reported in this manuscript support the conclusion that the phosphate-dependent glutaminase is associated with the inner membrane of the rat kidney mitochondria. Use of the digitonin-Lubrol procedure as originally described by Schnaitman and Greenawalt (12) could have led to the conclusion, based on a 10% recovery of activity, that this enzyme was contained within the matrix space. When borate is included in the procedure, 75% of the phosphate-dependent glutaminase activity is associated with the inner membrane fraction. The close association of the phosphate-dependent glutaminase activity with cytochrome oxidase and its separation from the malate dehydrogenase and rotenone-insensitive DPNH cytochrome c reductase activities by isopycnic centrifugation of the swell-shrink sonicated mitochondria proves that the association of this glutaminase with the inner membrane is not an artifact due to the borate treatment.

Results of efforts to purify the phosphate-dependent glutaminase are consistent with the conclusion that this enzyme is particulate (8). Use of mild treatments such as sonication, freezing and thawing, and osmotic shock never resulted in solubilization of more than 10% of this glutaminase activity. After inclusion of borate buffer, the enzyme could be partially solubilized with 1% deoxycholate or by resuspending lyophilized mitochondria. The applicability of this latter method suggests that the phosphate-dependent glutaminase is probably not as firmly associated with the inner membrane as cytochrome oxidase (22). This is consistent with our finding that a small fraction of glutaminase is solubilized in both the digitonin-Lubrol and the swell-shrink sonication procedures (13% and 7%, respectively).

Like the same enzyme from hog kidney (7), the rat kidney phosphate-dependent glutaminase polymerizes in the presence of borate into a very high molecular weight aggregate (>10^7 daltons) (8). In the presence of borate, the highly purified enzyme is extremely stable, and has a specific activity which is 3-fold greater than the labeled low molecular weight form of the enzyme which is obtained by removal of borate. At present we have not established whether the increase in phosphate-dependent glutaminase observed upon treatment of the inner membrane-matrix fraction with borate is due to activation of the enzyme or merely indicates that the glutaminase displays latency. The incubation with borate also results in an increase in the cytochrome oxidase and malate dehydrogenase activities (35% and 100%, respectively). If the phosphate-dependent glutaminase is latent then it must be associated with the inner surface of the inner mitochondrial membrane. (We are currently performing a detailed comparison of the kinetic properties of the particulate, borate and borate-free forms of the purified phosphate-dependent glutaminase to determine which form more closely approximates the enzyme in its native environment.)

If the phosphate-dependent glutaminase is contained within the inner mitochondrial membrane, its accessibility to substrate is restricted by the permeability characteristics of this membrane. Regulation of mitochondrial uptake of glutamine could very effectively regulate this glutaminase activity and its contribution to renal ammoniagenesis. Alternatively, its position within the inner membrane might suggest that the phosphate-dependent glutaminase is a component of a mitochondrial transport system for glutamine. This is consistent with the study of Kovačič et al. (23) who have shown that rat kidney mitochondria take up glutamine much more rapidly than glutamate and that during glutamine uptake, glutamate is accumulated within the mitochondria.

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1 N. P. Curthoys, unpublished results.
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