The Intramitochondrial Location of the Glutaminase Isoenzymes of Pig Kidney

By M. CROMPTON,* J. D. McGIVAN and J. B. CHAPPELL

Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

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1. The glutaminase activity of pig kidney is located almost entirely in the cortex. 2. Pig renal cortex contains two glutaminases, one phosphate-dependent and one phosphate-independent. Both isoenzymes are localized exclusively in the mitochondria. 3. After sonication of the mitochondria, the phosphate-dependent isoenzyme is entirely soluble, whereas approximately half the phosphate-independent isoenzyme is associated with the membranes. 4. In intact mitochondria, the activities of both isoenzymes respond to changes in the pH of the intramitochondrial compartment. 5. It is concluded that both glutaminase isoenzymes are situated in the intramitochondrial compartment, and that the phosphate-independent glutaminase may be bound to the inside of the inner mitochondrial membrane.

Glutaminase (L-glutamine amidohydrolase, EC 3.5.1.2) catalyses the deamidation of glutamine to yield ammonium glutamate. Glutaminase has been purified from kidney, liver and brain of the rat, and is reported to exist as two isoenzymes in each tissue (Katunuma et al., 1967). One isoenzyme displays a requirement for a high concentration of phosphate when isolated (at least 75 mM-phosphate is needed for maximum activity), whereas the other isoenzyme does not show this dependency; accordingly, the isoenzymes are referred to as P₁-dependent glutaminase and P₁-independent glutaminase.

The properties of the two isoenzymes in each tissue are quite distinct. In rat kidney, the P₁-dependent glutaminase has a high $K_m$ for glutamine (40 mM), a pH optimum of 8.5 and is inhibited by glutamate. The P₁-independent isoenzyme has a relatively low $K_m$ for glutamine (4 mM), a pH optimum of 7.5 and is not inhibited by glutamate (Katunuma et al., 1967). This P₁-dependent glutaminase may be identified with the P₁-activated glutaminase studied by several groups of workers. The P₁-dependent glutaminases from the kidneys of the pig (Klingman & Handler, 1958) and the dog (Sayre & Roberts, 1958) are inhibited by glutamate (competitive with phosphate) and ammonia (competitive with glutamine). Thus the regulation of this enzyme is thought to be effected in a feedback manner by the products of the reaction (see Balagura-Baruch, 1971; Lund et al., 1970).

To understand more fully how renal ammonia production by the glutaminases is regulated, it is essential to know the subcellular compartments in which these isoenzymes function. Katunuma et al. (1967) have reported that both the P₁-dependent and P₁-independent glutaminases from the kidney, liver and brain of the rat are recovered largely in the mitochondrial fraction. Also, the P₁-dependent glutaminases of pig kidney (Klingman & Handler, 1958) and guinea-pig liver (Guha, 1961) appear to be localized entirely in the mitochondria. However, since the inner mitochondrial membrane exhibits selective permeability properties towards metabolites, and encloses an intramitochondrial pool of metabolites and enzymes quite distinct from the extramitochondrial, or cytoplasmic, pool, it is important to establish the precise location of the glutaminase isoenzymes in the mitochondria. This information should enable the metabolic changes in the environment of the isoenzymes, and the possible implications of metabolite transport on the glutaminase activities, to be discussed with more certainty. The present paper deals with the location of the P₁-dependent and P₁-independent glutaminases in pig kidney, and evidence is presented that both isoenzymes are situated in the intramitochondrial compartment, and that the P₁-independent glutaminase may be bound to the inner mitochondrial membrane.

Methods

Isolation of pig kidney mitochondria

Kidneys were collected from pigs about 30 min after slaughter and were immediately sliced and chilled in ice-cold extraction medium: 0.25 M-sucrose,
3 mM-Tris–HCl, 1 mM-EGTA [ethanedioxybis(ethyl-
amine)tetra-acetic acid], final pH 7.4. The mito-
chondria were prepared from the chilled tissue as
described by Chappell & Hansford (1969), and
suspended finally in extraction medium (approx.
100 mg of mitochondrial protein/ml). The respiratory
control ratio (Lardy & Copenhaver, 1954), used as
an index of the integrity of the mitochondria, was
measured with an oxygen electrode (Chappell, 1964)
in a reaction medium containing 100 mM-choline
chloride, 45 mM-Tris–HCl, 5 mM-potassium phos-
phate, bovine serum albumin (0.05%, w/v) and
0.5 mM-EDTA, final pH 7.4; the value of this ratio
varied between 4 and 6 with succinate as substrate.

Fractionation
Preparation of cytoplasmic fractions. Cytoplasmic
fraction refers to the supernatant remaining after
sedimentation of the mitochondria (see the para-
graph above). This supernatant was centrifuged once
more to sediment residual mitochondria.

Preparation of membrane and soluble fractions of
pig kidney mitochondria. Mitochondria (3.0 ml, con-
taining about 300 mg of protein) were diluted with
17 ml of 100 mM-Tris–HCl, pH 8.0, containing 20 mM-
Tris–borate, which prevents inactivation of the P1-
dependent glutaminase (Klingman & Handler, 1958),
and sonicated for 2 min in an MSE ultrasonic dis-
integrator no. 1, set at 1.7 A, with a 1 cm-diameter
probe; the sonication was carried out with the vessel
containing the mitochondrial suspension in ice and
with intermittent cooling periods to ensure that the
sonicate remained cold. The sonicate was centrifuged
for 1 h at 4°C and 66 000 g (r,,. 3.69 cm) and the
pellet (designated the membrane fraction) and the
supernatant (soluble fraction) were separated and
retained.

Enzyme assays and mitochondrial measurements
Assay of glutaminase. Glutaminase activity was
measured by continuously monitoring the formation
of NH3 with an ion-selective electrode (type GKN 33;
Electronic Instruments Ltd., Richmond, Surrey,
U.K.). The electrode was calibrated in each experi-
ment by the addition of a known amount of NH4Cl.

Assay of marker enzymes. Malate dehydrogenase
activity was measured as described by Ochoa (1955).
Glutamate dehydrogenase was assayed in the forward
direction as described by Streeker (1955). Rotonene-
insensitive NADH–cytochrome c reductase was
measured as described by King & Howard (1967),
with the addition of rotenone (1 μg/mg of mito-
chondrial protein). Cytochrome a1–a3 was deter-
mined in a dual-wavelength spectrophotometer by
measuring the extinction change at 605 nm minus
630 nm after the addition of dithionite to a sus-
pension of the fraction in 50 mM-Tris–HCl, pH 7.5,
containing rotenone (1 μg/ml).

Measurement of mitochondrial swelling. This was
done with a Hilger–Watts recording spectrophoto-
meter by measuring changes in E610 as described by

Determination of mitochondrial protein. Protein
was measured by the method of Gornall et al. (1949),
with bovine plasma albumin (Sigma Chemical Co.)
as standard.

Results
Location of glutaminase in pig kidney
Table 1 shows that glutaminase was recovered only
in the mitochondrial fraction. No glutaminase was
detected in the cytoplasmic fractions from either
cortex or medulla.

The specific glutaminase activities of mitochondria
isolated from cortex and from medulla are presented
in line (b). The highest specific activity is in the
mitochondria from cortex. The cortex also contains
the highest concentrations of mitochondria (line c).
Hence, the glutaminase activity per g wet wt. of tissue
(line a) is very much higher in the cortex than in the
medulla. The low activity recovered in the medulla
is located in the outer zone, and may have resulted
from a small contamination of the medulla samples
by pieces of cortex. Experiments were also carried
out to assess the distribution of glutaminase through-
out the cortex. The activity was measured in mito-
chondria prepared from different parts of the cortex,
i.e. the outer, middle and inner regions. Approxi-
ately the same specific glutaminase activity (i.e.
2 μmol of NH3/min per g fresh wt. at pH 7.5; 6 μmol
of NH3/min per g fresh wt. at pH 8.5) was detected in
each of the three regions, indicating that glutamin-
ase is quite uniformly distributed throughout the
cortex. The pH values, 7.5 and 8.5, are close to the
pH optima of the glutaminase isoenzymes (Katunuma
et al., 1967), and the purpose of assaying at these pH
values was to discover whether the separation of
kidney tissue into the various regions of cortex and
medulla produced any separation of the two iso-
enzymes. The results show approximately the same
ratio of activities at pH 7.5 and pH 8.5 in the different
regions of cortex and medulla, which indicates a
similar gross distribution of the isoenzymes in pig
kidney.

Glutaminase activity of the membrane and soluble
fractions of sonicated pig renal-cortex mitochondria
Pig renal-cortex mitochondria were separated into
membrane and soluble fractions. The recoveries of
the glutaminases after sonication were determined
by measuring the activity of intact mitochondria in
RENAL GLUTAMINASE ISOENZYMES

Table 1. Glutaminase activity of the mitochondrial and cytoplasmic fractions from different regions of pig kidney

The reaction medium contained choline chloride (80 mM), Tris–HCl (40 mM), Tris–phosphate (30 mM), ADP (5 mM), KCl (50 µM) and glutamine (10 mM). The amount of tissue extract used corresponded to about 0.5 g of fresh kidney tissue. The final volume was 8.0 ml and the temperature was 25°C.

<table>
<thead>
<tr>
<th>Mitochondrial fraction</th>
<th>Medulla</th>
<th>Cytoplasmic fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cortex</td>
<td>Outer</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutaminase activity</td>
<td>7.5</td>
<td>8.5</td>
</tr>
<tr>
<td>(a) NH₃ produced (µmol/min per g fresh wt.)</td>
<td>2.0</td>
<td>6.2</td>
</tr>
<tr>
<td>(b) NH₃ produced (nmol/min per mg of mitochondrial protein)</td>
<td>109</td>
<td>327</td>
</tr>
<tr>
<td>(c) Mitochondrial protein (mg/g fresh wt.)</td>
<td>18.8</td>
<td>6.5</td>
</tr>
</tbody>
</table>

Table 2. Activity of marker enzymes in the membrane and soluble fractions of sonicated pig renal-cortex mitochondria

The assays were carried out at 25°C as described in the Methods section.

<table>
<thead>
<tr>
<th>Proportion of total activity recovered (%)</th>
<th>Malate dehydrogenase</th>
<th>Glutamate dehydrogenase</th>
<th>Cytochrome a+a₃</th>
<th>Rotenone-insensitive NADH–cytochrome c reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane fraction</td>
<td>4.4</td>
<td>4.7</td>
<td>85.4</td>
<td>82.8</td>
</tr>
<tr>
<td>Soluble fraction</td>
<td>94.2</td>
<td>96.3</td>
<td>4.1</td>
<td>8.6</td>
</tr>
</tbody>
</table>

30 mM-Tris–phosphate (which is sufficient to activate the P₇-dependent glutaminase maximally in intact mitochondria; Crompton & Chappell, 1973) and the activity of the total sonicate in 300 mM-Tris–phosphate; the recovery was 69% at pH 8.6 and 87% at pH 7.3.

These two fractions were assayed for glutaminase under various conditions designed to distinguish between the activities of the glutaminase isoenzymes. The purity of the fractions was estimated by assaying for certain marker enzymes (see Ernster & Kuylenstierna, 1970). Thus malate dehydrogenase and glutamate dehydrogenase were used as markers for the soluble fraction, cytochrome a+a₃ was used to detect the presence of the inner mitochondrial membrane, and rotenone-insensitive NADH–cytochrome c reductase was used as an indicator for the outer membrane. The separation of the marker enzymes by the fractionation procedure is given in Table 2. The soluble fraction contained 94–97% of the soluble marker activity and 4–9% of the membrane marker activity. The membrane fraction contained most of the inner-membrane (85%) and outer-membrane (83%) material, and 4–5% of the soluble marker activity, as judged by the criteria set out above.

Fig. 1 shows the glutaminase activity of the membrane and soluble fractions in the presence and the absence of added phosphate over a range of pH values. In the absence of phosphate, both fractions displayed maximum activity at about pH 7.5. In the presence of phosphate the activity of both fractions was increased, and maximum activity was observed at about pH 8.2–8.6. However, the inclusion of phosphate produced a much larger increase in the activity of the soluble fraction than for the membrane fraction. Specifically, the increases in glutaminase activity produced by the addition of phosphate to the soluble and membrane fractions were 5-fold and 0.25-fold respectively at pH 7.5, and 19-fold and 2.5-fold respectively at pH 8.4. Thus approx. 95% (calculated from values at pH 8.4) of the P₇-dependent activity was recovered in the soluble fraction of the mitochondrial sonicate. The 5% of total soluble...
activity remaining in the membrane fraction may be accounted for by contamination of membrane material by soluble protein, since about 4.5% of the total activity of glutamate dehydrogenase and malate dehydrogenase was also recovered in the membrane fraction (Table 2). If this contamination is taken into account, and the activity of the membrane fraction in the presence of phosphate is corrected for the presence of soluble activity (calculated to be 4.5%), then the corrected activity agrees reasonably well with the membrane activity in the absence of phosphate (Fig. 1, broken line). This indicates that the glutaminase activity associated with the membrane in sonicated mitochondria is solely Pi-independent. The Pi-dependent activity is entirely soluble and not bound to the membranes. Although the membrane activity is wholly Pi-independent, an equal amount of Pi-independent activity was also recovered in the soluble fraction. This cannot be attributed to membrane contamination of the soluble fraction, which was only about 4-9% as judged by the distribution of rotenone-insensitive NADH-cytochrome c reductase and cytochrome a+a3.

The degree to which phosphate activates the glutaminase activities of the membrane and soluble fractions of sonicated rat kidney mitochondria has been measured by McDermott & O'Donovan (1971). These workers observed that the addition of phosphate (170 mM) stimulated the soluble activity three-fold that of the membrane activity, which is consistent with the results reported here.

Fig. 1 shows that the glutaminase activity of pig renal-cortex mitochondria is largely Pi-dependent. If the values for the Pi-dependent and Pi-independent activities are calculated at pH 8.4 and 7.5 respectively, then about 95% of the total glutaminase activity is represented by the Pi-dependent form. This compares well with the value of 98% in whole pig kidney obtained by Katunuma et al. (1966).

Fig. 2 shows reciprocal plots (Lineweaver & Burk, 1934) for the glutaminase activity of the membrane and soluble fractions. Both fractions were assayed at pH 7.7. The \( K_m \) for glutamine of the soluble fraction in the presence of phosphate was approx. 5 mM. The \( K_m \) for glutamine of the membrane fraction with no phosphate added was approx. 0.4 mM. The reciprocal plot for the membrane activity in the presence of

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**Fig. 1. Glutaminase activity of the soluble (a) and membrane (b) fractions of sonicated pig renal-cortex mitochondria in the presence and absence of phosphate**

The reaction medium contained Tris–HCl (50 mM) and glutamine (10 mM), with and without tris–phosphate (300 mM). The amount of fraction used corresponded to about 5 mg of mitochondrial protein before sonication. The activities are expressed per mg of mitochondrial protein before sonication. The final volume was 8.0 ml and the temperature was 25°C. ○, Plus phosphate; ○, minus phosphate; △, glutaminase activity in the presence of phosphate corrected for the phosphate-dependent activity (see the text).
phosphate was not linear, since at high glutamine concentrations the curve diverged from the values obtained in the absence of phosphate. Presumably this was due to contamination of the membrane fraction by soluble activity with a higher \( K_m \) for glutamine. The \( K_m \) for the \( P_i \)-dependent activity (5 mM) is identical with the value reported for the \( P_i \)-dependent glutaminase purified from pig kidney by Klingman & Handler (1958) and from dog kidney by Sayre & Roberts (1958). The \( K_m \) values for glutamine of the \( P_i \)-dependent and \( P_i \)-independent glutaminase from rat kidney are 40 mM and 4 mM respectively (Katunuma et al., 1967), i.e. the \( K_m \) values are both about tenfold those of the corresponding constants for the pig kidney activities.

Fig. 3 shows the inhibition of the membrane and soluble glutaminase activities by glutamate. The soluble activity is inhibited markedly by glutamate, whereas the membrane activity is inhibited only slightly. Several groups of workers have shown that

Fig. 3. Inhibition by glutamate of the glutaminase activity of the membrane and soluble fraction of sonicated pig renal-cortex mitochondria

The reaction medium contained Tris–phosphate, pH 7.7 (50 mM), and glutamine (10 mM). The amount of fraction used was equivalent to about 5 mg of mitochondrial protein before sonication. Final volume, 8.0 ml; temperature, 25°C. *, Soluble glutaminase; o, membrane glutaminase.

Fig. 4. Inhibition by Bromocresol Purple of the glutaminase activity of the membrane and soluble fractions of sonicated pig renal-cortex mitochondria

The assay conditions were as described for Fig. 3. *, Soluble glutaminase; o, membrane glutaminase.
the purified P$_r$-dependent glutaminase from kidney tissue is inhibited by glutamate (e.g. Katunuma et al., 1967; Klingman & Handler, 1958), and the inhibition is competitive with phosphate (Sayre & Roberts, 1958). The P$_r$-independent glutaminase of kidney, however, is not inhibited by glutamate (Katunuma et al., 1967).

Fig. 4 shows the inhibition of the soluble and membrane glutaminase activities by Bromocresol Purple. It is evident that the soluble activity is much more sensitive to inhibition than the membrane activity. The inhibition of the purified P$_r$-dependent glutaminase from dog kidney by Bromocresol Purple and other phthalein dyes has been reported by Sayre & Roberts (1958). These authors showed that the inhibition is competitive with phosphate, and it is likely therefore that the difference in the degree of inhibition by Bromocresol Purple of the membrane and soluble activities results from their different requirements for phosphate.

Response of the glutaminase activities of pig renal-cortex mitochondria to pH changes in the intramitochondrial and extramitochondrial compartments

Because of the selectively permeable properties of the inner mitochondrial membrane the most important single question about the location of the glutaminase isoenzymes is whether they are located on the inside or the outside of the inner mitochondrial membrane. This question was approached by observing whether the activities of the two isoenzymes responded to changes in the pH of the intracellular or extra-mitochondrial compartment.

The glutaminase activity of intact mitochondria was measured in the presence of rotenone and antimycin to inhibit respiration. Under these conditions the oxidation of glutamate is prevented, and the glutamine that disappears can be accounted for entirely by the accumulation of glutamate in the external medium (Crompton & Chappell, 1973). Thus the only enzyme involved in this process is...
glutaminase. The glutaminase activity of respiration-inhibited mitochondria in the presence of the uncoupler carbonyl cyanide phenylhydrazone is shown as a function of pH in Fig. 5(a). The activities of both glutaminase isoenzymes, pH optima 7.6 (Pi-independent) and 8.4 (Pi-dependent), were detected. The experimental conditions (1 mm-glutamine and no added phosphate) were chosen to limit the Pi-dependent activity to a low value. The Pi-dependent activity observed may be attributed to the endogenous phosphate content of isolated mitochondria (about 5 nm: McGivan & Klingenberg, 1971).

The effect of changing the pH of the intramitochondrial compartment relative to the pH of the external medium was investigated by adding tris-nitrate to the reaction medium. The inner mitochondrial membrane is permeable to NO\textsubscript{3}\textsuperscript{-}, but impermeable to the tris cation. This is demonstrated in Fig. 6, where the rate of influx of various nitrate salts was followed by mitochondrial swelling in iso-osmotic nitrate solutions. Potassium nitrate enters rapidly in the presence of valinomycin, which permits rapid electrogenic movement of K\textsuperscript{+} across the inner mitochondrial membrane (Henderson et al., 1969). Ammonium nitrate enters rapidly only in the presence of the uncoupler, carbonyl cyanide phenylhydrazone, which allows rapid, electrogenic permeation of protons (Mitchell, 1965). Tris--nitrate, however, permeates very slowly. Thus an electrogenic influx of cations is accompanied by an influx of NO\textsubscript{3}\textsuperscript{-}, which indicates that NO\textsubscript{3}\textsuperscript{-} permeates the inner mitochondrial membrane electrogenically. Tris--nitrate influx is slow because the Tris cation permeates slowly. These ion movements are shown in Scheme 1.

Because of the large difference in the permeability of the inner membrane to the Tris cation and NO\textsubscript{3}\textsuperscript{-}, the addition of Tris--nitrate to the reaction medium would be expected to produce a diffusion potential (positive outside) across the inner mitochondrial membrane; the inclusion of carbonyl cyanide phenylhydrazone allows an influx of protons down the electrical gradient. That is, there is a net influx of HNO\textsubscript{3} and the matrix pH is decreased relative to the external pH.

Fig. 5(a) shows that the addition of Tris--nitrate to the reaction medium produces an apparent alkaline shift in the pH optima of both the Pi-independent and Pi-dependent activities in intact mitochondria. However, Tris--nitrate does not change the pH optima of either the Pi-independent or the Pi-dependent activity in broken mitochondria (Fig. 5b), although

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**Scheme 1. Permeation of nitrate salts through the inner mitochondrial membrane**

Valinomycin (V) and carbonyl cyanide phenylhydrazone (CCP) catalyse the permeation of K\textsuperscript{+} and H\textsuperscript{+} respectively.
the addition of Tris–nitrate does seem partially to inhibit the \( P_i \)-independent activity (pH optimum 7.6). These results may be interpreted to indicate that both the \( P_i \)-independent and \( P_i \)-dependent glutaminase activities are sensitive to pH changes occurring in the mitochondrial matrix, and that both activities are located in the intramitochondrial compartment.

**Discussion**

Pig renal-cortex mitochondria contain two types of glutaminase activity. On the basis of their requirements for phosphate, pH optima, \( K_m \) values for glutamine, and degree of inhibition by glutamate and Bromocresol Purple, the two types of activity may be identified with the \( P_i \)-dependent and \( P_i \)-independent glutaminase isoenzymes purified from kidney tissue by other workers.

After sonication of pig kidney mitochondria, the \( P_i \)-dependent glutaminase is recovered almost entirely in the soluble fraction. Further, the activity of this isoenzyme in intact mitochondria responds to variations in the pH of the intramitochondrial compartment. It is therefore proposed that the \( P_i \)-dependent glutaminase in pig kidney is a soluble enzyme localized in the matrix compartment of the mitochondrion.

The activity of the \( P_i \)-independent glutaminase in intact mitochondria is dependent on the pH of the matrix compartment, which indicates that this isoenzyme also is situated in the intramitochondrial compartment. The evidence relating to the precise location of the \( P_i \)-independent glutaminase within the matrix compartment is difficult to interpret, since approximately equal activities were recovered in the membrane and soluble fraction after sonication. This may mean that in unbroken mitochondria the enzyme exists in two states, i.e. partially bound to the inner membrane and partially free in the matrix. However, in view of the degree of mitochondrial disruption brought about during sonication, it is possible that the \( P_i \)-dependent glutaminase is wholly bound to the inner membrane but becomes partially dissociated from the membrane during sonication. The reverse situation, that this isoenzyme is entirely soluble in intact mitochondria and binds to the membrane as a result of sonication, seems less likely. On the basis of these considerations it is tentatively suggested that the \( P_i \)-independent glutaminase may be entirely bound to the inside of the inner mitochondrial membrane in vivo.

An important point, which merits re-emphasis, is that since both isoenzymes are situated in the inner mitochondrial compartment, the glutaminase activity in vivo will be subject to any restrictions imposed by the permeability of the inner mitochondrial membrane towards the substrate and products of the glutaminase reaction, and will be influenced by changes in the intramitochondrial, rather than cytoplasmic, concentrations of regulatory metabolites. The activity of the glutaminase isoenzymes in relation to the permeation of glutamine and glutamate across the inner membrane is the subject of the following paper (Crompton & Chappell, 1973).

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**References**


Chappell, J. B. (1964) *Biochem. J.* 90, 225–237


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