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Comparison of the phosphate-dependent glutaminase obtained from rat brain and kidney

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A phosphate-dependent glutaminase was purified 1200-fold from rat brain. In the absence of a polyvalent anion, the glutaminase exists as an inactive protomer which has an estimated M_r of 126000. The addition of 100mM-phosphate causes maximal activation and a dimerization (M_r 249000) of the glutaminase. The phosphate activation is sigmoidal, with a $K_{0.5}$ of 25mM and a Hill coefficient (h) of 1.5. Glutamate inhibition is competitive with respect to glutamine and is decreased by increasing the concentration of phosphate. Phosphate also decreases the K_m for glutamine. The purified glutaminase contains a predominant peptide (M_r 65000) and a minor peptide (M_r 68000) that are present in an approximate ratio of 4:1 respectively. The glutaminase immunoprecipitated from freshly solubilized brain tissue or from synaptosomal and non-synaptosomal brain mitochondria contains the same distribution of the two peptides. In contrast, the glutaminase purified from rat kidney contains five to seven peptides that range in M_r value from 59000 to 48000, and immunoprecipitates derived from freshly solubilized renal tissue contain only the M_r-65000 peptide. Partial proteolysis and size fractionation of the three immunoprecipitated peptides indicate that they are structurally related. The series of peptides characteristic of the purified renal glutaminase is generated on storage of the solubilized extract of kidney tissue. The glutaminase contained in the solubilized brain extract is not degraded unless a renal extract is added. Thus the difference in the pattern of peptides associated with the two purified enzymes is due to an endogenous renal proteinase that is not present in brain.

In mammals, the catabolism of glutamine occurs primarily in the small intestine, brain, kidney and liver (Haussinger & Sies, 1984), where it 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^+ ions and is activated by phosphate (Patel & McGivan, 1984). In contrast, the glutaminases isolated from the three other tissues are immunologically related (Curthoys *et al.*, 1976*a*). In the absence of a polyvalent anion, this type of glutaminase exists as an inactive protomer. The addition of phosphate or other polyvalent anion results in activation and dimerization of the glutaminase (Godfrey *et al.*, 1977). The subsequent addition of borate causes this form of the glutaminase to undergo extensive polymerization ($M_r > 10^7$).

The phosphate-dependent glutaminase has been purified from pig kidney (Kvamme *et al.*, 1970) and brain (Svenneby *et al.*, 1973), from rat kidney (Curthoys *et al.*, 1976*b*) and from cow brain (Chiu & Boeker, 1979). The purified enzymes exhibit significant differences in their kinetic properties and in their subunit M_r. With the glutaminase from pig kidney, inhibition by glutamate is non-competitive with glutamine but competitive with phosphate (Tveit *et al.*, 1970). In contrast, the glutaminases from rat kidney (Shapiro *et al.*, 1982) and from cow brain (Chiu & Boeker, 1979) exhibit a sigmoidal activation by phosphate and an inhibition by glutamate that is competitive with respect to glutamine. The glutaminase from pig kidney contains two peptides, of M_r 61000 and 53000 (Olsen *et al.*, 1973). The glutaminase from pig brain has been reported to contain a single polypeptide of M_r 64000 (Svenneby *et al.*, 1973) or 73000 (Nimmo & Tipton, 1980). In contrast, the glutaminase from rat kidney contains five to seven structurally related peptides (Clark & Curthoys,

1979). All of the peptides associated with the purified rat renal glutaminase bind the glutamine affinity labels, L-2-amino-5-chloro-4-oxo[5-^{14}C]-pentanoic acid (Shapiro *et al.*, 1978) and 6-diazo-5-oxo-L-[6-^{14}C]norleucine (Clark *et al.*, 1982), which stoichiometrically inactivate the glutaminase. The glutaminase, immunoprecipitated from freshly solubilized renal mitochondria, is composed primarily of a single peptide whose M_r is greater than that of any of the peptides contained in the purified glutaminase (Clark & Curthoys, 1979). When the solubilized mitochondria were stored at 4°C, full glutaminase activity was retained, but the series of smaller peptides were generated. These results indicate that the pattern of peptides contained in the purified rat renal glutaminase is due to its sensitivity to an endogenous proteinase that causes a partial and non-inactivating proteolysis of the native enzyme.

In an attempt to characterize its properties further, the phosphate-dependent glutaminase was purified from rat brain. This paper compares the physical and kinetic properties of the glutaminase purified from rat brain and kidney. It also compares the subunit composition of the purified glutaminases with that of the enzyme immunoprecipitated from solubilized homogenates of the two tissues.

Experimental

Materials

White male Sprague–Dawley rats (200–300g) were obtained from Zivic–Miller (Allison Park, PA, U.S.A.) and were maintained on Purina Rat Chow. White female New Zealand rabbits (3–4kg) were purchased from Hill Top Farms (Scotsdale, PA, U.S.A.) and complete Freund's adjuvant was obtained from GIBCO. L-[U-^{14}C]Glutamine (260Ci/mol) was purchased from New England Nuclear. DEAE Affi-Gel Blue, acrylamide and the standard proteins for the characterization of the subunit M_r were obtained from Bio-Rad. The enzymes β-galactosidase, catalase, fumarase, alcohol dehydrogenase, glutamate–oxaloacetate transaminase and glutamate dehydrogenase were purchased from Boehringer.

Enzyme assays

Phosphate-dependent glutaminase was assayed under the standard conditions developed to determine this activity in homogenates of rat renal tissue (Curthoys & Lowry, 1973). For the purification procedure and the M_r characterization, glutaminase activity was measured by using glutamate dehydrogenase to quantify the amount of glutamate formed (Curthoys & Weiss, 1974). The kinetics of the brain glutaminase were determined by using a radioactive assay that employs Dowex-1 columns to separate [^{14}C]glutamine and [^{14}C]-glutamate (Shapiro *et al.*, 1982). The enzymes used for M_r analysis were assayed by published procedures: β-galactosidase (Marchesi *et al.*, 1969), catalase (Beers & Sizer, 1952), fumarase (Hill & Bradshaw, 1969), alcohol dehydrogenase (Racker, 1955) and glutamate–oxaloacetate transaminase (Karmen, 1955). Protein was determined by the procedure of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Purification of brain glutaminase

The brain tissue from 50 rats was homogenized in 20vol. of 225mM-mannitol/75mM-sucrose/0.2mM-EDTA/5mM-Tris/HCl (MSET) buffer, pH7.4, and centrifuged at 25000*g* for 15min. The resulting pellet was resuspended in 2vol. of MSET buffer and diluted with an equal volume of MSET buffer containing sufficient digitonin to yield a final ratio of 0.15mg of digitonin/mg of protein. The mixture was stirred slowly for 30min at 4°C and then centrifuged at 25000*g* for 15min. The supernatant was discarded and the pellet was resuspended by homogenizing in 2vol. of MSET buffer. The suspension was re-centrifuged and the resulting pellet was again resuspended by homogenizing in 2vol. of MSET buffer. To this suspension was added 0.1vol. of buffer containing 0.1M-sodium tetraborate, 1M-potassium phosphate and 1M-potassium pyrophosphate, pH8.9. After standing at room temperature for 30min, the sample was vigorously homogenized, frozen in a solid-CO_2/acetone bath and freeze-dried overnight. The product was resuspended in 500ml of water and rehomogenized. The suspension was centrifuged at 25000*g* for 30min. The resulting pellet was resuspended with 200ml of 10mM-sodium tetraborate/0.1M-potassium phosphate/0.1M-potassium pyrophosphate (BPP) buffer, pH8.9, and then re-centrifuged at 25000*g* for 30min. The two supernatants were combined, made 30% saturated with $(NH_4)_2SO_4$ (16.4g/100ml) and incubated at 4°C for 30min. The sample was then centrifuged at 12000*g* for 15min. The precipitate was resuspended in approx. 40ml of 10mM-Tris acetate/10mM-potassium pyrophosphate/1mM-dithiothreitol (TPD) buffer, pH8.6, and the sample was dialysed for 4h against two 2-litre changes of TPD buffer. The sample was then applied to a 4cm × 100cm Sepharose 4B column that had been pre-equilibrated with TPD buffer. The brain glutaminase was eluted at a volume approx. 2.5 times the void volume of the Sepharose 4B column. The fractions containing glutaminase activity were pooled, made 30% saturated with $(NH_4)_2SO_4$ (16.4g/100ml), and incubated at 4°C for 30min. The sample was then centrifuged at 12000*g* for 30min and the

precipitate was resuspended in approx. 5ml of TPD buffer. The sample was dialysed for 90min against two 1-litre changes of TPD buffer. A volume of 20% Triton X-100 sufficient to yield a final concentration of 0.5% was added to the dialysed sample, which was then incubated at 4°C for 5min. Then 0.1 vol. of 0.1M-sodium tetraborate/1M-potassium phosphate/1M-potassium pyrophosphate buffer, pH8.9, was added to the sample. This mixture was incubated at 4°C for 1h and then applied to a 2.5cm × 70cm Sepharose 4B column that had been pre-equilibrated with BPP buffer. The brain glutaminase was eluted in the void volume of the second Sepharose 4B column. The fractions containing glutaminase activity were pooled, made 60% saturated with $(NH_4)_2SO_4$ (36.1g/ml), and the precipitate was collected as above. This material was resuspended in 1–2ml of BPP buffer, dialysed overnight against two 1-litre changes of BPP buffer, and then stored at 4°C.

M_r and kinetic analysis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed by the procedure of Laemmli (1970). The gels were stained for protein with 0.05% Coomassie Brilliant Blue in methanol/acetic acid/water (5:1:5, by vol.) and destained in 7.5% (v/v) acetic acid/5% (v/v) methanol. The Stokes radii of the purified glutaminase were determined by gel filtration on a Sephadex G-200 column (0.8cm × 100cm) equilibrated with either 10mM-Tris acetate, pH8.6, or 10mM-Tris acetate/100mM-potassium phosphate, pH8.6. The columns were run at a flow rate of 3ml/h and the elution data were plotted by the method of Siegel & Monty (1966). Sedimentation coefficients for the glutaminase in the presence and absence of phosphate were determined by sucrose-velocity-gradient centrifugation as described by Martin & Ames (1961). The gradients were centrifuged in an SW 50.1 rotor at 10°C for 15h at 85000*g*.

A rat brain was homogenized in 20vol. of MSET buffer and subjected to differential centrifugation and treatment with digitonin as described in the purification procedure. The solution was incubated at 4°C for 30min and then subjected to the swell–shrink sonication procedure that was used to prepare membrane-bound phosphate-dependent glutaminase from kidney (Shapiro *et al.*, 1982).

Preparation of anti-glutaminase IgG

Rabbit antiserum was prepared with the purified rat renal glutaminase as antigen (Curthoys *et al.*, 1976*a*); 1ml of the immune serum precipitated 20 units (i.e. 60 μg) of the glutaminase. Approx. 15ml of serum was dialysed against 20mM-Tris/HCl/28mM-NaCl/0.02% NaN_3 buffer, pH8.0, and applied to a 5cm × 15cm DEAE Affi-Gel Blue column pre-equilibrated with the same buffer. The eluent fractions containing IgG were combined and made 50% saturated with $(NH_4)_2SO_4$. The precipitated antibodies were resuspended and dialysed against 10mM-Hepes/150mM-NaCl buffer, pH7.4. The $F(ab)_2$ antibody fragments were prepared as described by Nisonoff *et al.* (1960) and purified by gel filtration on a 2cm × 50cm Bio-Gel P-200 column.

Triton X-100 solubilization of glutaminase

Glutaminase was solubilized from crude homogenates of various tissues or from isolated mitochondria with Triton X-100. The tissue was homogenized in 330mM-sucrose/20mM-Tris/HCl/1mM-EDTA buffer, pH7.4, and diluted to a volume 4 times the wet weight. Then 1vol. of 5% Triton X-100 was added and the suspension was incubated for 1h at 4°C. The samle was then centrifuged at 150000*g* for 30min, and the resulting supernatant contained the solubilized glutaminase. To isolate renal mitochondria, the initial homogenate was centrifuged at 600*g* for 10min, and the resulting pellet was resuspended in 2vol. of homogenate buffer and re-centrifuged. The combined supernatants were then centrifuged at 8000*g* for 10min to pellet the mitochondrial fraction. Brain tissue was excised and homogenized in 20vol. of homogenate buffer. The homogenate was centrifuged at 1900*g* for 5min and the resulting supernatant was centrifuged at 17000*g* for 10min. The final pellet, which contained total mitochondria, was resuspended to 2ml with homogenate buffer. Separation of synaptosomal and non-synaptosomal mitochondria was accomplished by the procedure of Booth & Clark (1978). The mitochondrial fractions were solubilized by dilution with an equal volume of 2% Triton X-100.

Immunoprecipitation of solubilized glutaminase

A sample containing 0.3unit of glutaminase activity was mixed with a portion of $F(ab)_2$ antibodies sufficient to immunoprecipitate 0.45unit of glutaminase activity. The mixture was made 150mM in NaCl and 10mM in Hepes buffer, pH7.4, and then incubated overnight at 4°C. Glutaminase activity was assayed before and after centrifugation at 12000*g* for 15min. The immunoprecipitate was washed twice by resuspension with 0.75ml of 10mM-Tris acetate buffer, pH8.6, and re-centrifuging. The final pellet was resuspended in 50 μl of sample buffer, heated at 100°C for 2min, and subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis by the procedure of Laemmli (1970).

Immunoprecipitation with IgG antibodies was performed by a slightly different procedure. The

enzyme/antibody mixture was diluted to 0.5ml with a buffer containing 330mM-sucrose, 20mM-Tris acetate, 150mM-NaCl, 0.2mM-EDTA and 1% Triton X-100, pH7.4. The sample was transferred to a 1.5ml Eppendorf Microfuge tube, underlayed with 0.5ml of 0.5M-sucrose and incubated at 4°C for 90min. The immune complex was then centrifuged through the 0.5M-sucrose at 12000*g* for 10min, and the immunoprecipitate was washed and analysed as described above.

The peptides isolated from immunoprecipitates of Triton X-100-solubilized renal and brain glutaminase were subjected to limited proteolytic digestion as described by Cleveland *et al.* (1977). The resulting profile of peptide fragments was detected with silver stain (Wray *et al.*, 1981).

Results

Renal and brain mitochondrial phosphate-dependent glutaminases undergo a reversible polymerization upon addition and removal of borate and phosphate anions. Successive gel filtration of the polymerized and unpolymerized forms of the enzyme has been used to purify the glutaminase solubilized from rat renal mitochondria (Curthoys *et al.*, 1976*b*). The direct application of this protocol proved inadequate for the purification of the glutaminase contained in rat brain. However, a reproducible procedure for the purification of this enzyme was developed by modifying the conditions and the sequence of steps. A summary of a typical purification is given in Table 1. The addition of borate and phosphate anions results in an increase in the glutaminase activity. This fact accounts for the increase in total activity that is associated with various steps in the purification. The overall procedure yields approx. 1mg of enzyme that has a specific activity of 250μmol/min per mg. This represents a 1200-fold increase in specific activity of the glutaminase compared with that observed in a crude homogenate of rat brain. The purified glutaminase has a specific activity that is similar to that of the glutaminase purified from rat kidney (Curthoys *et al.*, 1976*b*). Both enzymes are stable for several months when stored in BPP buffer at 4°C.

The purified rat renal glutaminase undergoes a phosphate-induced dimerization (Godfrey *et al.*, 1977) that is essential for activation of the enzyme (Morehouse & Curthoys, 1981). The effect of phosphate on the quaternary structure of the purified brain glutaminase was investigated by determining its Stokes radius and sedimentation coefficient. Both parameters were significantly increased by the addition of 100mM-potassium phosphate. The glutaminase is eluted from a Sephadex G-200 column equilibrated with 10mM-Tris acetate buffer, pH8.6, in a fraction equivalent to that of a protein with a Stokes radius of 52nm. When the column is equilibrated with buffer containing 100mM-potassium phosphate, the glutaminase activity is eluted in an earlier fraction that corresponds to a protein with a Stokes radius of 68nm. The glutaminase migrates in sucrose velocity gradients prepared in the absence and presence of 100mM-potassium phosphate with sedimentation coefficients of 5.8S and 8.8S respectively. If one assumes that the glutaminase has a normal partial specific volume of 0.73ml/g, these values can be used to calculate the M_r of the purified glutaminase (Siegel & Monty, 1966). The addition of phosphate increases the estimated M_r of the glutaminase from 126000 to 249000.

A partially purified mitochondrial membrane fraction was used to characterize the kinetic properties of the rat renal phosphate-dependent glutaminase (Shapiro *et al.*, 1982). A similar membrane fraction was prepared from rat brain and used to characterize the kinetic properties of the brain glutaminase. As shown in Fig. 1, the preparation exhibited no glutaminase activity in the absence of phosphate. Approx. 25mM-phosphate was required to produce half-maximal activation with 20mM-glutamine. A double-reciprocal plot of these data produced a curved line indicative of a sigmoidal activation profile. A replot of the data according to the Hill equation yielded a straight line with a slope of $h = 1.5$.

Table 1. *Summary of the purification of phosphate-dependent glutaminase from rat brain*

Step	Volume (ml)	Total protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min per mg)
Crude homogenate	1800	8640	1780	0.21
Digitonin-treated mitochondria*	250	5000	2600	0.52
Resuspended $(NH_4)_2SO_4$ pellet	40	520	650	1.25
First Sepharose 4B column*	6.6	131	680	5.2
Second Sepharose 4B column*	1.5	1.1	270	245

* Samples that contained 10mM-sodium tetraborate, 100mM-potassium phosphate and 100mM-potassium pyrophosphate.

In contrast, all of the gluamine-saturation profiles determined with the brain glutaminase were hyperbolic (Fig. 2). However, the K_m for glutamine was dependent on the phosphate concentration. Increasing the phosphate concentration from 10mM (Fig. 2*a*) to 150mM (Fig. 2*b*) decreased the K_m for glutamine from 14mM to 5mM. Glutamate is a competitive inhibitor with respect to glutamine at both low and high concentrations of phosphate. However, increasing the phosphate concentration increased the K_i for glutamate from 6mM to 45mM.

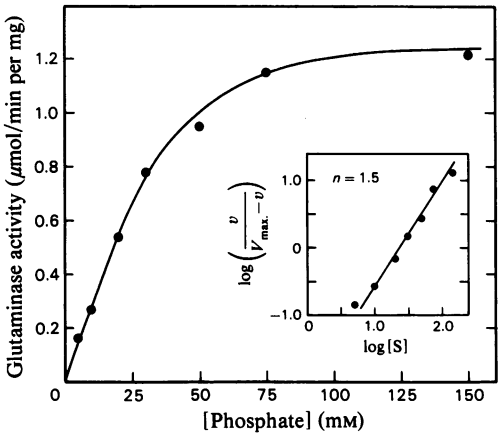

Fig. 1. *Phosphate-activation profile of mitochondrial membrane-bound glutaminase from rat brain*
The data are plotted as activity (v) versus phosphate concentration ([S]) determined in the presence of 20mM-glutamine. The insert illustrates the data replotted in the form of a Hill plot.

The phosphate-dependent glutaminases purified from rat renal and brain tissues were subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 3). The pattern of peptides associated with the two preparations of glutaminase differed significantly. The brain glutaminase contained two protein-staining bands with M_r values of 68000 and 65000, in an approximate ratio of 1:4 respectively (lane 1). The renal glutaminase contained five to seven protein-staining peptides that ranged in M_r from 59000 to 48000 (lane 4). The glutaminase was also immunoprecipitated from freshly prepared Triton X-100-solubilized extracts of brain and kidney tissue and subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 3). The protein-staining bands observed in the 53000- and 23000-M_r range are due to the heavy and light chains of the IgG molecule. The immunoprecipitate of brain glutaminase contained the 68000- and 65000-M_r peptides that are associated with the purified enzyme, and a third peptide, of M_r 63000 (lane 2). The third protein-staining band is likely to be a non-specific contaminant, since it also appeared in a control precipitate obtained from a Triton X-100 supernatant of a brain homogenate that was incubated in the absence of anti-glutaminase IgG (lane 3). The immunoprecipitated renal glutaminase contained

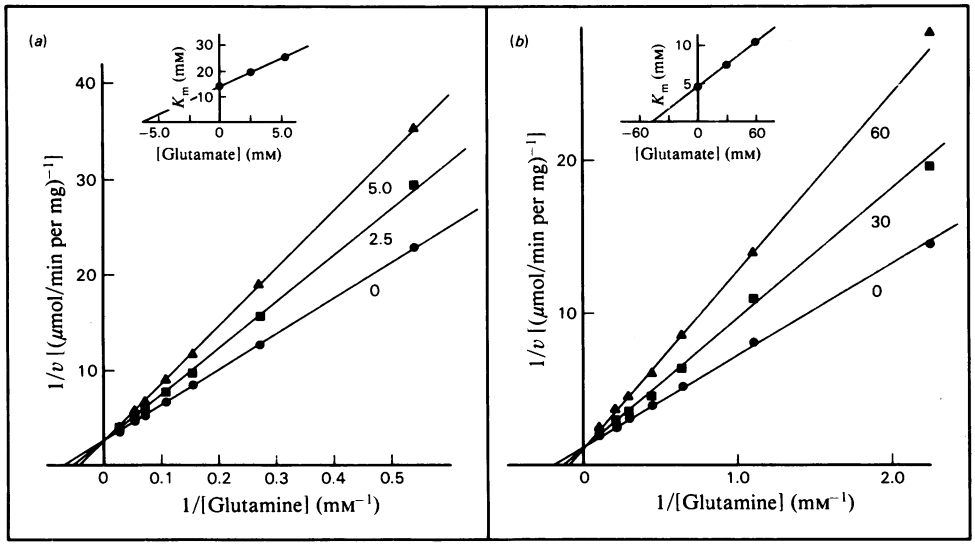

Fig. 2. *Glutamate inhibition of mitochondrial mmbrane-bound glutaminase from rat brain*
The data are plotted as double-reciprocal plots of activity (v) versus glutamine concentration determined in the presence of either 10mM-phosphate (*a*) or 150mM-phosphate (*b*) and the indicated concentration (mM) of glutamate. The inserts are replots of the apparent K_m for glutamine versus the corresponding concentration of glutamate.

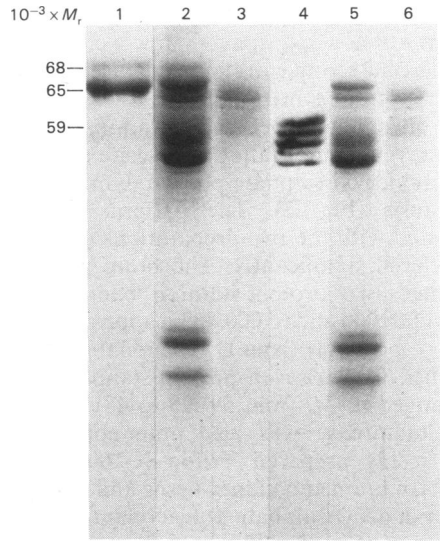

Fig. 3. *Comparison of the peptide structure of phosphate-dependent glutaminase purified or immunoprecipitated from crude homogenates of rat brain and kidney*

The following samples were subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and stained for protein: lane 1, 6 μg of purified rat brain glutaminase; lane 2, 0.3 unit of glutaminase immunoprecipitated from a Triton X-100-solubilized homogenate of rat brain; lane 3, control precipitate of Triton X-100-solubilized rat brain homogenate obtained in the absence of anti-glutaminase IgG; lane 4, 6 μg of purified rat renal glutaminase; lane 5, 0.3 unit of glutaminase immunoprecipitated from a Triton X-100-solubilized homogenate of rat kidney; lane 6, control precipitate of Triton X-100-solubilized rat renal homogenate obtained in the absence of anti-glutaminase IgG.

two major protein-staining bands, of M_r 65000 and 63000 (lane 5). The M_r-63000 peptide was again identified as a non-specific contaminant (lane 6), which precipitated in the absence of IgG. A M_r-68000 peptide was not observed even when a 4-fold greater amount of the renal glutaminase activity was immunoprecipitated (results not shown).

It has been suggested that the glutaminase from pig brain exists in two forms which differ kinetically and which may reside in separate populations of brain mitochondria (Kvamme & Olsen, 1979). This hypothesis suggests the possibility that the two peptides associated with the rat brain enzyme may represent different forms of the glutaminase that are localized in different types of mitochondria. Therefore, total brain mitochondria were separated into synaptosomal and non-synaptosomal fractions. The crude mitochondria, the synaptosomal and the non-synaptosomal fractions were solubilized with Triton X-100 and the

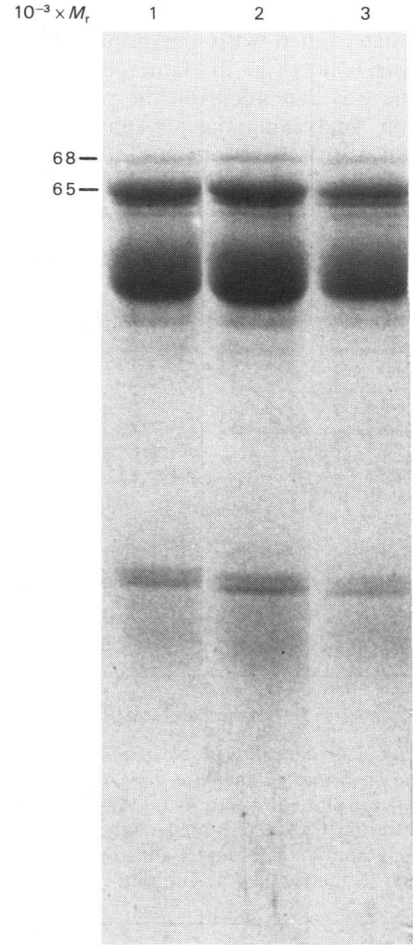

Fig. 4. *Comparison of the glutaminase peptides immunoprecipitated from brain synaptosomal and non-synaptosomal mitochondria*

Brain mitochondria were isolated by differential centrifugation and then centrifuged through a discontinuous Ficoll gradient to separate synaptosomal and non-synaptosomal mitochondria. The mitochondrial fractions were treated with 1% Triton X-100, and the solubilized glutaminase was immunoprecipitated with anti-glutaminase IgG. The precipitates were subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and stained for protein. The samples correspond to glutaminase immunoprecipitated from unfractionated brain mitochondria (lane 1), from non-synaptosomal mitochondria (lane 2), and from synaptosomal mitochondria (lane 3).

glutaminase activity was immunoprecipitated (Fig. 4). The three samples contained an identical pattern of M_r-68000 and -65000 peptides.

The various peptides contained in the immunoprecipitates of the brain and kidney glutaminase were isolated and subjected to peptide mapping by

using *Staphylococcus aureus* V8 proteinase (Fig. 5). When the isolated peptides were re-subjected to electrophoresis in the absence of V8 proteinase, they migrated as individual peptides (lanes 1–3 and 7). The pattern of peptides produced by partial proteolysis of the M_r-68000 and -65000 peptides of the brain (lanes 4 and 5) and renal glutaminase (lane 6) were nearly identical. Partial proteolysis of the M_r-63000 peptide isolated from the immunoprecipitate of the renal glutaminase (lane 8) yielded a very different pattern of peptides. Therefore the M_r-63000 contaminant is not structurally related to the M_r-68000 and -65000 glutaminase peptides. The peptide maps were repeated by using a 2-fold higher concentration of the V8 proteinase, and the silver-stained gels were analysed by densitometry. The increased amount of proteinase did not produce any additional peptides, but merely decreased the intensity of the slower-migrating peptides and increased the intensity of the faster-migrating peptides (results not shown).

In order to compare further the sensitivity of the brain and renal phosphate-dependent glutaminase to endogenous proteinases, mitochondria from both tissues were isolated and solubilized with Triton X-100. The two samples were stored at 4°C for 3 days, and on each day 0.3unit of the glutaminase was immunoprecipitated (Fig. 6). For this experiment, $F(ab)_2$ fragments of the rabbit anti-glutaminase IgG were used in order to avoid the co-migration of potential degradation products of the glutaminase with the IgG heavy chain. Storage for 3 days had no effect on the structure of the brain glutaminase. All four samples contained the identical pattern of M_r-68000 and -65000 peptides (lanes 2, 4, 6 and 8). In contrast, the immunoprecipitate of the freshly prepared renal mitochondrial extract contained only the M_r-65000 glutaminase peptide (lane 1). After 1 day (lane 3) the renal glutaminase was partially degraded to the faster-migrating peptides. The intensity of the faster-migrating bands increased on further storage. By day 3 (lane 7), the immunoprecipitate contained a pattern of peptides similar to that exhibited by the purified renal glutaminase. The glutaminase activity contained in the solubilized brain and renal mitochondrial extracts was unaltered by storage for 3 days at 4°C.

In order to determine if the brain glutaminase was susceptible to a similar proteolytic degrada-

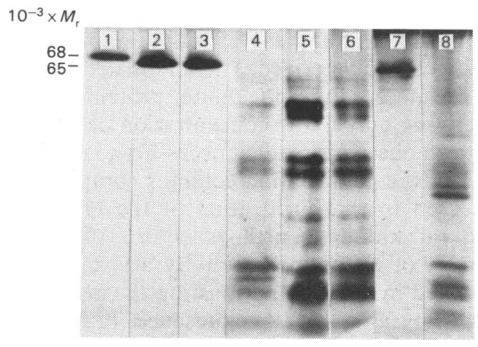

Fig. 5. *Limited proteolytic digestion of the peptides associated with the phosphate-dependent glutaminase immunoprecipitated from Triton X-100-solubilized rat brain and kidney tissue*

Homogenates of brain and renal tissues were solubilized with Triton X-100 and centrifuged at 150000*g* for 30min. Approx. 0.3unit of glutaminase activity was immunoprecipitated from the resulting supernatants and subjected to sodium dodecyl sulphate/polyacrylamide (10%)-gel electrophoresis. The resulting peptides were eluted and subjected to re-electrophoresis (12% acrylamide) in the absence (lanes 1, 2, 3 and 7) or presence (lanes 4, 5, 6 and 8) of 0.025 μg of *Staphylococcus aureus* V8 proteinase. The samples contain: the M_r-68000 peptide from brain tissue (lanes 1 and 4); the M_r-65000 peptide from brain tissue (lanes 2 and 5); the M_r-65000 peptide from kidney tissue (lanes 3 and 6); and the M_r-63000 peptide from kidney tissue (lanes 7 and 8).

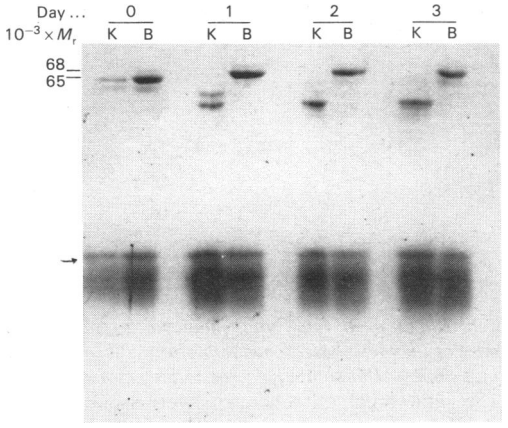

Fig. 6. *Comparison of the sensitivity of phosphate-dependent glutaminase contained in Triton X-100-solubilized rat brain and kidney mitochondria to endogenous proteolysis*

Renal and brain mitochondria were isolated by differential centrifugation, solubilized with 1% Triton X-100 and centrifuged at 150000*g* for 30min. The supernatants were recovered and stored at 4°C. Samples containing 0.3unit of renal and brain glutaminase were immunoprecipitated by using $F(ab)_2$ fragments of anti-glutaminase IgG on the day of solubilization (0) and 1, 2 and 3 days later. The samples were subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and stained for protein. Kidney and brain samples are indicated as K and B respectively. The $F(ab)_2$ fragments are indicated by the arrow.

tion, the effect of adding a glutaminase-depleted renal extract on the structure of the solubilized brain glutaminase was studied (Fig. 7). As observed previously, the structure of the brain glutaminase was unaltered during storage in the absence of the renal extract. However, the addition of the renal extract resulted in degradation of the brain glutaminase to yield increasing amounts of the smaller peptides. By day 3, the peptide pattern was again similar to that observed with the purified renal glutaminase. This degradation also occurred without loss of glutaminase activity. Thus the proteinase that is responsible for the partial proteolysis of the renal glutaminase is apparently not present in brain tissue.

In an attempt to find a proteinase inhibitor that would prevent the degradation of the glutaminase, the following compounds were added individually and in combinations to Triton X-100-solubilized renal mitochondria: phenylmethanesulphonyl fluoride (1mM); benzamidine (10mM); ε-aminohexanoic acid (10mM); leupeptin (50μg/ml); pepstatin (50μg/ml); chymostatin (50μg/ml); antipain (50μg/ml); sodium vanadate (0.1mM); *o*-phenanthroline (2mM). None of these compounds prevented the partial proteolysis of the glutaminase. Furthermore, the addition of the inhibitors had no effect on the ratio (1:4) of the M_r-68000 and -65000 peptides observed in immunoprecipitates of the brain glutaminase.

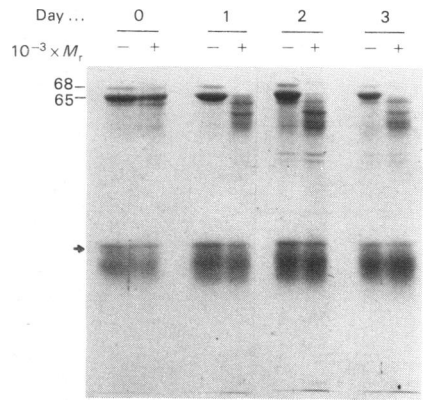

Fig. 7. *Effect of a renal mitochondrial extract on the stability of the phosphate-dependent glutaminase contained in Triton X-100-solubilized rat brain mitochondria*

Brain and renal mitochondria were isolated and solubilized with 1% Triton X-100. A sample of the renal mitochondrial supernatant was incubated with sufficient $F(ab)_2$ fragments of anti-glutaminase IgG to precipitate the glutaminase activity completely. Samples of the glutaminase-depleted renal extract, equivalent to 0.3unit, were added to four samples of solubilized brain mitochondria containing 0.3unit of glutaminase. The combined samples ('+' lanes) were stored at 4°C and immunoprecipitated by using $F(ab)_2$ fragments of anti-glutaminase IgC on the initial day of mixing (0) and 1, 2 and 3 days later. Similar samples were immunoprecipitated from untreated brain mitochondrial supernatants ('−' lanes). The precipitates were subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and stained for protein. The $F(ab)_2$ fragments are indicated by the arrow.

Discussion

The specific activity of phosphate-dependent glutaminase in a crude homogenate of rat brain is about 2-fold greater than that observed in a crude homogenate of normal rat kidney tissue. The differential centrifugation procedure used to isolate rat renal mitochondria yielded a very low recovery of the phosphate-dependent glutaminase when applied to the fractionation of a crude homogenate of brain tissue. Thus it was necessary to begin the purification by using a membrane fraction that was obtained by centrifuging the crude homogenate at 25000*g* for 15min. The inclusion of the digitonin treatment was necessary to remove sufficient lipid in order to make feasible the solubilization of the glutaminase by freeze-drying in the presence of borate, phosphate and pyrophosphate anions. Under these conditions, the solubilized glutaminase is both stabilized and extensively polymerized. The solubilized glutaminase is quite labile in dilute protein solutions containing only a low concentration of pyrophosphate anions. This made it necessary to reverse the order of the gel-filtration columns compared with that used in the purification of the glutaminase from rat kidney (Curthoys *et al.*, 1976*b*). The recovery of glutaminase activity was significantly improved by initially performing the gel-filtration chromatography in the presence of the TPD buffer. The high protein concentration associated with this step apparently stabilizes the glutaminase activity. In the subsequent chromatography step, the highly purified glutaminase is recovered in a dilute solution, but it is stabilized by the presence of the BPP buffer.

The M_r characterization indicates that the glutaminase purified from rat brain also undergoes a phosphate-induced dimerization. The Stokes radii determined for the brain glutaminase in the absence and presence of 100mM-phosphate are significantly greater than the corresponding values previously reported for the glutaminase purified from rat kidney (Godfrey *et al.*, 1977). This is consistent with the fact that the kidney glutaminase is subjected to partial proteolysis during its solubilization and purification (Clark & Curthoys, 1979). Sedimentation of the purified rat renal

glutaminase in sucrose gradients prepared in water and in ^2H_2O indicated that this enzyme had an unusual partial specific volume of 0.76ml/g (Godfrey *et al.*, 1977). The sedimentation coefficient previously reported for the protomeric and dimeric forms of the renal glutaminase were calculated in a way that takes into account its abnormal partial specific volume. Without this correction, the sedimentation coefficients determined for the renal glutaminase would have been very similar to those reported here for the glutaminase from rat brain. ^2H_2O-sedimentation-velocity analysis of the glutaminase purified from rat brain did not indicate that this enzyme had an abnormally high partial specific volume (results not shown).

The kinetic properties of the phosphate-dependent glutaminase from rat brain are identical with those previously reported for the glutaminase from rat kidney (Shapiro *et al.*, 1982). The glutaminase is inactive in the absence of phosphate or other activating polyvalent anion. This is consistent with the previous characterization that the protomeric form of the rat renal glutaminase is catalytically inactive (Morehouse & Curthoys, 1981). The concentration of phosphate (100mM) that is sufficient to produce dimerization is also sufficient to produce maximal activation. For both enzymes, the phosphate activation is sigmoidal and inhibition by glutamate is competitive with respect to glutamine. Similarly, for both enzymes increasing concentrations of phosphate decrease the K_m for glutamine and increase the K_i for glutamate. Furthermore, the K_m and K_i values measured at 10mM- and 150mM-phosphate are nearly identical for the particulate glutaminase preparations derived from the two tissues.

The purified brain glutaminase contains peptides of M_r 68000 and 65000. These results suggest that the protomeric form of the glutaminase may contain two subunits. By contrast, the purified renal glutaminase contains a series of peptides that range in M_r from 59000 to 48000. The M_r range previously reported for the proteolysed subunits of the renal glutaminase (Clark & Curthoys, 1979) was significantly greater than that determined in the present study. This difference is due to the inaccurate assignment of M_r for β-galactosidase, one of the standard proteins used previously, and the use of a large amount of the standard proteins, which produced broad bands. The M_r values reported in this study were obtained after correcting these procedural errors and should more accurately reflect the true M_r of the glutaminase peptides.

Immunoprecipitates of the phosphate-dependent glutaminase obtained from a freshly solubilized kidney homogenate or isolated renal mitochondria contain a single specific peptide, of M_r 65000. The storage of either supernatant leads to the appearance of the same lower-M_r peptides. Thus the proteinase responsible for degradation of the renal glutaminase is retained in the isolated mitochondrial fraction. However, this fraction is heavily contaminated with lysosomes. The various inhibitors that were tested and found to be ineffective should have blocked the action of many of the known proteinases. Thus the subcellular origin and the specificity of the proteinase responsible for partial degradation of the renal glutaminase are unknown. The M_r-65000 form of the glutaminase observed in the initial precipitate may represent the native structure of the renal enzyme. However, these studies cannot exclude the possibility that significant proteolysis of the renal glutaminase occurs during the 2–3h that is required to immunoprecipitate the enzyme.

The 1:4 ratio of M_r-68000 and -65000 peptides observed in the purified brain glutaminase was initially interpreted to suggest that the native brain glutaminase exists as a single M_r-68000 peptide that is partially proteolysed to the M_r-65000 form. However, the glutaminase immunoprecipitated from freshly solubilized brain tissue or isolated brain mitochondria exhibits an identical pattern of the two peptides. This pattern was unaffected by storage of the isolated supernatant for 3 days at 4°C. Therefore, if the brain glutaminase does undergo degradation by an endogenous proteinase, it must occur only during the 2h required for solubilization and formation of an immune complex. It is unlikely that the product of a partial cleavage reaction would be resistant to further proteolysis. Therefore both peptides may be associated with the native form of the brain glutaminase.

The peptide mapping of the isolated brain and kidney peptides indicates that they are structurally related. This conclusion is also supported by the observation that addition of a renal extract causes degradation of both the M_r-68000 and -65000 brain peptides to a pattern identical with that observed in the purified renal glutaminase. These observations, along with the immunological identity (Curthoys *et al.*, 1976*a*), suggest that the brain and kidney glutaminase may be derived from the same structural gene. The 4:1 ratio of brain glutaminase peptides could result from a difference in post-translational processing. As with other mitochondrial proteins, the glutaminase may be initially synthesized as a larger cytoplasmic precursor that is processed during its import into the mitochondria (Schatz & Butow, 1983). The present study indicates that the difference in glutaminase peptides is not due to a difference in distribution between synaptosomal and non-synaptosomal mitochondria. However, the two forms of brain

glutaminase could reflect a difference in cellular or submitochondrial distribution.

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