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Biosynthesis and processing of mitochondrial glutaminase in HTC hepatoma cells

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Rat HTC hepatoma cells were used to characterize the biosynthesis and processing of the renal isoenzyme of the mitochondrial glutaminase. Immunoblot analysis indicated that mitochondria isolated from HTC cells contained two prominent glutaminase peptides of 68 and 65 kDa and two minor peptides of 61 and 58 kDa. When the cells were labelled with [^{35}S]methionine, the glutaminase-specific antibodies precipitated the same four polypeptides. However, when labelled in the presence of 5 μM-carbonyl cyanide *m*-chlorophenylhydrazone, an uncoupler of oxidative phosphorylation, only a 72 kDa cytoplasmic precursor of the mitochondrial glutaminase was immunoprecipitated. A comparison of the peptides generated by partial proteolysis of the precursor and the fully processed peptides indicates significant structural similarity. A 71 kDa form of the glutaminase was also observed when HTC cells were pulse-labelled for 2–6 min with [^{35}S]methionine. Pulse–chase experiments indicate that the cytoplasmic precursor is quantitatively converted into the mature forms of the glutaminase. In addition, the observed kinetics established that the 71 kDa peptide is a true intermediate in the import of the mitochondrial glutaminase.

INTRODUCTION

Renal extraction and metabolism of plasma glutamine is increased in response to metabolic acidosis [1,2]. The primary pathway for this metabolism is initiated by a mitochondrial glutaminase, which is asymmetrically associated with the matrix surface of the mitochondrial inner membrane [3]. In response to chronic acidosis, the glutaminase activity within the proximal convoluted segment of the rat nephron is increased 20-fold [4]. The renal isoenzyme of the mitochondrial glutaminase is also abundant in brain, intestine and hepatoma tissues, but is absent from adult liver. The glutaminase contained within rat brain and renal mitochondria consists of two structurally related peptides that have molecular masses of 68 and 65 kDa [5,6]. Activation of this enzyme requires the presence of phosphate or other polyvalent anions and the formation of a tetrameric structure [7]. However, translation of brain and renal polyadenylated RNA in a rabbit reticulocyte lysate yields a 72 kDa protein that is precipitated by glutaminase-specific IgG [8]. Thus the mitochondrial glutaminase may be synthesized as a single precursor that is processed to yield two structurally related peptides.

A general mechanism for the synthesis and translocation of nuclear encoded mitochondrial proteins has been elucidated [9–11]. The precursor of mitochondrial proteins which are synthesized and released from cytoplasmic ribosomes generally contain an *N*-terminal targeting sequence which interacts with a proteinaceous receptor on the mitochondrial surface. Translocation into the mitochondrial matrix requires the presence of a membrane potential across the inner membrane. The presequence is removed within the matrix by specific processing peptidases, and the newly imported polypeptide undergoes refolding to assume its active conformation. Two specific processing peptidases have been partially purified and characterized [12,13]. The sequential processing of the presequence may be necessary to redirect the peptide back across the inner membrane [14,15] or for the protein to form its proper quaternary structure.

The biosynthesis of the mitochondrial glutaminase may proceed by the general mechanism described above. However, the observation that the initial translation is apparently processed to yield two different subunits of the glutaminase is unusual. Thus, in the present study, rat HTC hepatoma cells, which express a significant level of the renal glutaminase, were used to characterize further its biosynthesis.

MATERIALS AND METHODS

Reagents for PAGE, DEAE-Affi-Gel Blue and protein molecular-mass standards were products of Bio-Rad. ^{14}C-labelled protein molecular-mass standards were purchased from Bethesda Research Laboratories.

[^{35}S]Methionine (sp. radioactivity 1100 Ci/mmol) was obtained from New England Nuclear. Aqueous counting scintillant (ACS) and NCS solubilizer were products of Amersham. SDS was purchased from Gallard/Schlesinger. *Staphylococcus aureus* V8 proteinase was purchased from Miles Laboratories. Ultra-pure sucrose was obtained from Schwarz–Mann. X-ray photographic film (XAR-S) was a product of Kodak. DME/F12, MEM Select-amine kit, BSA, cell-culture dishes, penicillin G and $NaHCO_3$ were obtained from Gibco. Streptomycin was purchased from Calibochem. All other biochemicals were obtained from Sigma.

Cell cultures and immunoprecipitations

HTC-SR cells were grown in 60 mm-diameter culture dishes, in Dulbecco's modified Eagle's/Ham's F12 nutrient solutions (DME/F12; 1:1, v/v) containing penicillin G (12 μg/l), streptomycin (200 μg/l), $NaHCO_3$ (1.2 g/l) and 15 mM-Hepes, pH 7.4, supplemented with 10% (v/v) fetal-calf serum. Plates containing near-confluent cells were used for experiments, and confluent cells were subcultured 1:9 using 0.25% trypsin/0.1% EDTA in Krebs–Ringer phosphate-buffered saline.

Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; ACS, aqueous counting scintillant; DME, Dulbecco's modified Eagle's medium; F12, Ham's F12 medium.

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To immunoprecipitate specifically the labelled glutaminase, a 60 mm-diameter plate of HTC cells was incubated for 15 min at 37 °C in minimal essential medium (MEM) minus methionine supplemented with 50 μCi of [^{35}S]methionine. This resulted in the incorporation of 2×10^7 c.p.m. of radioactivity/mg of trichloroacetic acid-precipitable protein. The cells were solubilized by adding a portion of a 210000 *g* supernatant from a crude rat brain homogenate [5] containing 1% Triton X-100, 0.7 unit of glutaminase activity, proteinase inhibitors [25 mM-benzamidine, 1 mM-phenylmethanesulphonyl fluoride, aprotinin (200 μg/ml), chymostatin, antipain, leupeptin, pepstatin and bestatin (all at 80 μg/ml)] and chelators (2 mM-EDTA and -EGTA). The samples were incubated for 10 min at 4 °C, then centrifuged for 5 min at 4 °C at 158000 *g* in an Airfuge. The resulting supernatants were incubated with 1 unit of anti-glutaminase IgG for 1.5 h at 4 °C. The resulting immunoprecipitates were collected by centrifugation through 0.5 ml of 0.5 M-sucrose at 10000 *g* for 10 min at 4 °C. The pellets were then washed twice with 10 mM-Tris/acetate (containing 1% Triton X-100), pH 7.4, and once with 0.1% lithium dodecyl sulphate, pH 7.4, in homogenate buffer. The washed pellets were then subjected to SDS/PAGE [16], followed by fluorography [17]. The sizes of the labelled polypeptides were characterized relative to the mobility of ^{14}C-labelled protein molecular-mass standards. The co-precipitation of added carrier enzyme greatly improved the recovery of the labelled glutaminase and the removal of non-specific proteins.

In order to generate the precursor of the glutaminase, the labelling was carried out in the presence of 5 μM-carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) [18]. For 'chase' experiments, MEM containing 10 mM-methionine plus 10 mM-cysteamine was used. The density of bands on the fluorographs were quantified with a model-620 Video densitometer.

A 1 μl aliquot of the labelled protein, obtained from the supernatant of the Airfuge spin, was precipitated by adding 100 μl of 5% (w/v) trichloroacetic acid/1% (w/v) phosphotungstic and incubating for 5 min at 4 °C. The resulting pellets were washed twice with 100 μl of the same solution, followed by centrifugation at 2000 *g* for 5 min at 4 °C. Pellets were then dissolved by incubating with 0.6 ml of NCS tissue solubilizer at 37 °C and counted for radioactivity in 2.4 ml of ACS scintillant using a Packard model-3L55 scintillation counter.

Enzyme analysis

The phosphate-dependent glutaminase activity was measured by quantifying the amount of glutamate formed from glutamine [19]. Protein was assayed by the method of Lowry *et al.* [20], using BSA as the standard.

Rabbit polyclonal anti-glutaminase IgG was prepared using rat kidney enzyme as antigen [21]. The IgG fraction was purified from rabbit antiserum by chromotography on DEAE-Affi-Gel Blue. The specificity of the antibodies was established by characterizing the proteins which are precipitated from a Triton X-100-solubilized supernatant of a rat brain homogenate [5] and by immunoblot analysis [6]. In both analyses, the only peptides detected were the 68 and 65 kDa proteins that are characteristic of the purified brain glutaminase [5]. The glutaminase solubilized from either rat brain or kidney produces the same immunological titre [22]. Thus the antibody titre was determined by using a freshly prepared crude rat brain supernatant fraction. The purified IgG fraction had a titre of 20 units/ml. Absorbed glutaminase antibodies were prepared by incubating the IgG with an excess of purified brain glutaminase [5] and were used in control experiments.

Rats were decapitated and brains were immediately removed. The tissue was homogenized in a Potter–Elvehjem homogenizer with 3 vol. of 0.3 M-sucrose/0.2 mM-EDTA/10 mM-Tris, pH 7.4. The crude homogenate was diluted with one-quarter its volume of 5% Triton X-100, incubated for 1 h at 4 °C and then centrifuged for 30 min at 210000 *g* in a 50Ti rotor. The glutaminase activity in the resulting supernatant was approx. 3–4 units/ml.

Immunoblot analysis was carried out essentially as described in [6]. Briefly, the protein samples were subjected to SDS/PAGE and then transferred to nitrocellulose for immunostaining. After blocking with 3% (w/v) gelatin for 4 h at room temperature, the nitrocellulose was incubated with a 1:200 dilution of the purified antibodies. It was then incubated with a anti-rabbit IgG–horseradish peroxidase conjugate and stained with 4-chloro-1-naphthol.

Labelled precursor and mature forms of glutaminase were immunoprecipitated from cells and subjected to limited proteolytic digestion as described by Cleveland *et al.* [23]. The partially digested products were subjected to SDS/PAGE and revealed by fluorography. Immunoprecipitated samples were mixed with *S. aureus* V8 proteinase suspended in SDS sample buffer containing 10% (v/v) glycerol. The samples were electrophoresed until the Bromophenol Blue dye migrated 2 cm into the stacking gel. Proteolytic digestions continued while the current was turned off. After 30 min of digestion, electrophoresis was re-initiated. In order to make equivalent the amount of label incorporated into the precursor and the processed protein, precursor immunoprecipitated from multiple cell plates was pooled and then subjected to proteolytic digestion.

RESULTS

Various hepatomas express the kidney type of glutaminase at a level which correlates with the growth rate of the tumour [24]. HTC-SR hepatoma cells have a specific activity for the mitochondrial glutaminase which is approximately one-third of that measured in a crude homogenate of normal rat kidney. Antibodies prepared against the purified renal glutaminase do not precipitate the liver isoenzyme [22]. They were used to compare the glutaminase contained in HTC cells and in rat brain. The two activities exhibit an identical response in a quantitative precipitant analysis (Fig. 1). In addition, the glutaminase contained in HTC cells is completely inactive in the absence of phosphate (Fig. 2). Increasing concentrations of phosphate cause a hyperbolic activation with a K_m of 18 mM. At saturating phosphate, glutamate is a competitive inhibitor with respect to glutamine and has a K_I of 26 mM. Thus the glutaminase contained in HTC

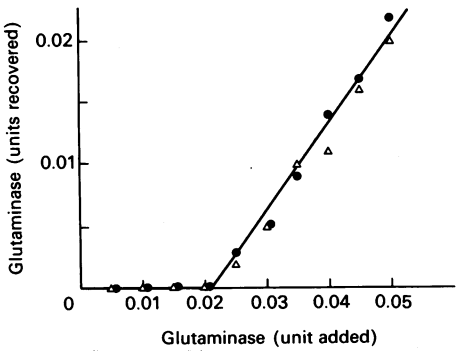

Fig. 1. Quantitative immunoprecipitant analysis of the glutaminase activity contained in rat brain and in HTC cells

Aliquots of a 1% Triton X-100-solubilized supernatant of homogenates of rat brain (△) or of HTC cells (●) containing the indicated units of glutaminase activity were added to 0.025 unit of rabbit anti-(rat renal glutaminase) IgG and incubated overnight at 4 °C. The samples were then centrifuged at 10000 *g* for 10 min and the final supernatants were assayed for glutaminase activity.

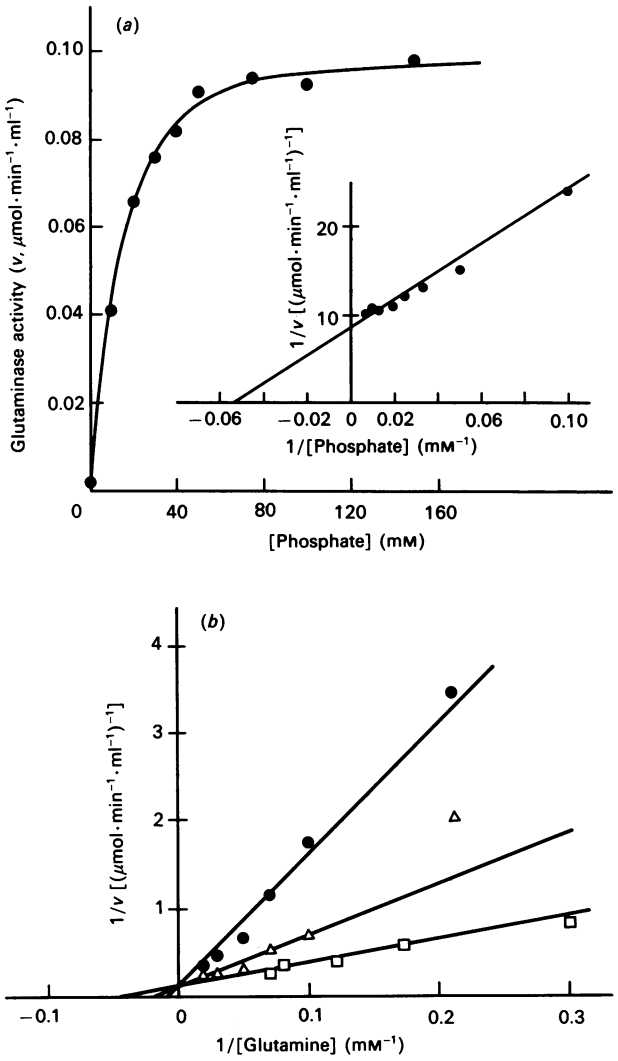

Fig. 2. Kinetic properties of the glutaminase contained in HTC cells

(*a*) Phosphate activation. The glutaminase activity was measured as a function of increasing phosphate concentration. In the inset, data are presented as a double-reciprocal plot of activity versus phosphate concentration. (*b*) Glutamate inhibition. The glutaminase activity was measured as a function of increasing glutamine concentration in the absence (□) or presence of 45 mM- (△) or 90 mM- (●) glutamate. Data are presented as a double-reciprocal plot of activity versus glutamine concentration.

cells exhibits an immunological titre, a phosphate-activation profile and glutamate inhibition that are characteristic of the kidney isoenzyme.

Immunoblot analysis of mitochondria isolated from HTC cells was carried out in order to identify the forms of the glutaminase contained in the hepatoma cells (Fig. 3). The two major immunoreactive peptides present in intact HTC mitochondria (lane 2) are identical in size with the glutaminase contained in rat brain (lane 1). Larger aliquots of HTC mitochondria were solubilized with Triton X-100 and incubated with the anti-glutaminase IgG. The resulting immunoprecipitates contained the same two predominant peptides and two additional minor peptides of 61 and 58 kDa (lane 3). However, none of the four peptides were precipitated when solubilized mitochondria were incubated with pre-absorbed antibodies (lane 4). When the supernatant of the latter sample was re-incubated with anti-glutaminase IgG (lane 5), all four immunoreactive peptides were re-precipitated. Therefore, the hepatoma cells may also contain the more highly

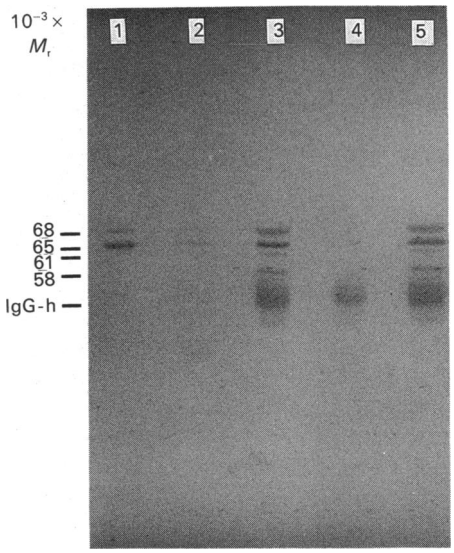

Fig. 3. Immunoblot analysis of the mitochondrial glutaminase contained in HTC cells

Cells obtained from a 60 mm-diameter plate were homogenized with 100 μl of buffer containing 0.3 M-sucrose, 10 mM-Tris and 0.2 mM-EDTA, pH 7.4, and the mitochondria were isolated by differential centrifugation. Aliquots of the mitochondria were either analysed directly or solubilized with Triton X-100 and immunoprecipitated with anti-glutaminase IgG. The various samples contained: 6 μg of partially purified brain glutaminase (lane 1), 0.15 μg of HTC mitochondrial protein (lane 2), the precipitates obtained by incubating 1.5 μg of Triton X-100-solubilized HTC mitochondria with anti-glutaminase IgG (lane 3) or with pre-absorbed anti-glutaminase IgG (lane 4), and the immunoprecipitate derived by incubating the supernatant from the preceding sample with anti-glutaminase IgG (lane 5). The positions of the glutaminase peptides are indicated by M_r values. The diffuse lower band in lanes 3, 4 and 5 corresponds to the heavy subunit of the anti-glutaminase antibodies (IgG-h).

processed forms of glutaminase that were previously observed in intestinal and renal papillary mitochondria [6].

Immunoprecipitates derived from HTC cells that were labelled with [^{35}S]methionine contained the same four immunoreactive peptides as identified by immunoblot analysis (Fig. 4, lane 1). Previous studies have established that the addition of an uncoupler of oxidative phosphorylation blocks the uptake and processing of mitochondrial precursor proteins. Thus the effect of increasing concentrations of CCCP on the biosynthesis of the mitochondrial glutaminase was studied (Fig. 4, lanes 2–4). The addition of 5 μM-CCCP blocks the synthesis of the mature forms of the glutaminase and causes the concomitant appearance of a 72 kDa peptide. This protein co-migrates with the primary translation product encoded by the mitochondrial glutaminase mRNA (lane 5). Thus the addition of the uncoupler results in the accumulation of the precursor of the mitochondrial glutaminase.

In order to establish the subcellular localization of the various forms of the glutaminase, cells were labelled with [^{35}S]methionine in the presence or absence of 5 μM-CCCP and subjected to subcellular fractionation. In the absence of proteinase K, the 72 kDa form of the glutaminase is found in the cytosolic fraction, whereas the mature forms of the glutaminase are found in the mitochondrial fraction (Fig. 5*a*). In addition, the 72 kDa form is degraded by proteinase K, whereas the smaller forms are resistant to added proteinase (Fig. 5). Thus the 72 kDa form represents the primary translation product and the cytoplasmic precursor of the glutaminase, whereas the other peptides represent imported and processed forms of the glutaminase.

In order to verify that the 72 kDa protein is a precursor to the

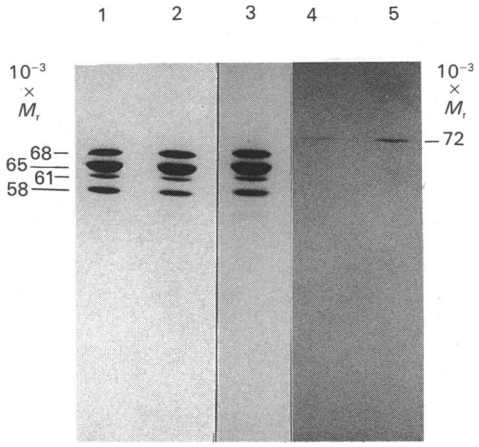

Fig. 4. Effect of increasing CCCP concentrations on biosynthesis of the mitochondrial glutaminase

HTC cells were preincubated for 5 min in the presence of various concentrations of CCCP (see below) and then labelled with [^{35}S]methionine for 15 min in the presence of the same concentration of CCCP. The cells were then solubilized and incubated with anti-glutaminase IgG and the resulting precipitates were analysed by SDS/PAGE and fluorography. The cells were treated with 0 μM- (lane 1), 0.2 μM- (lane 2), 1 μM- (lane 3) or 5 μM-CCCP (lane 4). Lane 5 contains the immunoprecipitate of the products synthesized by translation *in vitro* of rat renal polyadenylated RNA in a rabbit reticulocyte lysate. The M_r values of the various peptides are indicated.

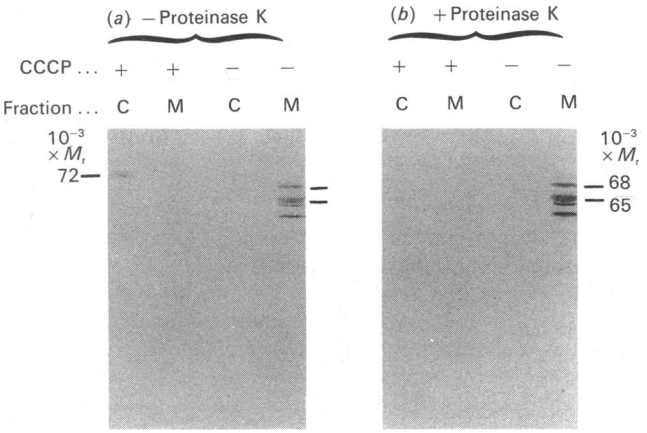

Fig. 5. Subcellular distribution of precursor and processed forms of glutaminase synthesized in HTC cells

Cells were labelled with [^{35}S]methionine for 15 min in the presence (+) or absence (−) of 5 μM-CCCP. Cells were homogenized and separated into cytosolic (C) and mitochondrial (M) fractions by differential centrifugation. The resulting fractions were then incubated in the absence (−Proteinase K) or presence (+Proteinase K) of proteinase K (15 μg/ml) for 30 min at 4 °C. Proteolysis was stopped by the addition of 1 mM-phenylmethane sulphonyl fluoride. Glutaminase was then solubilized, immunoprecipitated, then analysed by SDS/PAGE and fluorography. M_r values for the resulting peptides are indicated.

mature forms of the glutaminase, a structural comparison of this peptide and the mature forms was accomplished by limited proteolytic digestion. Preliminary experiments were carried out in order to optimize the concentrations of *S. aureus* V8 proteinase necessary to generate a peptide map. In order to equate the amount of [^{35}S]methionine contained in the precursor and mature peptides, a greater number of plates were labelled in the presence of CCCP. As Fig. 6 shows, the digestion maps of the precursor

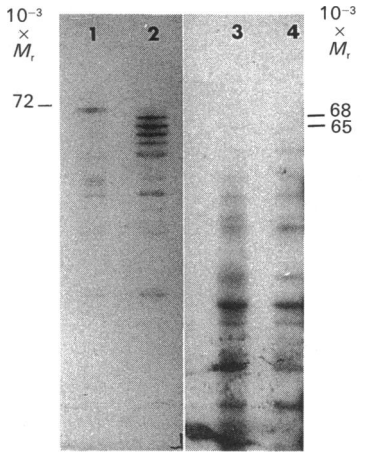

Fig. 6. Comparison of peptide fragments generated by partial proteolysis of the 72 kDa precursor and the mature forms of the glutaminase synthesized in HTC cells

In order to generate sufficient precursor (lanes 1 and 3), four plates of HTC cells were labelled with [^{35}S]methionine in the presence of 5 μM-CCCP, whereas a single plate was labelled in the absence of CCCP to produce the fully processed peptides (lane 2 and 4). The cells were then solubilized and glutaminase immunoprecipiatates were prepared and incubated with either 0.025 μg (lanes 1 and 2) or 0.050 μg (lanes 3 and 4) of *S. aureus* V8 proteinase. The resulting peptides were subjected to SDS/PAGE and revealed by fluorography.

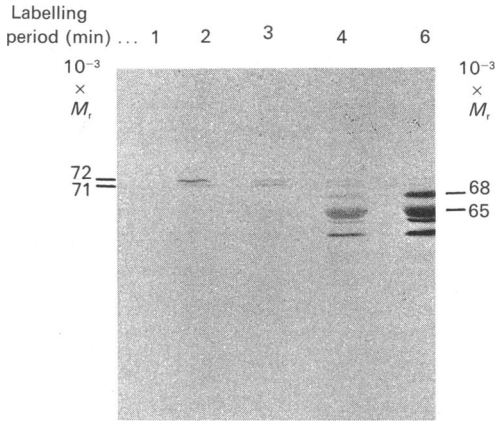

Fig. 7. Pulse-labelling of HTC cells

Cells were labelled with [^{35}S]methionine for the indicated periods of time at 37 °C in the absence of an uncoupler. The cells were solubilized and the glutaminase was immunoprecipitated and analysed by SDS/PAGE and fluorography. The M_r values of the resulting peptides are shown.

and mature forms of the glutaminase produced with two concentrations of proteinase were nearly identical. Thus the apparent cytosolic precursor is both structurally and antigenically related to the mature mitochondrial glutaminase.

When the HTC cells were labelled at 37 °C for short periods of time (i.e. 1–6 min), the 72 kDa precursor and a 71 kDa form of glutaminase were observed (Fig. 7). The 71 kDa species was detectable with only 2–3 min of labelling. Its formation appeared to occur more slowly than that of the 72 kDa precursor, but preceded the appearance of the mature forms of the glutaminase. By 6 min the predominant peptides detected were of the mature enzyme. Therefore, the 71 kDa form of the glutaminase appears to be an intermediate of the processing reaction.

In order to establish the relationship between the various

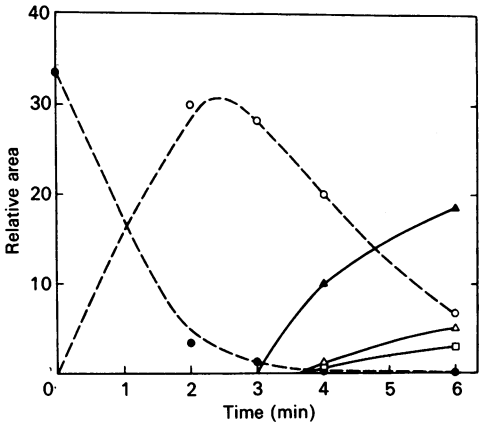

Fig. 8. Pulse–chase labelling of HTC cells at 37 °C

Cells were labelled with [^{35}S]methionine for 10 min at 37 °C in the presence of 5 μM-CCCP and then chased with excess unlabelled methionine in the presence of cysteamine. The cells were then solubilized, and the glutaminase immunoprecipitated and analysed by SDS/PAGE and fluorography. The resulting fluorography was quantified by densitometry, and the relative intensities associated with each of the glutaminase peptides were plotted against the period of chase. Symbols: ●, 72 kDa precursor; ○, 72 kDa intermediate; ▲, △, □, mature forms of glutaminase (▲, 65 kDa; △, 68 kDa; □, 58 kDa).

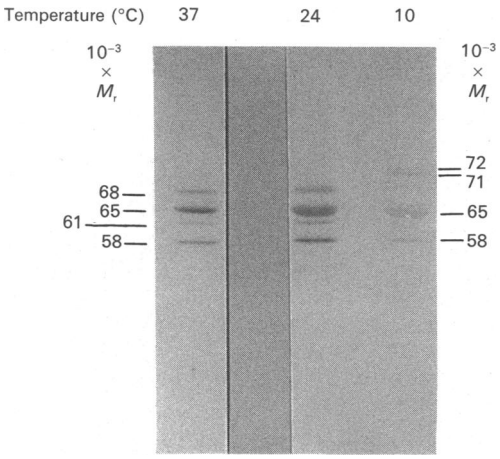

Fig. 9. Effect of temperature on the products of pulse–chase labelling of glutaminase in HTC cells

Cells were labelled with [^{35}S]methionine for 10 min at 37 °C in the presence of 5 mM-CCCP and then chased for 15 min at 37, 24 or 10 °C in the presence of excess unlabelled methionine and cysteamine. The cells were then solubilized, and the glutaminase was immunoprecipitated and analysed by SDS/PAGE and fluorography. M_r values of the resulting peptides are shown.

forms of the glutaminase, pulse–chase experiments were carried out (Fig. 8). When HTC cells were pulse-labelled for 10 min in the presence of 5 μM-CCCP, only the 72 kDa precursor was detected. When cells were subsequently chased at 37 °C for 2 and 3 min, both the precursor and the 71 kDa intermediate were observed. Subsequent chases for 4 and 6 min resulted in the disappearance of the 72 kDa form and a decrease in the 71 kDa peptide, with the concurrent appearance of the mature forms of the glutaminase. The data establish that (*a*) the 72 kDa peptide is rapidly converted into the 71 kDa form, (*b*) that the amount of the 71 kDa peptide peaks between 2–3 min, and (*c*) the subsequent disappearance of the intermediate leads to the concomitant formation of the mature forms of the glutaminase. Thus the observed kinetics confirm a precursor–product relationship between the 72 kDa peptide and the processed glutaminase and establish that the 71 kDa peptide is a true intermediate in the important pathway.

The processing of mitochondrial glutaminase occurs rapidly at 37 °C. Therefore attempts were made to perform the pulse–chase experiments at lower temperatures. However, the kinetics of processing at 24 °C were still very rapid, and the use of lower temperatures resulted in incomplete processing. For example, a chase of 15 min at 10 °C results in the production of the 72, 71, 65 and 58 kDa forms of the glutaminase (Fig. 9). Longer chases of up to 2 h at 10 °C did not result in a complete chase of the precursor into products (results not shown). This could be accomplished only by increasing the temperature above 24 °C. Thus it was not possible to reduce sufficiently the kinetics of the mitochondrial processing to examine more thoroughly the temporal sequence of the appearance of the various forms of the glutaminase.

DISCUSSION

The 68 and 65 kDa subunits are normal components of the native enzyme within the rat kidney and brain mitochondria. It is unknown whether both peptides are catalytically active. However, the more degraded forms of glutaminase isolated from rat kidney retain full enzymic activity and the ability to bind glutamine affinity analogues [25,26]. The separated 68 and 65 kDa proteins produce a similar pattern of peptides when subjected to limited proteolytic digestion [27]. In addition, anti-glutaminase IgG, which was affinity-purified versus a chimaera of *Escherichia coli* β-galactosidase and the *C*-terminal half of the glutaminase, reacts with both the 68 and 65 kDa peptides [28]. Thus the two peptides are likely to be derived from a common precursor.

The minor peptides of 61 and 58 kDa apparently result from the additional proteolytic cleavage of the 68 and 65 kDa peptides respectively. An identical pattern of four peptides was previously detected by direct immunoblot analysis of mitochondria isolated from rat small intestine and renal papilla. However, owing to the lower specific radioactivity and the limits of protein loading on the SDS/polyacrylamide gel, the minor peptides were detectable only in immunoprecipitates of solubilized extracts of the HTC cells. Thus it remains possible that, in this system, the minor peptides may be generated by a non-mitochondrial proteinase that remains active in the presence of the added proteinase inhibitors.

Tong *et al.* [8] have previously shown that translation *in vitro* of rat renal polyadenylated RNA produced a 72 kDa protein that is specifically immunoprecipitated by anti-glutaminase IgG. This polypeptide was not detected in translations of liver mRNA, nor was it precipitated by antibodies preabsorbed with purified brain glutaminase. In order to establish that the 72 kDa protein is the precursor of miochondrial glutaminase, HTC cells were labelled with [^{35}S]methionine in the presence of increasing concentrations of CCCP. Addition of an uncoupler causes the accumulation of mitochondrial precursors. With 5 μM-CCCP, a form of glutaminase was observed which had the same electrophoretic mobility as the 72 kDa peptide that was synthesized by translation *in vitro*. Thus, consistent with most other mitochondrial proteins, the glutaminase is initially synthesized as a precursor that is larger than the mature enzyme.

Experiments performed to identify the subcellular location of the precursor of the mitochondrial glutaminase indicated that it was contained in the cytosol and was suspectible to externally added proteinase. By contrast, the mature forms of the glutaminase were found within the mitochondria, where they are

protected from externally added proteinase. Thus endoproteolytic processing must occur either coincident with, or immediately after, the translation of the precursor. The half-lives of many mitochondrial precursor proteins *in vivo* are less than 1–2 min [29–31]. The half-life of the precursor of the mitochondrial glutaminase appears to be equally rapid. However, the stabilities of the precursor molecules that accumulate in the presence of an uncoupler vary significantly. The mitochondrial glutaminase retains the ability to be translocated into the mitochondria for at least 15 min after its synthesis. Thus, in the presence of CCCP, the glutaminase precursor is relatively stable and it remains translocation-competent.

The [^{35}S]methionine contained in the pulse-labelled precursor can be quantitatively chased into the mature glutaminase. This firmly establishes a precursor–product relationship between the two forms of the glutaminase. Furthermore, the 71 kDa peptide is formed and disappears with kinetics that are consistent with that of a true intermediate in the import pathway of the mitochondrial glutaminase. The pulse–chase experiments indicate that the 65 kDa peptide is formed more rapidly than the 68 kDa peptide. The two predominate forms of the glutaminase could be synthesized by competing proteolytic reactions. Alternatively, the 68 and 61 kDa forms could result from covalent modification of the 65 and 58 kDa peptides respectively. Interestingly, the 68 and 61 kDa peptides are not detected when the cells were labelled at 10 °C.

Many other mitochondrial proteins have been shown to be processed to their mature forms via intermediates. As with the glutaminase, the intermediates produced during the synthesis of cytochrome b_2 [32], the Fe–S protein [14] and malate dehydrogenase [33] are all 1 kDa smaller than their initial precursors. The significance of intermediates in the biogenesis of mitochondrial enzymes has been investigated [14,15]. The two-step processing reactions described for the precursors of yeast cytochrome b_2 and the Fe–S protein of *Neurospora* have been shown to be important in determining their localization within the intermembrane space.

Given the submitochondrial localization of the glutaminase, it is unlikely that the formation of the 71 kDa intermediate exposes a sorting signal that further directs its translocation. Thus the sequential proteolysis of the glutaminase precursor may merely reflect a slow step in a concerted series of leader-peptide cleavages. Alternatively, the formation of an intermediate may be essential to initiate the assembly of active tetramers. This process may require the release from a translocator-bound intermediate or the formation of a precise *N*-terminus. The ensuing delay in folding of the translocated protein might allow time for interaction with additional subunits in order to facilitate tetramer formation.

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