Biochimica et Biophysica Acta, 567 (1979) 216–224

BBA 68671

MOLECULAR FORM AND KINETIC PROPERTIES OF PHOSPHATE-DEPENDENT GLUTAMINASE IN THE MITOCHONDRIA ISOLATED FROM THE KIDNEYS OF NORMAL AND ACIDOTIC RATS

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(Received September 11th, 1978)

Key words: Glutaminase; Acidotic rat; Isoenzyme; (Kinetics)

Summary

1. Investigation of the mechanism of renal ammoniagenesis revealed that the K_m of phosphate-dependent glutaminase (L-glutamine amidohydrolase, EC 3.5.1.2) for glutamine was significantly decreased, whereas V was increased if the mitochondria from both normal and acidotic rats were suspended in a hypotonic medium. These and other data indicate that the inner membrane regulates glutaminase activity and that in metabolic acidosis the rate of glutamine influx is increased.

2. The study of molecular form and kinetic properties of glutaminase contained in the mitochondria showed that only in the case of acidotic Wistar rats the enzyme exhibits unusual stability if heated 10 min at 50°C. Furthermore, 3 min heating at 50°C resulted in a strong activation of the enzyme. This suggests that in acidosis the enzyme is in a different molecular form or in a different conformational state.

3. The enzyme is equally sensitive to the inhibition by glutamate and NaCl in both kinds of mitochondria, whereas the value of the Hill coefficient for phosphate is 2. This indicates that glutaminase is in the form of a monomer. It is most probable that in vivo there is an equilibrium between monomer and dimer forms of the enzyme.

4. Determination of the activation energy of glutaminase showed that in the case of normal and acidotic Wistar rats a break in the Arrhenius plot at 28°C and 24°C occurs. This difference results probably from changes in the enzyme microenvironment during adaptation to acidosis.

Introduction

Although the importance of glutamine as the principal source of urinary ammonia is generally accepted [1], the mechanism of complex regulation of

renal ammoniagenesis, especially on subcellular and molecular level, remains unclear. The problem becomes even more complex due to the fact that phosphate-dependent glutaminase (L-glutamine amidohydrolase, EC 3.5.1.2) is an allosteric protein susceptible to a variety of anionic activators and inhibitors [2,3]. Besides, since glutaminase is located inside mitochondria [4], the inner mitochondrial membrane might be another important factor in regulation of ammonia production. In connection with this, it was proposed that the transport of glutamine but not glutaminase activity controls the rate of mitochondrial glutamine deamidation in acidosis [5,6,7]. At the present moment three key questions may be put forward concerning increased ammoniagenesis during acidosis: Does the increased synthesis of ammonia, as an adaptive response to metabolic acidosis, result from (a) a greater amount of the enzyme, (b) the activation of preexisting enzyme, or (c) the increased permeability of the inner mitochondrial membrane for glutamine?

Phosphate-dependent glutamine was thoroughly studied especially by the research groups of Kvamme, Curthoys and Katunuma [8—11]. These studies are related to the molecular and kinetic properties of the purified enzyme. The aim of our investigation was to give a new insight into the molecular and kinetic properties of glutaminase in isolated mitochondria where the enzyme is in a more physiological environment.

Materials and Methods

Rats of Wistar and Sprague-Dawley-derived strains (200—300 g) of both sexes were used. They were fed ad libitum on regulatory laboratory chow. Chronic metabolic acidosis was induced by providing the rats with a 1.5% NH_4Cl solution as their sole source of drinking water for periods of 7—9 days.

Kidney mitochondria were isolated as described previously [12]. This preparation is usually more or less contaminated with microsomes which possess a high activity of the phosphate-independent glutaminase [4]. In order to avoid interference of the activity of this enzyme with the activity of the phosphate-dependent glutaminase the mitochondria were purified by velocity Ficoll step gradient centrifugation [13]. They were essentially free from contaminating phosphate-independent glutaminase and had good respiratory control. Hydrolysis of glutamine was virtually absent in a phosphate free medium. Other kinetic data indicate also that the activity of the phosphate-dependent enzyme was specifically measured. The activity of alkaline phosphatase, which is a microsomal enzyme, was ten times decreased in purified mitochondrial preparation.

Phosphate-dependent glutaminase activity was determined by measuring the formation of [^{14}C]glutamate from [U-^{14}C]glutamine or spectrophotometrically. In the first case, intact mitochondria were incubated 20—30 min at 30°C in isotonic or hypotonic medium of the following composition: isotonic medium 120 mM KCl, 10 mM Tris-HCl, 30 mM Tris-phosphate, 2.5 mM $MgCl_2$, rotenone (1 μg/ml); final pH 7.6. Hypotonic medium 30 mM Tris-phosphate, rotenone (1 μg/ml); final pH 7.6. In both cases the incubation mixture contained 0.9 ml of incubation medium, 50 μl mitochondrial suspension (1—1.5 mg protein) and 50 μl [U-^{14}C]glutamine (0.2 M) (except when the concentra-

tion of the substrate was varied). The reaction was started by adding [U-^{14}C]-glutamine (10 mM final conc.) and finished by 0.25 ml trichloroacetic acid (20%). Radioactive glutamine and glutamate were separated on Dowex 1X8 (acetate form) as described previously [14], and the radioactivity was measured by using a gas-flow Geiger-Muller counter. The quantity (nmol) of glutamate formed was calculated from the amount and specific activity of [U-^{14}C]glutamine added. All assays were done in triplicate and the mean of three determinations was recorded.

The spectrophotometric assay of glutaminase activity was performed as described by Kvamme et al. [2] except that 0.015% Triton X-100 was present. In this case NH_3 production was continuously measured. There was always a linear relationship betwen the amount and activity of the enzyme.

The incubation of the mitochondria in the hypotonic medium was sufficient to alter permeability of the inner membrane but it did not result in the leakage of glutaminase. This is important since inside the mitochondria the enzyme is in a much higher concentration so that interaction between its subunits and their aggregation to catalitically active dimer and polymer forms is easier. Evidence that the enzyme did not leak out was obtained by measuring its activity in the supernatant fraction after incubation and centrifugation of the mitochondria. All the activity was recovered in the pellet fraction.

Oxygen uptake was measured polarographically by means of a Clark oxygen electrode. Mitochondrial proteins were determined by the biuret reagent [15] which contained 1.5% deoxycholate.

[U-^{14}C]Glutamine was obtained from the Radiochemical Centre Amersham. The other chemicals were from Merck (Darmstadt) or from Sigma Co. (St. Louis).

Results

Latency of phosphate-dependent glutaminase

If the transport of glutamine across the inner membrane is the rate-limiting step for glutaminase activity, a change in the K_m and V would be expected [16, 17]. This was really found with both normal and acidotic mitochondria. When the normal mitochondria were suspended in a hypotonic medium a decrease of the K_m from 3.0 to 1.5 mM and an increase of the V from 30 to 40 nmol · min^{-1} · mg^{-1} protein were observed indicating the phenomenon of latency. An increase of the concentration of the substrate, decreased enzyme latency. Similar results were obtained with acidotic mitochondria except that the decrease of the K_m was less pronounced (from 2.9 to 2.2 mM), whereas V was more increased (from 100 to 202 nmol · min^{-1} · mg^{-1} protein). The kinetic constants were calculated by the method of least squares. It should be mentioned that under our experimental conditions (rotenone present) the existence of a significant phosphate and pH gradient between the inside and the outside of the mitochondria suspended in the isotonic medium and their influence on enzyme kinetics should not be expected.

Heat stability of glutaminase

Experimental data reported by Katunuma and collaborators showed that

phosphate-dependent glutaminase is very heat labile [3]. Curthoys and coworkers found that in the absence of phosphate, the half-life of activity of the purified enzyme at 37°C was 12 min [11]. In whole rat kidney homogenate the phosphate-dependent glutaminase is completely denaturated by heating at 50°C for 10 min while the phosphate-independent glutaminase is completely stable to such treatment. This provided a method to prepare phosphate-independent glutaminase free of phosphate-dependent isoenzyme activity [18]. We found, however, that phosphate-dependent glutaminase in intact mitochondria isolated from kidney of acidotic Wistar rats has unusual stability so that heating at 50°C for 10 min had little or no effect on its activity (Fig. 1). The enzyme in normal mitochondria was inactivated 70—80%. It is interesting that 3 min heating at 50°C resulted in a strong activation of glutaminase in acidotic mitochondria which in some cases amounted to more than 100%. Similar investigation with Sprague-Dawley-derived rats showed that there is no significant difference in enzyme stability between normal and acidotic mitochondria and that phosphate-dependent glutaminase is generally more heat-labile in this strain of rats (Fig. 1). This finding suggests that the increase of heat stability observed in Wistar rats is not essential for renal adaptation to acidosis, at least in some strain of rats. However, this opens several interesting questions of which the following are especially important: is this heat stability the result of existence of glutaminase in acidotic mitochondria in a different molecular form or is it the consequence of the difference of the enzyme environment?

Protection of glutaminase from heat inactivation in normal mitochondria

Preincubation of normal kidney mitochondria with phosphate, phosphate-borate or Bromothymol blue which induce aggregation of enzyme molecules,

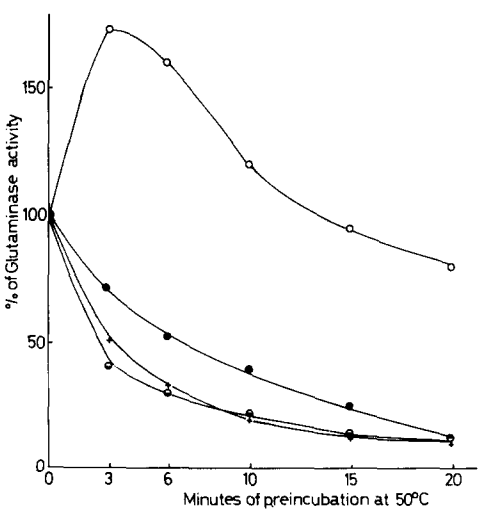

Fig. 1. The activity of phosphate-dependent glutaminase after preincubation of intact kidney mitochondria at 50°C. The mitochondria were isolated from normal (●) or acidotic (○) Wistar rats and from Sprague-Dawley-derived normal (⊜) or acidotic (+) rats. They were preincubated in the isolation sucrose (approx. 20—30 mg protein/ml). The enzyme activity was determined by measuring the formation of [^{14}C]glutamate from [U-^{14}C]glutamine after incubation of heated mitochondria in the standard hypotonic medium.

fully protected the enzyme from heat inactivation (Table I). This indicated that the percentage of the enzyme which is denaturated at 50°C is probably present as monomer (Tris-form) or that already aggregated complex has low stability. It has been reported that there is a correlation between phosphate concentration, heat stability and dimer formation [10,11]. Does it mean, that in acidotic mitochondria the enzyme is already present as a stable dimer or polymer?

Effect of glutamate and NaCl on enzyme activity in normal and acidotic mitochondria

If glutaminase is already present as oligomer or polymer, the inhibitory effect of glutamate will be much less pronounced [2], whereas the inhibition by NaCl will be virtually absent [10,11,19]. However, as can be seen from Table I and Figs. 2 and 3 the enzyme is almost equally sensitive to glutamate and NaCl inhibition in both normal and acidotic mitochondria. Strong and nearly equal activation of glutaminase in both kind of mitochondria with tetraphenyl borate, which induces association of the enzyme molecules [20], also suggests that the enzyme is not in a polymeric form.

Mode of activation of glutaminase by phosphate

There is question whether the enzyme is in the form of monomer or dimer, or whether there is an equilibrium between these two molecular forms. Determination of glutaminase activity in normal and acidotic mitochondria against phosphate concentration gives a sigmoidal curve in both cases indicating that the enzyme is in a Tris-form. Further evidence for this conclusion comes from the analysis of these data by means of Hill plot (Fig. 4). In both cases the n

TABLE I

HEAT STABILITY, PROTECTION AND ACTIVATION OF PHOSPHATE-DEPENDENT GLUTAMINASE

The mitochondria were isolated from normal and acidotic Wistar rats. They were heated 10 min at 50°C in the sucrose isolation medium or in the medium containing 100 mM Tris-phosphate plus 10 mM sodium borate, 100 mM Tris phosphate or 30 mM Tris phosphate plus 0.125 mM Bromothymol blue. In the cases where the mitochrondria were preincubated at 50°C with activators the same mixture was used for the enzyme assay. In other cases the standard hypotonic medium was employed. Before heating the mitochondria were allowed to swell for several minutes and then preincubated 15 min with activators. The enzyme assay was performed as described in Fig. 1.

Experimental conditions	Glutaminase activity (nmol · min^{-1} · mg^{-1} protein)			
	Normal		Acidotic	
	Activity	%	Activity	%
Control	45.2	100	98.6	100
Heated	12.3	27	83.3	85
Phosphate + borate	107.6	236	207.4	210
Phosphate	83.7	187		
Heated with phosphate	87.2	192		
Tetraphenyl borate (0.5 mM)	89.6	196	251.6	254
Heated with bromomethyl blue	68.4	150		
Glutamate (5 mM)	7.0	15	12.0	12
Glutamate (10 mM)	4.5	10	7.0	7

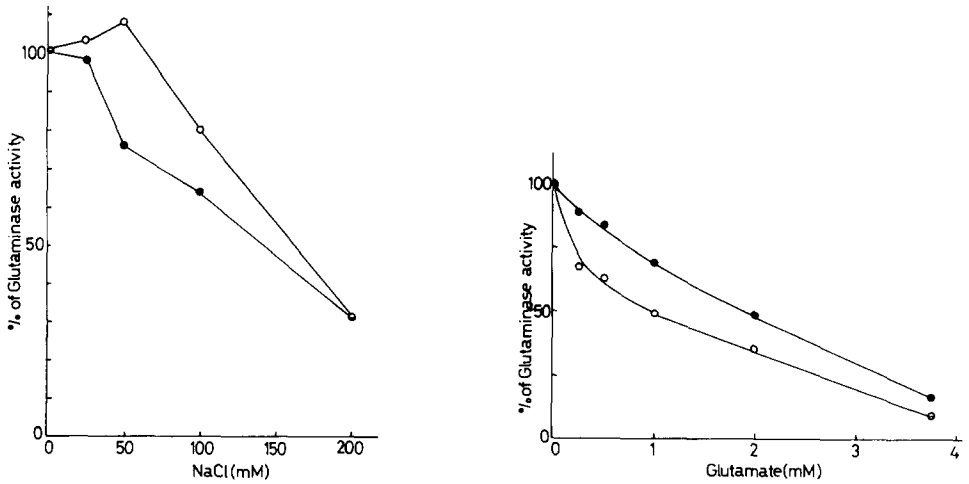

Fig. 2. Effect of NaCl on the activity of phosphate-dependent glutaminase in mitochondria isolated from kidneys of normal (●) and acidotic (○) Wistar rats. The standard hypotonic medium was used as an assay solution. The mitochondria were allowed to swell for several minutes in the hypotonic medium before NaCl was added. The enzyme activity was assayed as described in Fig. 1.

Fig. 3. Inhibition of glutaminase activity by glutamate in mitochondria isolated from kidneys of normal (●) and acidotic (○) Wistar rats. The enzyme activity was estimated in the hypotonic medium as described in Fig. 1.

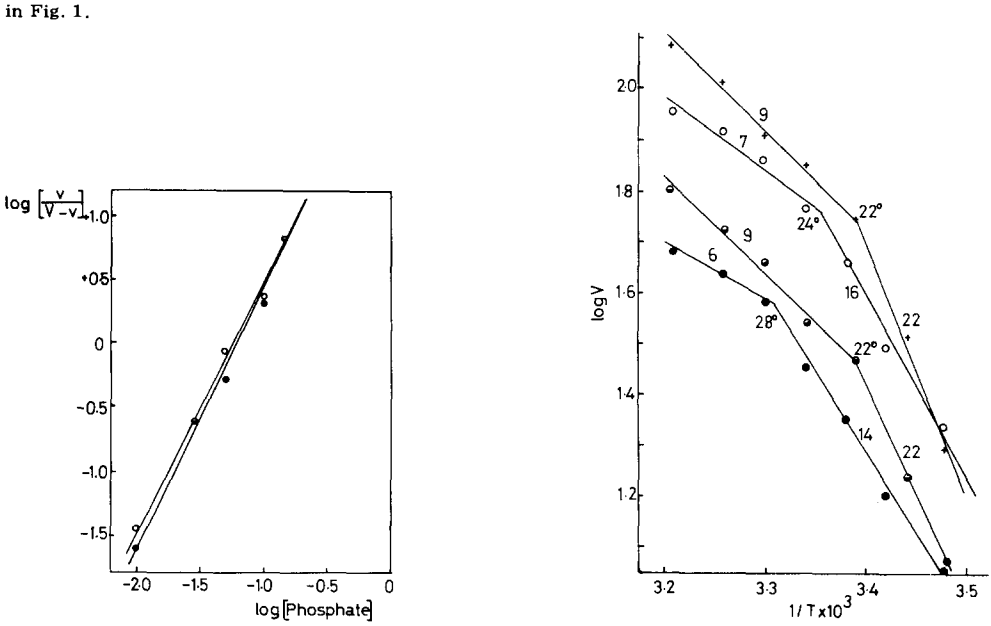

Fig. 4. Hill plots of data derived from reciprocal plots of glutaminase activity against phosphate concentration (0.010—0.150 M). n values (for phosphate) were calculated from the slopes. ●, normal rats, n = 2.04; ○, acidotic rats, n = 2.06. In the case where the concentration of phosphate was more than 30 mM the mitochondria were allowed to swell for several minutes in the standard hypotonic medium before concentration of the activator was adjusted. The same results were obtained when Sprague-Dawley rats were used.

Fig. 5. Arrhenius plots of glutaminase activities. The mitochondria isolated from kidneys of normal (●) and acidotic (○) Wistar rats or normal (⊖) and acidotic (+) Sprague-Dawley rats were incubated in the standard hypotonic medium. Enzyme assay was performed as described in Fig. 1. Values for energy of activation (kcal/mol) are shown above each plot. T, absolute temperature.

values for phosphate equal 2 indicating again that the enzyme is in monomeric form.

Time-dependence of enzyme activation

The time-dependent activation occurs following the incubation of the Tris-enzyme with phosphate or phosphate plus borate. It is suggested that this activation reflects a transition of the Tris enzyme to higher molecular forms [8,9]. Our investigation showed that there is no time-dependent activation of glutaminase. Instead, a linear relationship between time and activity of the enzyme was found. This suggests that the enzyme is already present as a dimer which is contrary to the previous conclusion. Experiments carried out in the presence of 150 mM Tris-phosphate showed the same linear relationship. There was no strain difference. When the initial rate of the enzyme activity was measured spectrophotometrically in the presence of 30 mM Tris-phosphate the same results were obtained. Yet, we assume that the time-dependent activation does occur, but since the enzyme is in a very high concentration inside the mitochondria, the aggregation of glutaminase molecules is too fast to be recorded by our technique. It is interesting to note that only in the case of acidotic mitochondria the time-dependent activation of glutaminase did occur if the enzyme was heated at 50°C which probably weakened interaction between glutaminase subunits. This finding suggests that glutaminase molecules display a higher degree of co-operativity in acidotic than in normal mitochondria.

Activation energy change of glutaminase in normal and acidotic mitochondria

It was reported that glutaminase is associated with the inner mitochondria membrane [14,21]. Determination of the activation energy of the enzyme showed a break in the Arrhenius plot indicating that physical state of the phospholipid membrane modulates glutaminase activity [14]. We assumed in relation to this that the high heat stability of glutaminase in acidotic mitochondria of Wistar rats is a consequence of a specific phospholipid composition of the membrane. It has been shown, for instance, that activity of mitochondrial ATPase is regulated by length and degree of unsaturation of phospholipid acyl chains [22]. In accordance with the previous finding, a break or discontinuity in the Arrhenius plot and a decrease of the energy above the break was observed (Fig. 5). In the case of acidotic mitochondria isolated from the kidneys of Wistar rats the discontinuity at 24°C was found, whereas the enzyme from normal rats showed the break at 28°C. Glutaminase from both normal and acidotic Sprague-Dawley rats showed the break at 22°C. These significant differences suggest that, probably due to a different phospholipid composition of the membrane, the phase transition from crystalline to liquid crystalline occurs at different temperatures.

Discussion

It was proposed a few years ago that during acidosis there is an increase in the capacity of kidney mitochondria to take up glutamine, probably as the result

of an adaptive increase in the specific glutamine carrier [5,6]. However, the transport data obtained in these studies do not measure initial rates of glutamine influx but the steady state level of ^{14}C label from [^{14}C]glutamine which is represented mainly as [^{14}C]glutamate. Therefore, they do not represent a direct measure of glutamine transport. Even if the measurement of the initial rate is achieved by applying rapid Millipore filtration technique there is still the problem of glutamine hydrolysis which can not be completely inhibited in intact mitochondria. For this reason we assumed that the study of enzyme latency could be a useful approach to the problem. Higher K_m for glutamine which was found in the isotonic medium suggests that the influx of the substrate was one of the factors controlling glutaminase activity in intact mitochondria. This finding implies that the increase in the amount of the enzyme during acidosis must be followed by the increase of the rate of glutamine transport. The decrease of V in isotonic medium could be the consequence of a lower rate of glutamine influx or/and glutamate efflux. It was reported that the inner membrane of kidney mitochondria has very low permeability for glutamate [23]. Also, the study of the kinetics of purified enzyme showed that V is decreased if glutamate is present [2]. Glutaminase latency would be probably much less pronounced if glutamate was metabolised.

Transformations of glutaminase from one molecular form to another due to protein-protein interaction suggests that this enzyme might represent, according to Frieden, slowly equilibrating association-dissociation system [24]. An increase of the amount of the enzyme in the mitochondria during acidosis should favour faster interaction and better co-operativity of its subunits. This should have an influence on its molecular form and kinetics. It was suggested in relation to this that in acidotic mitochondria self-association of glutaminase may occur in the absence of ligand at high protein concentration (2 mg/ml) and that in this situation the enzyme may already be present as a polymer [10]. Our finding of an unusual heat stability of the enzyme in acidotic mitochondria suggested the same assumption. However, kinetic analysis of the inhibition of glutaminase by glutamate and NaCl and the mode of its activation by phosphate strongly suggest that the enzyme is in the form of a monomer.

Another explanation of the unique behavior of glutaminase in acidotic Wistar rats could be that in this case the enzyme is in a different environment or in the presence of a specific ligand so that it is in a different conformational state which is much less sensitive to heat inactivation. The results obtained by the analysis of the activation energy of glutaminase indicate that there is a change in the enzyme environment in acidosis. It is possible that changes of phospholipids of the inner membrane, especially of the composition of its acyl chains, are responsible for the specific behavior of the enzyme. All the regulatory effects which are possible in intact mitochondria may be missed under more or less artificial enzyme assay conditions. It is found, for instance that there is no any difference in molecular and kinetic properties between purified glutaminase from normal and acidotic rat kidney [25]. However, there are a number of possibilities in vivo for reactivation of preexisting enzyme which under physiological conditions does not display full capacity. This conclusion holds especially for the acute acidosis where ammonia synthesis is increased without a concommitant increase of the amount of glutaminase [1].

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