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IMPORTANCE OF THE FLUX OF PHOSPHATE ACROSS THE INNER MEMBRANE OF KIDNEY MITOCHONDRIA FOR THE ACTIVATION OF GLUTAMINASE AND THE TRANSPORT OF GLUTAMINE

ZORAN KOVAČEVIĆ

Department of Biochemistry, Medical School, 21000 Novi Sad (Yugoslavia)

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SUMMARY

The effect of mersalyl, an inhibitor of phosphate transport across the inner mitochondrial membrane, was investigated on the uncoupled respiration of pig kidney mitochondria in the presence of glutamine as substrate and on the activity of the phosphate-dependent glutaminase in the intact organelles. In addition, the submitochondrial location of the enzyme was reinvestigated.

(1) It was found that mersalyl completely inhibits uncoupled respiration of the mitochondria in the presence of glutamine as substrate, whereas respiration with glutamate was not affected. The same amount of mersalyl which inhibits coupled oxidation of glutamine also inhibits coupled oxidation of glutamate and some other substrates.

(2) Mersalyl strongly inhibited the activation of glutaminase in intact mitochondria only in the presence of inhibitors of electron transport or of an uncoupler. The addition of a detergent prevented or fully released the inhibition. The effect of mersalyl was observed even when the mitochondria were pre-incubated with phosphate or incubated in the phosphate-free medium. If mersalyl and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were added 3 min after pre-incubation with phosphate the same intramitochondrial concentration of the anion as in control experiments was found, whereas the activity of glutaminase was severely inhibited. These findings suggest that the activation of the enzyme by phosphate in intact nonenergized mitochondria occurs only if the activator moves across the inner mitochondrial membrane.

(3) Mersalyl (plus CCCP) markedly decreased [^{14}C]glutamine- and [^{32}P]-phosphate-permeable mitochondrial spaces. A close correlation between the decrease of phosphate and glutamine permeable spaces and the inhibition of glutaminase activity was found.

(4) If the activation energy of the enzyme was determined with frozen mitochondrial preparations, a discontinuity or break in the Arrhenius plot was observed, whereas the presence of a detergent completely abolished the break. Digitonin or ultra-

Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

sonic treatment of the mitochondria followed by separation of the membrane and the soluble fraction revealed that glutaminase is a membrane-bound enzyme.

On the basis of these findings it is concluded that there is an association between the transport of phosphate on one side and the transport of glutamine and glutaminase activity on the other. It is possible that the movement of phosphate across the membrane activates the enzyme which facilitates diffusion of glutamine down a concentration gradient. However, the existence of a specific glutamine-phosphate carrier is not ruled out.

INTRODUCTION

Phosphate dependent glutaminase (EC 3.5.1.2), which is one of the key enzymes in glutamine and ammonia metabolism, has been thoroughly investigated, especially in the last ten years [1–4]. The investigations carried out by Kvamme and collaborators [4, 5] were particularly important in the elucidation of the molecular properties of pig kidney glutaminase. This group of investigators has clearly shown that glutaminase exists in different interconvertible molecular forms. In the absence of phosphate, a so-called Tris · Cl form appears, which is virtually inactive. Addition of phosphate induces aggregation of the enzyme molecules resulting in the appearance of the active P-form which is a dimer of the Tris · Cl form. If borate is present together with phosphate, 25 or more Tris · Cl enzyme molecules aggregate to the phosphate-borate form. The process of the polymerization is time dependent. These molecular forms of glutaminase differ in respect to solubility, kinetic properties and susceptibility to a variety of anionic activators and inhibitors. It has been suggested that in addition to phosphate, acetyl-CoA and some intermediates of the citric acid cycle may be physiological activators of glutaminase [6, 7]. Glutamate is known as a powerful inhibitor of the enzyme.

There is no doubt that the phosphate-dependent glutaminase is a mitochondrial enzyme [8–10], although its submitochondrial location is still uncertain. According to some investigators [10, 11] kidney glutaminase is located in the mitochondrial matrix, whereas others suggest that the enzyme is contained in the inner mitochondrial membrane [9]. We have been interested for a long time in the regulation of glutaminase activity in intact mitochondria [12]. The aim of this investigation was to obtain some new data that will help us to understand the mode of activation and regulation of glutaminase in its physiological environment, especially in relation to the transport of glutamine, and to elucidate the submitochondrial location of the enzyme.

MATERIALS AND METHODS

In this communication we present only the data which were obtained with pig renal cortex mitochondria, although in many cases the same was observed using rat kidney mitochondria. The mitochondria were isolated as described elsewhere [12].

Oxygen uptake was measured polarographically by means of a Clark electrode.

Swelling of the mitochondria in isosmotic solutions of glutamine and ammonium phosphate was recorded at 520 nm on a Unicam 1800 spectrophotometer.

The activity of glutaminase was measured by continuously recording the production of ammonia with an NH_4^+-sensitive glass electrode as described elsewhere [12] or by measuring production of [^{14}C]glutamate from [U-^{14}C]glutamine. The standard incubation medium for these experiments and for determination of oxygen consumption was of the following composition: 100 mM Tris · Cl, 30 mM Tris phosphate, 2.5 mM $MgCl_2$. The final pH was 7.4 and the final volume was 5 ml; the temperature was 30 °C. The amount of the mitochondria used for assay was usually 6–7.5 mg protein.

Mitochondrial spaces available to sucrose, glutamine and phosphate were determined by a gravimetric-isotopic technique [13]. Mitochondria (13–15 mg protein in 0.2 ml) were suspended in 0.9 ml of the standard Tris · Cl medium described above. After 3 min pre-incubation at 30 °C [^{14}C]sucrose was added followed by the addition of cold glutamine (8 mM). Incubation was carried out for 1 min (after addition of glutamine) and the suspension was centrifuged at 15 000 × *g* for 2 min in a tared centrifuge tube (Eppendorf 3200). The supernatant fluid was decanted and fluid adhering to the walls of the centrifuge tubes was removed with a cotton swab. After weighing, the mitochondrial pellet (solubilized with 0.5 ml of 3 % deoxycholate) and the supernatant solution were quantitatively transferred into 10-ml cylinders. Radioactivity was measured by using a gas-flow Geiger-Muller counter. The same procedure was employed in order to determine [^{14}C]glutamine and [^{32}P]phosphate spaces. In the first case almost all radioactivity was found in glutamine and glutamate. The radioactivity found in the inner space which corresponds to [^{14}C]glutamate was identified as [^{14}C]glutamine, since for such glutamate formation to occur glutamine must penetrate the inner mitochondrial membrane and reach the site of glutaminase.

Mitochondrial proteins were determined by the biuret reagent [14], which contained 1.5 % deoxycholate.

Mersalyl was a gift from Sigma Co., and avenaciolide was a generous gift of Dr. W. B. Turner (I.C.I. Ltd., Macclesfield, U.K.).

RESULTS

Inhibition of uncoupled respiration of kidney mitochondria by mersalyl in the presence of glutamine as substrate

Mersalyl, a well known inhibitor of the transport of inorganic phosphate across the inner mitochondrial membrane [15, 16], inhibited coupled respiration of mitochondria in the presence of glutamine as substrate, as expected (Fig. 1). However, the addition of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) did not release the inhibition. The same effect was found if glutamine was oxidized in the presence of an uncoupler (Fig. 1b). If glutamate was used as a substrate, mersalyl did not have any effect on the uncoupled respiration of the mitochondria. Furthermore, glutamate completely released the inhibition observed in the presence of glutamine. It can be seen from the same figure that avenaciolide inhibited respiration in the presence of glutamate but not of glutamine.

Effect of mersalyl on the swelling of kidney mitochondria in the isosmotic solution of glutamine and ammonium phosphate

As may be expected, mersalyl, at the concentration that inhibited respiration

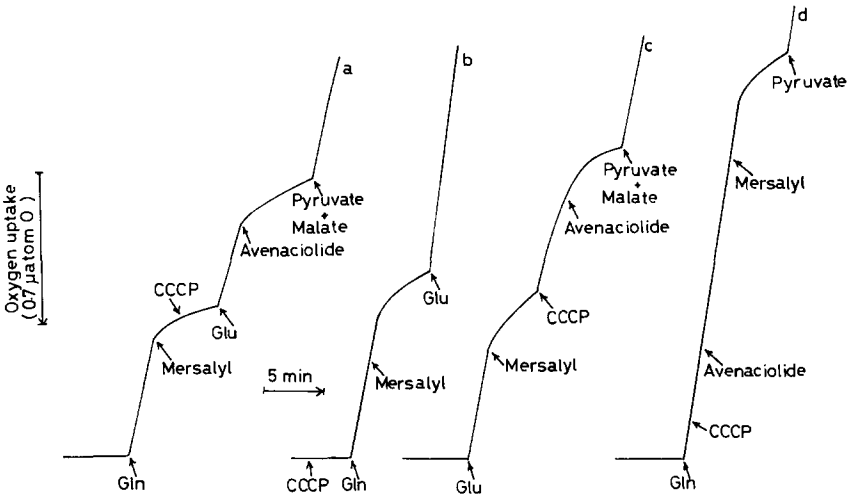

Fig. 1. Inhibition of the respiration of pig kidney mitochondria by mersalyl in the presence of glutamine as substrate. The mitochondria (about 6–7.5 mg protein) were incubated in the standard Tris · Cl medium described in the Materials and Methods section. In all cases 0.5 mM ADP was present. Additions (where indicated): 1 mM glutamine, 40 μM mersalyl, 1 μg/ml CCCP, 4 mM glutamate, 30 μM avenaciolide, 1.3 mM malate and 0.2 mM pyruvate. The same effect was observed in the KCl medium.

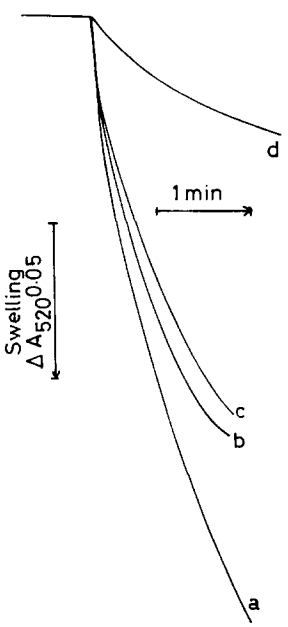

Fig. 2. Effect of mersalyl on the swelling of mitochondria in an isosmotic solution of glutamine or ammonium phosphate. The mitochondria (1.2 mg protein) were suspended in (a) 100 mM ammonium phosphate, (b) 220 mM glutamine with 40 μM mersalyl, (c) 220 mM glutamine and (d) 100 mM ammonium phosphate with 40 μM mersalyl. In all cases 20 mM Tris · Cl, 1 mM EDTA and 1 μg/ml rotenone were present. The final volume was 2.5 ml and the final pH was 7.5; the temperature was 22 °C. Swelling of the mitochondria was followed by measuring decrease of absorbance at 520 nm.

in the presence of glutamine, strongly inhibited swelling of the mitochondria in an isosmotic solution of ammonium phosphate (Fig. 2). However, we could not observe inhibition of swelling of the mitochondria in isosmotic glutamine.

Effect of mersalyl on the activation of glutaminase in intact mitochondria

The experiments with the oxygen electrode suggested that mersalyl strongly inhibits glutaminase in intact mitochondria (Fig. 1). This was confirmed by experiments using the NH_4^+ electrode which showed that the addition of mersalyl brought about complete inhibition of glutaminase in the preparation of intact mitochondria (Fig. 3). This inhibition was observed only in the presence of inhibitors of the respiratory chain or an uncoupler and was fully released by adding Triton X-100. In these experiments rotenone was added after the addition of the mitochondria and before mersalyl, whereas CCCP was always added after mersalyl. Since the mitochondria were pre-incubated 3–5 min in phosphate medium before the addition of mersalyl, in order to reach the steady-state concentration of the activator of glutaminase in the matrix space, this suggests that the effect of mersalyl is not due to prevention of accumulation of phosphate but to inhibition of the movement of phosphate across the inner mitochondrial membrane.

The same inhibitory effect of mersalyl was observed if experiments were performed in phosphate-free medium, probably because of inhibition of the cycling of endogenous phosphate (Fig. 3).

In some experiments succinate was added before mersalyl in order to load the mitochondria with phosphate. It was found that the mitochondria swell quite extensively under these conditions. However, the addition of CCCP after mersalyl

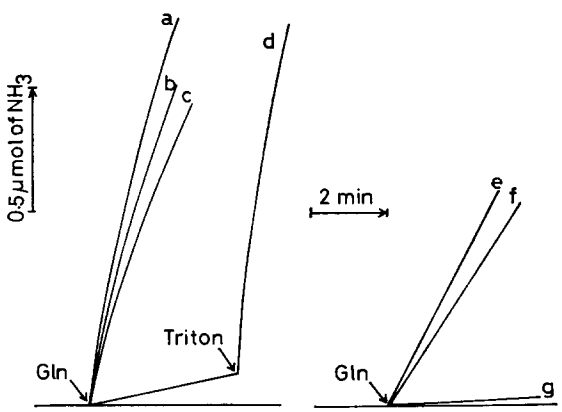

Fig. 3. Effect of mersalyl on the activity of glutaminase in the preparation of intact pig kidney mitochondria. The mitochondria approximately (6–7.5 mg protein) were pre-incubated 5 min in the standard Tris · Cl medium before the addition of mersalyl. Additions: (a) none; (b) 1 µg/ml CCCP or 1 µg/ml rotenone; (c) 40 µM mersalyl; (d) 1 µg/ml rotenone (added before mersalyl) or 1 µg/ml CCCP (added after mersalyl), 40 µM mersalyl and 0.02 % Triton X-100 (where indicated); (e) none or 40 µM mersalyl; (f) 1 µg/ml rotenone and (g) 1 µg/ml rotenone (added before the mitochondria) or 1 µg/ml CCCP (added after the mitochondria) plus 40 µM mersalyl. In the cases under e, f and g the standard Tris · Cl medium without phosphate was used and the mitochondria were added after mersalyl. In each case the reaction was started by adding 8 mM glutamine.

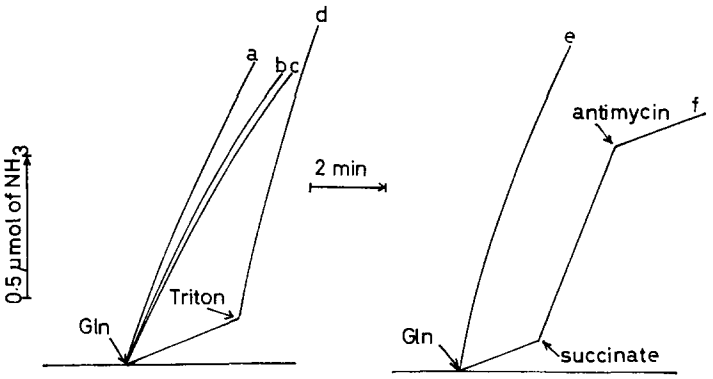

Fig. 4. Effect of mersalyl on the activity of glutaminase in the presence of succinate. In all these experiments the standard Tris · Cl medium was used. Additions: (a) 3 mM succinate; (b) 3 mM succinate and 40 μM mersalyl; (c) 3mM succinate and 1 μg/ml CCCP; (d) 3 mM succinate, 40 μM mersalyl, 1 μg/ml CCCP (added after mersalyl) and, where indicated, 0.02 % Triton X-100; (e) 1 μg/ml rotenone, 3 mM succinate and 40 μM mersalyl; (f) 1 μg/ml rotenone, 40 μM mersalyl and, where indicated, 3 mM succinate and 1 μg/ml antimycin. In all the cases, except under f, succinate was added before the mitochondria, and the mitochondria were pre-incubated 3 min before the addition of mersalyl.

TABLE I

PENETRATION OF MITOCHONDRIAL SPACE BY [^{14}C]SUCROSE, [U-^{14}C]GLUTAMINE AND [^{32}P]PHOSPHATE

The experimental conditions and technique for the determination of solute spaces are described in the Material and Methods section. The concentration of [U-^{14}C]glutamine was 8 mM, and the metabolite was always added after mersalyl (23 nmol/mg protein) and CCCP (1 μg/ml). In the case where [^{32}P]phosphate-permeable space was measured a phosphate-free standard Tris · Cl medium was used. The final concentrations of phosphate were 30 mM and 3 mM. The results are expressed as the means ±S.E. and the number of experiments is given in the parentheses.

Solute and conditions	Solute spaces (% of total pellet volume)
[^{14}C]Sucrose	
control	84±0.5 (8)
+mersalyl+CCCP	87±0.6 (5)
[^{14}C]Glutamine	
control	96±2.0 (20)
+mersalyl+CCCP	63±3.0 (20)
[^{32}P]Phosphate (30 mM external concn.)	
control	85±0.8 (5)
+mersalyl+CCCP (added after P_i)	85±1.2 (5)
+mersalyl+CCCP (added before P_i)	72±1.3 (5)
[^{32}P]Phosphate (3 mM external concn.)	
control	119±0.5 (5)
+mersalyl+CCCP (added after P_i)	115±1.3 (5)
+mersalyl+CCCP (added before P_i)	85±1.6 (5)

caused complete inhibition of glutaminase (Fig. 4). In the case where rotenone and mersalyl were present, addition of succinate (after mersalyl) activated the enzyme, whereas antimycin inhibited it (Fig. 4). Since mersalyl also inhibits dicarboxylate-phosphate exchange we assume that in this case succinate entered the inner space on the dicarboxylate-dicarboxylate carrier providing that the mitochondria contained a catalytic amount of malate [15]. This is supported by the finding that succinate released the inhibition of uncoupled glutamine oxidation by mersalyl.

Effect of mersalyl and CCCP on penetration of mitochondrial spaces by [^{14}C]sucrose, [^{14}C]glutamine and [^{32}P]phosphate

Table I shows that mersalyl and CCCP markedly decreased glutamine and phosphate-permeable spaces, whereas sucrose-permeable space was slightly increased. It should be noted that in this case the glutamine-permeable space was lower compared to sucrose space. The same was found for phosphate if its external concentration was high (30 mM). These results are not compatible with the two-space model but can be explained by a one-space model [18]. They are consistent with the explanation that these solutes penetrate mitochondrial spaces by a saturable process. The activity of glutaminase, measured under the same conditions, was more than 90 % inhibited in the presence of mersalyl plus CCCP.

If mersalyl and CCCP were added 3 min after pre-incubation of the mitochondria with phosphate, the same intramitochondrial concentration of the anion as in control experiments was found, whereas the activity of glutaminase was severely inhibited. It should be noted that at 3 mM external concentration of phosphate the anion accumulated in the mitochondria.

Correlation between the transport of phosphate, glutaminase activity and the transport of glutamine

By increasing the amount of mersalyl it was possible to show a very close correlation between the inhibition of transport of phosphate on one side and inhibition of glutaminase activity and transport of glutamine on the other (Table II).

A correlation was also found between the inhibition of coupled respiration of the mitochondria with glutamine and the inhibition of glutaminase activity (Fig. 5). The same amount of mersalyl was needed to inhibit coupled oxidation of glutamate, indicating that the inhibition of the transport of phosphate is the primary event.

Activation energy change of glutaminase

Some mitochondrial enzymes have discontinuities or breaks in the Arrhenius plots [19, 20]. The breaks were abolished when the mitochondria were solubilized with detergents. This phenomenon was explained on the basis that a lipid-phase change induces a conformational change in the membrane-bound enzymes, resulting in the alteration of the activation energy of the enzymes. Recently, Matlib and O' Brien [21] reported that a lipid-phase change in the inner membrane would not only affect the firmly bound inner membrane enzymes but also those loosely attached to the membrane. In agreement with this we found a break in the Arrhenius plot of glutaminase if the activation energy of the enzyme was determined with frozen mitochondrial preparations. The presence of Triton X-100 completely abolished the break (Fig. 6).

TABLE II

PENETRATION OF MITOCHONDRIAL SPACE BY [^{32}P]PHOSPHATE AND [U-^{14}C]-GLUTAMINE, AND THE ACTIVITY OF GLUTAMINASE IN THE PRESENCE OF DIFFERENT AMOUNTS OF MERSALYL

Phosphate space was determined as described in the Material and Methods. The mitochondria (13 mg protein) were suspended in 0.9 ml phosphate-free Tris · Cl medium with increasing amounts of mersalyl, plus CCCP (1 μg/ml), followed by the addition of 10 mM [^{32}P]phosphate. After 3 min, 8 mM glutamine was added and incubation was continued for another 1 min. In the case of the estimation of the glutamine-space the same amounts of the mitochondria were suspended in the Tris · Cl medium with 10 mM unlabelled phosphate. After a 3 min pre-incubation, mersalyl and CCCP were added followed by the addition of 8 mM [U-^{14}C]glutamine. 1 min later the mitochondria were spun down. The activity of glutaminase was determined under the same experimental conditions as the glutamine space (the mitochondria were pre-incubated with phosphate) except that incubation was stopped by adding 0.3 ml trichloroacetic acid (20 %). The results are expressed as the mean ±S.E. and the number of experiments are given in the parentheses.

Amount of mersalyl (nmol/mg protein)	Solute spaces (% of total pellet volume)		% of glutaminase activity
	[^{32}P]phosphate	[^{14}C]glutamine	
0	102±1.9 (5)	103±2.0 (5)	100
11.5	104±2.0 (5)	103±1.4 (5)	106
15.4	98±0.5 (5)	95±3.0 (5)	84
19.2	94±1.6 (5)	89±2.1 (5)	19
23.0	87±0.5 (5)	84±1.9 (5)	5

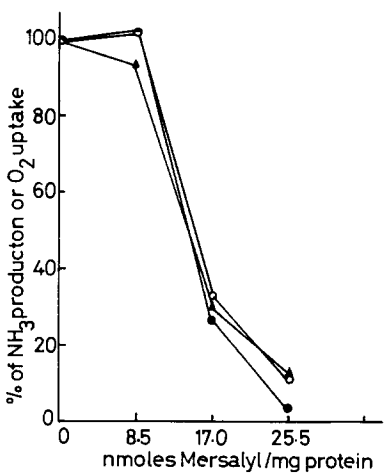

Fig. 5. Inhibition by mersalyl of glutaminase activity (○) and coupled respiration of the mitochondria in the presence of glutamine (●) or glutamate (▲). The mitochondria (6 mg protein) were incubated in the standard Tris · Cl medium. Additions: (a) O_2 electrode experiments, 0.4 mM ADP, 1 mM glutamine or 2.5 mM glutamate, mersalyl was added before the substrates; (b) NH_4^+ electrode experiments, 8 mM glutamine and 1 μg/ml CCCP. Mersalyl was added 3 min after the mitochondria and before CCCP. The final volume of the incubation medium was 5 ml and the temperature was 30 °C in both cases.

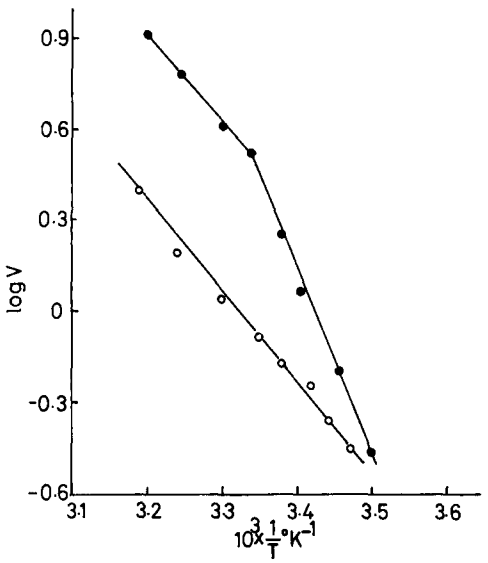

Fig. 6. Arrhenius plots of the phosphate-dependent glutaminase. Initial rates of the enzyme reaction were determined with an NH_4^+ electrode. The incubation medium was 100 mM Tris/phosphate (pH 8.0). The final volume was 5 ml and the amount of the mitochondria was about 6 mg protein. Isolated mitochondria were stored at −18 °C for 24 h prior to use. (●) Frozen mitochondria; (○) Triton X-100 (0.05 %) lysed frozen mitochondria.

TABLE III

DIGITONIN AND ULTRASONIC TREATMENT OF KIDNEY MITOCHONDRIA AND DISTRIBUTION OF ENZYMES BETWEEN THE MEMBRANE (PELLET) AND THE SOLUBLE FRACTION

Treatment of the mitochondria	Percentage of activity recovered								
	Glutaminase			Glutamate dehydrogenase			Cytochrome oxidase		
	pellet	soluble	recovery	pellet	soluble	recovery	pellet	soluble	recovery
With digitonin (mg digitonin/mg protein)									
0	92	6	98	54	34	88	88	3	91
0.03	81	10	91	41	59	100	91	3	94
0.06	75	10	85	33	67	100	97	6	103
0.12	2	15	17	14	86	100	108	2	110
Sonication (s)									
0	110	0	110	80	12	92	95	3	98
1×15	81	4	85	44	56	100	102	2	104
2×15	60	9	69	27	85	112	93	5	98
3×15	35	8	43	16	84	100	97	3	100

Distribution of glutaminase between the membrane and the soluble fraction after digitonin and ultrasonic treatment of kidney mitochondria

Before treatment with digitonin (Merck) the mitochondria were stored overnight at −18 °C and then suspended in ice-cold 100 mM Tris · Cl (pH 7.4) with increasing concentration of the detergent (Table III). After 20 min the suspension was diluted six times with the same buffer and centrifuged for 10 min at 20 000 × *g*. The pellet was resuspended in the same buffer.

In the case of ultrasonic treatment the freshly prepared mitochondria were suspended in 100 mM Tris · Cl, pH 7.4 (approx. 10 mg protein/ml) and sonicated with an MSE ultrasonic disintegrator at 3 μm in aliquots of 3 ml (1.7 mm-diameter probe). The duration of sonication was increased from 15 to 45 s (Table III). The sonicate was centrifuged for 30 min at 100 000 × *g*. The pellet was resuspended in the same buffer.

The activities of glutaminase, glutamate dehydrogenase and cytochrome oxidase were determined in all fractions (total, pellet and soluble). The last two enzymes were markers for the matrix and the inner membrane respectively. Glutaminase was assayed in 100 mM Tris/phosphate buffer (pH 8.0) by measuring formation of [^{14}C]glutamate after its separation from [U-^{14}C]glutamine [22]. Glutamate dehydrogenase and cytochrome oxidase were assayed as described elsewhere [8]. Table III shows that after mild treatment of the mitochondria with digitonin or ultrasonic vibration 80 % of the glutaminase is found in the membrane fraction, compared to 40 % of the glutamate dehydrogenase. By increasing the digitonin/protein ratio or by more vigorous ultrasonic treatment of the mitochondria, glutaminase was detached from the inner membrane and inactivated. The inactivation appears, probably, to be due to dissociation of the enzyme to its subunits. The activity can be

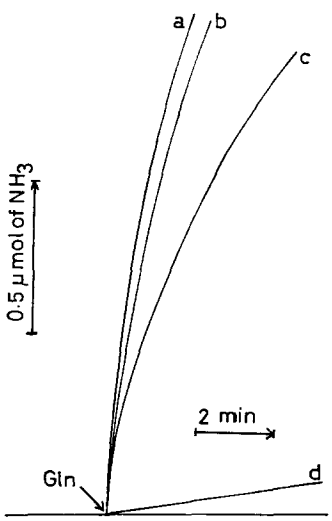

Fig. 7. Inhibition of glutaminase activity by glutamate in the preparation of intact and lysed mitochondria. Mitochondria (approx. 6–7.5 mg protein) were suspended in the standard Tris · Cl medium. Additions: (a) 0.05 % Triton X-100; (b) none; (c) 5 mM glutamate and (d) 0.05 % Triton X-100 and 5 mM glutamate. The reaction was started by adding 8 mM glutamine. In each case 1 μg/ml rotenone was present.

fully recovered in the soluble fraction if the enzyme is pre-incubated with Bromothymol blue.

Inhibition of glutaminase by glutamate in the preparation of intact and broken mitochondria

These experiments clearly show that glutaminase is inhibited by glutamate much more strongly in preparations of broken kidney mitochondria than in the intact organelles (Fig. 7). This suggests that externally added glutamate must penetrate the inner mitochondrial membrane in order to reach its binding site on the enzyme.

DISCUSSION

It has been shown that phosphate-dependent glutaminase is the enzyme which enables mitochondria from different sources to respire in the presence of glutamine as substrate [22, 23]. Therefore, it was reasonable to expect that the inhibition of the transport of phosphate by mersalyl might result in the inhibition of uncoupled respiration in the presence of glutamine. Since the addition of glutamate completely abolished the effect of mersalyl, it does not appear to be due to inhibition of the pathways of glutamate oxidation, but to specific inhibition of glutaminase. This was confirmed by the NH_4^+ electrode experiments.

It is known that glutaminase is quite sensitive to mercurials [1, 2]. However, the complete release of the inhibition by Triton X-100 and the absence of the inhibition in the presence of the detergent or in the preparation of freeze-thawed mitochondria revealed that the effect of mersalyl is not due to direct inhibition of glutaminase. It is, therefore, obvious that a mechanism which is important for the activity of glutaminase in intact mitochondria is inhibited.

The complete inhibition of glutaminase by mersalyl (plus CCCP or rotenone) in intact mitochondria which were pre-incubated with phosphate and the same effect of mersalyl in phosphate-free medium indicate that phosphate must move across the inner mitochondrial membrane in order to activate the enzyme. This is supported by the finding that intramitochondrial concentration of phosphate is the same regardless of the presence of mersalyl, provided that the mitochondria were pre-incubated in the medium with phosphate before addition of the inhibitor. It appears, therefore, that the inhibitory effect of mersalyl is not caused by a change in intramitochondrial concentration of phosphate. It is interesting to note that in the oxygen electrode experiments the addition of glutamate after mersalyl, in an amount that completely inhibits glutamine oxidation, produces a short burst of respiration followed by a strong inhibition due to utilization of endogenous phosphate. If endogenous phosphate is important for the activation of glutaminase the same phenomenon would be observed in the case of coupled glutamine oxidation, but it is not.

It must be mentioned that we were not able to observe inhibition of swelling of the mitochondria by mersalyl (plus rotenone) in an isosmotic solution of glutamine. This concentration of glutamine is very high and unphysiological, so that the metabolite could penetrate the inner membrane in an unspecific manner. However, determination of [^{14}C]glutamine- and [^{32}P]phosphate-permeable spaces in the presence of mersalyl and CCCP revealed a close correlation between the activity of glutaminase, movement of phosphate across the inner mitochondrial membrane and

the transport of glutamine. The amount of mersalyl that inhibits the transport of phosphate also inhibits the transport of glutamine and glutaminase activity. At this moment the question of the mechanism of the transport of glutamine and its association with transport of phosphate becomes particularly important. The simplest explanation would be that the inhibition of the transport of phosphate causes primarily inhibition of the activation of the phosphate-dependent glutaminase, which consequently results in the inhibition of the transport of glutamine decreasing the rate of its diffusion down a concentration gradient. It has already been suggested that glutaminase might play an important role in the transport of glutamine [24, 25]. A high activity of the enzyme would be needed to keep the intramitochondrial concentration of glutamine low and to facilitate diffusion of the substrate. However, a possibility that glutaminase is at the same time the glutamine carrier, or that a specific glutamine-phosphate carrier exists is not excluded.

It should be pointed out that mersalyl inhibits glutaminase only in the presence of the inhibitors of respiratory chain or an uncoupler. This suggests that the transport of glutamine and activation of phosphate-dependent glutaminase are possible due to the high-energy state of the mitochondria if the movement of phosphate through the inner membrane is inhibited.

There is a very small possibility that some other factors are responsible for the inhibitory effect of mersalyl. It should be mentioned in connection with this that under the given experimental conditions significant intramitochondrial pH changes are not expected. Besides there are several lines of evidence which suggest that we are not dealing with the inhibition of the enzyme activity due to the inhibition of the transport of glutamate and its subsequent accumulation in the matrix space. First, mersalyl does not inhibit uncoupled respiration in the presence of glutamate. Second, the inhibition of the efflux of glutamate from mitochondria by avenaciolide or Bromocresol purple [25] did not result in the inhibition of the respiration in the presence of glutamine as substrate.

It has already been mentioned that conflicting reports have appeared concerning the submitochondrial location of kidney glutaminase [9–11]. It must be pointed out in relation to this that the employment of either the digitonin-Lubrol procedure [26] or the swell-shrink sonication procedure [27] for subfractionation of mitochondria may result in perturbation of membrane components and also in the solubilization of the enzymes which are less firmly associated with the membrane. This is supported by the findings presented in Table III. It is accepted that there is a class of so-called peripheral membrane proteins, which are loosely bound to the membrane and may be readily released by gentle procedures such as changing the ionic strength, composition or pH of the medium [28, 29]. In addition to solubilization of membrane enzymes, the presence of some ions, for example borate and phosphate in the case of glutaminase [5], may result in the precipitation of an originally soluble enzyme which was detached from the membrane. It seems that the appearance of glutaminase in different interconvertible molecular forms with different kinetics, activity and solubility may be the reason for the difficulties in attempting to determine the exact submitochondrial location of the enzyme. Therefore, it is important to search for ways for the determination of the enzyme location in intact mitochondria. Estimation of the activation energy of glutaminase in frozen and lysed mitochondria, and the results obtained by digitonin and ultrasonic treatment of the mitochondria

strongly suggest that the enzyme is intimately, although not very firmly, associated with the inner mitochondrial membrane.

If the activity of glutaminase is of primary importance for the transport of glutamine then it must be assumed that the binding site of the enzyme for phosphate is embedded in the membrane since the movement of the anion is needed for the activation of glutaminase and the transport of glutamine. The other part of the enzyme with binding sites for the substrate and glutamate is probably exposed toward the matrix space. This conclusion is based on the finding that avenaciolide, Bromocresol purple and Bromothymol blue inhibit respiration of the mitochondria with glutamate but not with glutamine, indicating that glutamate, formed by the activity of glutaminase, is released directly into the matrix space and does not have to penetrate the membrane in order to be oxidized in mitochondria [25]. Furthermore, the inhibition of the efflux of glutamate by avenaciolide or Bromocresol purple results in the inhibition of glutaminase [25], whereas externally added glutamate inhibits the enzyme very feebly, indicating that the binding site for glutamate is exposed toward the inner space. It seems possible that, if not activated, the enzyme exists dissociated as its protomers (Tris · Cl form according to Kvamme and coworkers). The movement of phosphate across the membrane or energization of mitochondria may bring about association of the protomers, resulting in allosteric activation of glutaminase and facilitated diffusion of glutamine down a concentration gradient.

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