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POSSIBLE MECHANISMS OF THE EFFLUX OF GLUTAMATE FROM KIDNEY MITOCHONDRIA GENERATED BY THE ACTIVITY OF MITOCHONDRIAL GLUTAMINASE

ZORAN KOVAČEVIĆ

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

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SUMMARY

The transport of glutamate across the inner membrane of kidney mitochondria and the influx of glutamine into the mitochondria was studied using an oxygen electrode, the swelling technique and by continous recording of the activity of the mitochondrial glutaminase by an NH_4^+-sensitive electrode. It is well known that the enzyme is activated by inorganic phosphate and strongly inhibited by glutamate.

1. Avenaciolide, Bromocresal purple and Bromothymol blue inhibited the respiration of the mitochondria almost completely in the presence of glutamate as substrate but not in the presence of glutamine. Production of aspartate during the oxidation of glutamine was not significantly inhibited by avenaciolide but it was markedly suppressed by Bomocresol purple and Bromothymol blue.

2. Swelling of kidney mitochondria in an isosmotic solution of glutamine and ammonium phosphate was not inhibited by avenaciolide or Bromocresol purple indicating that these substances do not inhibit the penetration of the mitochondrial membrane by glutamine or phosphate.

3. The activity of the mitochondrial glutaminase was strongly inhibited by avenaciolide or Bromocresol purple in the presence of inhibitors of respiration or an uncoupler but not in their absence. Experimental data suggest that this was caused by the inhibition of glutamate efflux. The addition of a detergent removed this inhibition.

On the basis of these observations it was concluded that two mechanisms exist which enable glutamate to leave the inner space of kidney mitochondria: (a) an electrogenic efflux coupled to the respiration-driven proton translocation and the presence of a membrane potential (positive outside) and (b) an electroneutral glutamate-hydroxyl antiporter which is inhibited by avenaciolide and which operates in both directions. Our observations do not support the existence of the electrogenic glutamine-glutamate antiporter or glutamate-aspartate exchange in the mitochondria studied.

INTRODUCTION

Many attempts have been made, especially in the last few years, to elucidate the problem of the glutamate translocation across the inner mitochondrial membrane [1–5]. Two mechanisms have already been postulated by means of which glutamate enters mitochondria: (a) an electroneutral exchange between glutamate and hydroxyl ions [2] which is inhibited by avenaciolide [6] and *N*-ethylmaleimide [2], and (b) an influx of glutamate coupled to the efflux of aspartate which is energy-dependent and unidirectional [7]. However, there are few papers dealing with the mechanism of the efflux of glutamate from mitochondria, although this phenomenon must be of great importance, especially for the kidney cells since the phosphate-dependent glutaminase (one of the main generators of glutamate) has an exclusively mitochondrial location [8, 9, 10]. In relation to this problem it was suggested that the efflux of glutamate, produced inside kidney mitochondria by the activity of glutaminase, occurs by an obligatory exchange with glutamine entering the intramitochondrial space on an electrogenic glutamine-glutamate antiporter [11]. At the same time, this proposal suggests a possible mechanism for the transport of glutamine in kidney mitochondria, a mechanism which has not been clarified as yet. However, the experiments reported here, in which the oxidation of glutamate and glutamine was followed in the presence of avenaciolide, revealed that the latter inhibits the respiration of kidney mitochondria in the presence of glutamate but not in the presence of glutamine. This throws some doubt on the existence of the glutamine-glutamate antiporter. The main purpose of the present study was therefore to obtain more information concerning the mechanism of glutamine entry and glutamate efflux from kidney mitochondria during mitochondrial glutaminase activity.

MATERIAL AND METHODS

Rat kidney and pig renal cortex mitochondria were prepared as described elsewhere [12].

The oxygen electrode and swelling experiments were carried out with mitochondria from both sources, while in the experiments in which the activity of the mitochondrial glutaminase was followed with an NH^+_4-sensitive glass electrode, pig kidney mitochondria only were used. The reason was that the phosphate-independent glutaminase, which is a microsomal enzyme [13], has a very low activity in pig kidney so that it does not interfere with the activity of the mitochondrial phosphate-dependent glutaminase in the case when the mitochondrial preparation is slightly contaminated with microsomes. Besides, the K_m of pig kidney phosphate-dependent enzyme for glutamine is about 4 mM, being ten-fold lower compared to the corresponding rat kidney enzyme [14, 15].

Oxygen consumption was measured polarographically by means of a Clark oxygen electrode. The standard media for the incubation of mitochondria were (a) 120 mM KCl/10 mM Tris · Cl/10 mM phosphate/2.5 mM $MgCl_2$, and (b) 100 mM Tris · Cl/30 mM phosphate (Tris salt)/2.5 mM $MgCl_2$; in both cases the pH was 7.4. Final volume was 5 ml, and the amount of mitochondria was usually 5–6 mg protein. The temperature was 30 °C.

Swelling of mitochondria in isosmotic solution of metabolites was followed by

measuring the decrease of absorbance of mitochondrial suspension at 520 or 640 nm on a Unicam 1800 spectrophotometer.

The activity of the mitochondrial glutaminase was measured by continuously recording the formation of ammonia with an NH^+_4-sensitive glass electrode (Electronic Instruments Ltd, Surrey, U.K.) as described elsewhere [12]. The incubation medium was always the standard Tris · Cl medium as described above, unless otherwise indicated. All other experimental conditions were the same as in the case of oxygen uptake determination.

Aspartate originating from the oxidation of glutamine was determined enzymically [16] and by means of a Geiger-Muller counter after separation on a column of Dowex 1×8 (1×10 cm; acetate form). In the latter case, [U-^{14}C]glutamine was used (The Radiochemical Centre, Amersham, U.K.).

Mitochondrial proteins were determined with the biuret reagent [17] to which 1.5 % deoxycholate was added. Avenaciolide was a generous gift of Dr W. B. Turner (I.C.I. Ltd., Macclesfield, U.K.).

RESULTS

Oxygen electrode experiments

The effect of avenaciolide, Bromocresol purple and Bromothymol blue on the respiration of kidney mitochondria in the presence of glutamate and glutamine as substrates was investigated.

McGivan and Chappell have shown that avenaciolide inhibits respiration of rat liver mitochondria in the presence of glutamate as substrate [6]. Despite some undesirable effects, it is one of the most specific and effective inhibitors of glutamate transport and has no effect on phosphate penetration [4, 18]. Starting from the assumption that the transport of glutamine and glutamate across the mitochondrial membrane occurs by an obligatory exchange between the two metabolites [11], we expected that avenaciolide would also inhibit the respiration in the presence of glutamine. However, at the concentration which inhibited the oxidation of glutamate (stimulated by ADP plus P_i) completely, avenaciolide did not have any effect on the oxidation of glutamine (Fig. 1). It should be noted that the oxidation of malate, pyruvate, succinate and 2-ketoglutarate was not inhibited. The same effect was obtained using either rat kidney or pig kidney mitochondria.

Since it was found earlier [19] that the inhibition of aspartate aminotransferase with aminooxyacetate brings about inhibition of respiration in the presence of glutamate but not of glutamine, it was important to see whether there is any inhibitory effect of avenaciolide on the transamination pathway of glutamate oxidation. The determination of aspartate production during the oxidation of glutamine revealed that there is no significant inhibition of the transamination pathway of glutamine oxidation (Table I). In contrast to avenaciolide, Bromocresol purple and Bromothymol blue had a very strong inhibitory effect. Besides, avenaciolide did not inhibit respiration in the presence of a limiting concentration of aspartate (Fig. 1d). At the concentration used, avenaciolide did not have the effect of an uncoupler. In the case of pig renal cortex mitochondria it had the same effect whether respiration was stimulated by ADP plus P_i or by carbonyl cyanide *m*-chlorophenylhydrazone (CCCP).

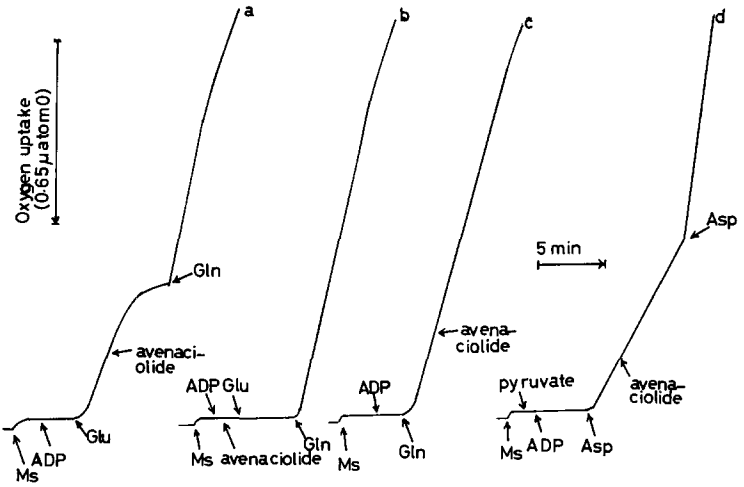


Fig. 1. Effect of avenaciolide on the respiration of kidney mitochondria in the presence of glutamate (Glu), glutamine (Gln) and aspartate (Asp) as substrates. Rat kidney mitochondria (approx. 6 mg protein) were incubated in the standard KCl medium described in Material and Methods. The same was found if the standard Tris · Cl medium was used. Where indicated, 0.3 mM ADP was added. Additions: (a) 5 mM glutamate, 20 μM avenaciolide and 1 mM glutamine; (b) 20 μM avenaciolide, 5 mM glutamate and 1 mM glutamine; (c) 1 mM glutamine and 20 μM avenaciolide and (d) 0.15 mM pyruvate, 0.5 mM aspartate, 20 μM avenaciolide and 5 mM aspartate.

TABLE I

EFFECT OF AVENACIOLIDE, BROMOCRESOL PURPLE AND BROMOTHYMOL BLUE ON THE RESPIRATION OF PIG KIDNEY MITOCHONDRIA IN THE PRESENCE OF GLUTAMATE AS SUBSTRATE, AND ON THE PRODUCTION OF ASPARTATE DURING THE OXIDATION OF GLUTAMINE

The mitochondria (7 mg protein) were incubated in the standard Tris · Cl medium in the presence of 1 mM ADP with or without the inhibitors. The reaction was started by adding 2 mM glutamate or 1.3 mM [U-^{14}C]glutamine. The inhibitors were present at the following concentrations: 40 μM avenaciolide, 0.4 mM Bromocresol purple and 40 μM Bromothymol blue. The final volume of the incubation mixture was 5 ml and the temperature 30 °C. Oxygen uptake was measured by a Clark electrode. In the case of glutamine oxidation the incubation was carried out for 15 min. After this time the reaction was stopped by mixing 4 ml of the sample with 1 ml of 20 % perchloric acid. After neutralization with K_2CO_3 the extract was used for enzymatic and isotopic determination of aspartate. These two analyses were correlated very well. The presence of the inhibitors did not interfere with the enzymatic assay of aspartate. The results are expressed as the mean ±S.E. and the number of experiments are given in the parentheses.

	Respiration in the presence of glutamate		Production of aspartate from glutamine	
	Oxygen uptake (natomO/min)	% of inhibition	Aspartate production (nmol/sample)	% of inhibition
Control	700		912±43(7)	
Avenaciolide	19	97	816±79(7)	10
Bromocresol purple	10	99	137±21(7)	85
Bromothymol blue	20	97	152±12(7)	84

Bradford and McGivan found [3] that Bromocresol purple strongly inhibits the translocation of glutamate across the rat liver mitochondrial membrane in both directions. In the case of kidney mitochondria we found a strong inhibition of respiration in the presence of glutamate, while the respiration, in the presence of even a limiting concentration of glutamine (0.2 mM), was negligibly inhibited (Fig. 2a, b, c).

Fig. 2 also shows that Bromothymol blue inhibited mitochondrial respiration very markedly in the presence of glutamate, malate and succinate, whereas glutamine abolished this inhibition completely.

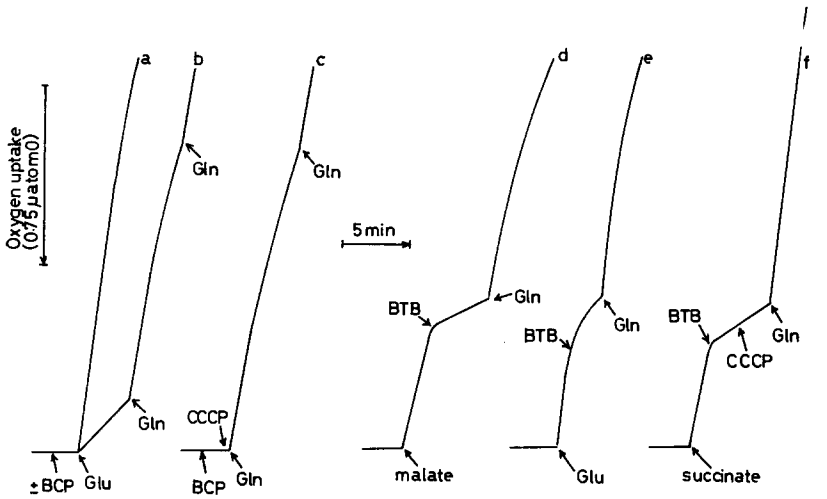


Fig. 2. Effect of Bromocresol purple (BCP) and Bromothymol blue (BTB) on the respiration of pig kidney mitochondria in the presence of glutamate, malate, succinate and glutamine. The mitochondria (5–6 mg protein) were incubated in the standard Tris · Cl medium (in the case of Bromocresol purple) or in the standard KCl medium (the experiments with Bromothymol blue). In each case, 0.3–0.4 mM ADP was present. Additions (where indicated): (a) 2 mM glutamate; (b) 0.4 mM Bromocresol purple, 2 mM glutamate and 0.2 mM glutamine (limiting concentration); (c) 0.4 mM Bromocresol purple, 1 µg/ml CCCP and 0.2 mM glutamine; (d) 1.2 mM malate, 0.1 mM Bromothymol blue and 1 mM glutamine; (e) 4 mM glutamate, 0.1 mM Bromothymol blue and 1 mM glutamine and (f) 0.5 mM succinate, 0.1 mM Bromothymol blue, 1 µg/ml CCCP and 1 mM glutamine.

Swelling experiments

Swelling of kidney mitochondria in an isoosmotic solution of glutamine, ammonium glutamate, ammonium phosphate and in the standard Tris · Cl medium in the presence of avenaciolide, Bromocresol purple and Bromothymol blue was investigated.

These experiments revealed that avenaciolide and Bromocresol purple did not inhibit the swelling of pig kidney and rat kidney mitochondria in an isosmotic solution of glutamine (Fig. 3b). Both kinds of mitochondria swell rapidly and extensively in isoosmotic solution of ammonium phosphate and this swelling is not inhibited by avenaciolide or by Bromocresol purple (Fig. 3a). It should be noted that avenaciolide and Bromocresol purple, at the concentration used, did not produce swelling of the mitochondria in the standard Tris · Cl medium, while Bromothymol blue brought about quite a large increase in mitochondrial volume (Fig. 3f, g).

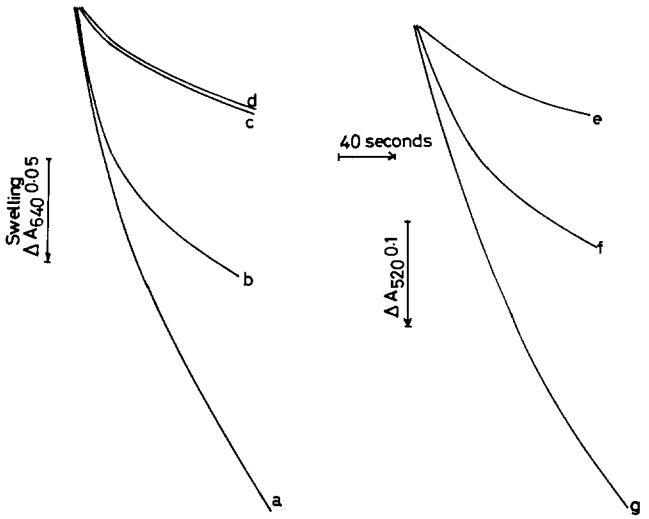


Fig. 3. Swelling of pig kidney mitochondria in isosmotic solutions of ammonium phosphate, glutamine, ammonium glutamate and in the standard Tris · Cl medium. The mitochondria (1.3 mg protein) were suspended in the solutions of the following composition: (a) 100 mM ammonium phosphate with or without 20 μM avenaciolide or 0.1 mM Bromocresol purple; (b) 220 mM glutamine in the presence or absence of 20 μM avenaciolide or 0.1 mM Bromocresol purple; (c) 100 mM ammonium glutamate; (d) 100 mM potassium glutamate plus 1 μg/ml valinomycin with or without CCCP; (e) Tris · Cl medium; (f) Tris · Cl medium with 40 μM Bromothymol blue and (g) Tris · Cl medium with 100 μM Bromothymol blue. In each case (except where Tris · Cl medium was used) 1 mM EDTA, 20 mM Tris · Cl and 1 μg/ml rotenone were present. The final pH was adjusted at 7.5, and the final volume was 2.5 ml; the temperature was 22 °C. The absorbance was recorded at 640 or 520 nm.

As was found earlier [11, 12], kidney mitochondria do not swell in an isosmotic solution of ammonium glutamate or potassium glutamate in the presence of valinomycin with or without CCCP (Fig. 3c, d).

NH^+_4-sensitive electrode experiments

The changes in ammonia production brought about by the activity of the mitochondrial glutaminase in the presence of avenaciolide and Bromocresol purple were measured.

If glutaminase has to operate in intact mitochondria, glutamine must penetrate the inner mitochondrial membrane, while NH_3 and glutamate have to be extruded into the extramitochondrial space. Phosphate is known to be an activator of the enzyme. Although it was found that glutaminase may be activated by dicarboxylic acids [20] and acetyl-CoA [21], our unpublished experimental data suggest that this does not happen under physiological conditions. Ammonia, as a product of the enzymatic reaction, penetrates the mitochondrial membrane rapidly and, besides, it is not an inhibitor of glutaminase. Glutamate, however, is a very potent inhibitor of glutaminase (6–7 mM glutamate inhibits glutaminase completely at 30 mM phosphate concentration and at pH 7.4) so that any inhibition of its efflux from mitochondria would result in the inhibition of the enzyme. It must be noted that under the given experimental conditions a significant influence of intramitochondrial pH

changes should not be expected. On the basis of these considerations, it was assumed that the continous recording of glutaminase activity by an NH_4^+sensitive electrode might give useful information concerning the mechanism of glutamate efflux from mitochondria taking into account all other factors which could affect the enzyme.

Fig. 4. shows that avenaciolide, together with rotenone, at a concentration which inhibits the respiration of mitochondria in the presence of glutamate, brought about a strong inhibition of glutaminase activity, probably by inhibiting the efflux of glutamate from the organelles. It should be noted that if endogenous respiration in the presence of rotenone is high, then antimycin must be added. By increasing the concentration of avenaciolide the inhibitory effect disappeared, presumably as a result of mitochondrial swelling [6]. It is interesting to note that the addition of aspartate did not release this inhibition. In the absence of rotenone, or in the presence of the inhibitor of respiration plus succinate, avenaciolide did not inhibit glutaminase activity. However, the addition of an uncoupler under these conditions brought about a very strong inhibition. Avenaciolide did not have any effect, even at twice the concentration, on the enzyme activity in the presence of a detergent or in the preparation of frozen and thawed mitochondria.

Fig. 4 shows also that the addition of Bromocresol purple in the presence of rotenone or CCCP, before or after glutamine, caused almost complete inhibition of glutaminase activity, probably by inhibiting the efflux of glutamate. In the absence of inhibitors of respiration, or an uncoupler, a negligible inhibition was observed. Although Bromocresol purple has been reported to inhibit glutaminase directly [22],

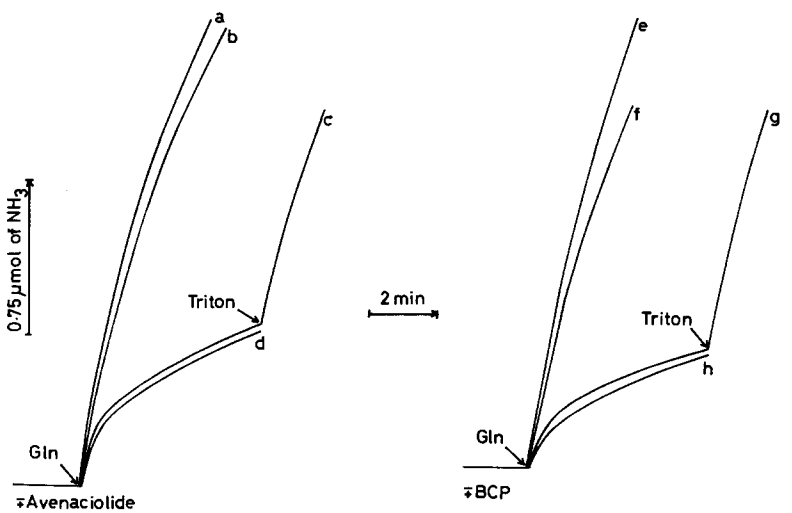


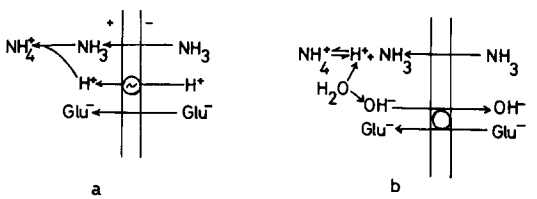
Fig. 4. Production of NH_3 by the activity of pig kidney mitochondrial glutaminase. The mitochondria (5–6 mg protein) were incubated in the standard Tris · Cl medium described in the Material and Methods section. The final volume was 5 ml and temperature 30 °C. Additions: (a) none or 1 µg/ml rotenone, 2.5 mM succinate and 20 µM avenaciolide; (b) 1 µg/ml rotenone or 1 µg/ml CCCP; (c) 1 µg/ml rotenone, 20 µM avenaciolide and 0.02 % Triton X-100 (where indicated); (d) 1 µg/ml rotenone, 2.5 mM succinate, 20 µM avenaciolide and 1 µg/ml CCCP; (e) none; (f) 0.4 mM Bromocresol purple (BCP); (g) 1 µg/ml rotenone, 1 µg/ml antimycin, 0.4 mM Bromocresol purple and 0.02 % Triton X-100 (where indicated) and (h) 0.4 mM Bromocresol purple and 1 µg/ml CCCP. In each case the reaction was started by adding 8 mM glutamine.

we found that it did not inhibit the enzyme in broken mitochondrial preparations, even at a higher concentration than that used for the inhibition of the enzyme in the intact organelles. It can be seen from Fig. 4g that the addition of Triton X-100 eliminated the inhibition caused by Bromocresol purple. Besides, according to Colonna et al. [23], one should not expect penetration of this dye into the mitochondrial matrix.

Preliminary experiments showed that *N*-ethylmaleimide has the same effect as avenaciolide and Bromocresol purple.

DISCUSSION

The experimental observations described above suggest two possible mechanisms for the efflux of glutamate from kidney mitochondria: (a) an electrogenic efflux coupled to the respiration-driven proton translocation and the presence of an electrical potential gradient across the inner mitochondrial membrane (positive outside; Scheme 1a); this mechanism is sensitive, of course, to the inhibitors of the respiratory



Scheme1

Scheme 1. Mechanisms of the efflux of glutamate from kidney mitochondria: (a) electrogenic efflux of glutamate coupled to the respiration driven proton translocation and the presence of a membrane potential (positive outside) and (b) electroneutral glutamate-hydroxyl antiporter.

chain and uncouplers, and (b) an electroneutral glutamate-hydroxyl antiporter which is inhibited by avenaciolide (Scheme 1b). The proposal of the existence of second mechanism is based on the inhibitory effects of avenaciolide. However, it must be pointed out that we were not able to demonstrate the swelling of kidney mitochondria in an isosmotic ammonium glutamate. Therefore, the problem of the electroneutral glutamate-hydroxyl exchange in kidney mitochondria needs further clarification. Yet, we believe that the finding of more suitable experimental conditions will help to demonstrate the carrier using also the swelling technique.

Bromocresol purple inhibits the glutamate-hydroxyl exchange as avenaciolide does, but the mechanism of the inhibition is probably different. This dye, which has a negative charge, binds to the membrane so that there may be an electrostatic repulsion of anions [24]. Bromothymol blue is similar to Bromocresol purple but at the concentration used it produced swelling of mitochondria. Besides, this dye is known as a very strong activator of glutaminase [14]. This, together with swelling, may be a reason that we did not observe the inhibition of glutamate efflux in the presence of Bromothymol blue.

Experiments with avenaciolide and Bromocresol purple showed that in the presence of CCCP almost complete inhibition of glutaminase activity occurs by the accumulation of glutamate, whereas the influx of glutamine is not blocked since the

respiration of mitochondria proceeded at almost a maximal rate in the presence of the limiting concentration of this substrate. Besides, kidney mitochondria swell in an isosmotic solution of glutamine in the presence of avenaciolide or Bromocresol purple plus rotenone, although there is a strong inhibition of glutamate efflux under these conditions. It is interesting to note that preincubation of the mitochondria with avenaciolide or Bromocresol purple did not result in an immediate inhibition of glutaminase since during the first 10–20 s the enzyme activity is quite high followed by a very strong inhibition caused by the accumulation of glutamate. This means that in this interval a normal influx of glutamine occurs, whereas the efflux of glutamate is severely inhibited. By calibrating an NH^+_4-sensitive electrode with a known amount of ammonia and from the data corresponding to the volume of the inner mitochondrial space, it could be calculated that during this interval approx. 5–6 mM glutamate accumulates inside the mitrochondria which is enough to cause a very strong inhibition of glutaminase at 30 mM phosphate and at pH 7.4. All these observations suggest that the influx of glutamine is not obligatory and strictly coupled to the efflux of glutamate, which throws doubt on the existence of an electrogenic glutamine-glutamate carrier [11]. The proposal of an electrogenic glutamine-glutamate antiporter was based on the finding that the increase in the amount of intramitochondrial glutamine is approximately equal to the decrease of intramitochondrial glutamate. However, it must be pointed out that under the given experimental conditions the amount of glutamine and glutamate inside the mitochondria is a result of not only the transport of the metabolities but also of glutaminase activity which was not taken into account. It should be mentioned in connection with this that a direct measurement of glutamine transport is very difficult unless intramitochondrial glutaminase is completely inhibited. This is probably the reason why the influx of glutamine into the mitochondria has not been fully elucidated. Welbourne suggested a passive glutamine uptake coupled to mitochondrial glutaminase [25]. In other words, glutamine appears to diffuse down a concentration gradient in gaining access to the utilizing enzyme. The diffusion may be facilitated by mitochondrial swelling which usually appears due to energization.

The complete inhibition of the respiration of kidney mitochondria by avenaciolide in the presence of glutamate as substrate, and the removal of this inhibition by glutamine, even in the presence of an uncoupler, suggests that, in this case, the electrogenic glutamate-aspartate antiporter did not operate as a mechansim for the efflux of aspartate from the mitochondria. This is supported by the finding that avenaciolide did not inhibit the transamination pathway of glutamine oxidation. Since avenaciolide does not inhibit glutamate-aspartate exchange [5], then the inhibition of glutamate-hydroxyl antiporter should have no effect on the inhibition of respiration.

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