The effect of inhibitors of glutamate transport on the pathway of glutamate oxidation in rat liver mitochondria

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1. INTRODUCTION

Glutamate can be oxidized in isolated rat liver mitochondria by two major metabolic routes. The deamination pathway, in which glutamate dehydrogenase (EC 1.4.1.3) is the key enzyme, leads to the formation of ammonia; the transamination pathway, involving aspartate aminotransferase (EC 2.6.1.1) causes aspartate formation [1–3]. The mitochondrial inner membrane is equipped with two transport systems for glutamate [4,5] that can be considered functionally connected with these metabolic pathways:

(a) The glutamate–aspartate antiporter brings about a 1:1 exchange of glutamate (plus H^+) *vs* aspartate. The transport is electrophoretic and, hence, is effectively unidirectional under conditions where a membrane potential (negative inside) is maintained across the mitochondrial membrane [5–8];

(b) The glutamate–H^+ symporter (alternatively described as a glutamate hydroxyl antiporter or a glutamic acid uniporter) brings about a reversible, electroneutral, proton-compensated transport of glutamate [9–13].

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Abbreviations: FCCP, *p*-trifluoromethoxy-(carbonyl-cyanide)-phenylhydrazone

Despite intensive work on isolated mitochondria as well as in intact cells and in vivo, the factors controlling hepatic glutamate metabolism are not well understood. Several authors have argued that the rate of ammonia formation is controlled at the level of glutamate dehydrogenase, e.g., by the redox state of NADP [1–3], or by a mass action effect of the reactants of the enzyme [14,15] (see also [16,17]). In contrast in [11], on the basis of kinetic studies on glutamate uptake, the authors suggested that the activity of the glutamate translocator limits the rate of deamination of added glutamate (see also [18]). The kinetic data in [10] have also been interpreted as supporting a rate control at the glutamate transport step [5]. We have argued [12] that these previous estimates of glutamate transport may have seriously underestimated the initial rate of glutamate uptake, due to the limited capacity of mitochondria for uptake of ions by a proton-compensated mechanism. This interpretation is further supported by our recent work [13,19] which emphasizes the role of matrix and medium pH on the kinetic characteristics of glutamate transport. Here, we have studied the effect of inhibitors of glutamate transport on mitochondrial glutamate metabolism, in order to assess whether under different metabolic conditions in isolated mitochondria, the rate of deamination of glutamate may be limited by the activity of the glutamate translocator.

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2. MATERIALS AND METHODS

Rat liver mitochondria were isolated in 0.25 M sucrose, essentially as in [20]. Mitochondria were incubated at 25°C in a medium (final vol. 1.2 ml) containing 45 mM KCl; 50 mM Tris–HCl (pH 7.4), 10 mM potassium phosphate, 5 mM $MgCl_2$, 2 mM EDTA, 20 mM glucose and 5 IU hexokinase (EC 2.7.1.1). Unless otherwise indicated, glutamate was added to a concentration of 10 mM. Further additions are indicated in the figure legends. Reactions were started by addition of mitochondria, and were quenched with $HClO_4$ (final concentration 3.3%). After removal of protein, the extracts were neutralized with KOH. Ammonia, aspartate and glutamate were measured spectrophotometrically in the neutralized extracts by standard enzymic methods (see [21]).

Protein was determined with the biuret method, using bovine serum albumin as a standard. Radiochemicals were obtained from New England Nuclear. Enzymes were purchased from Boehringer Mannheim. Hexokinase was dialyzed against 10 mM potassium phosphate, 1% glucose in order to remove ammonium sulfate. FCCP was a gift from Dr P. Heytler, of DuPont DeNemours (Wilmington); Avenaciolide was donated by Dr W.B. Turner, ICI. Other chemicals and biochemicals were purchased from Sigma (St Louis) and from Howse and McGeorge (Nairobi) (agent for British Drug Houses).

3. RESULTS

If glutamate transport would limit the rate of mitochondrial glutamate deamination, inhibitors of the glutamate translocator would be expected to affect ammonia formation from extramitochondrial glutamate, but not from internally generated glutamate. Proline oxidation produces intramitochondrial glutamate [22], which can be deaminated without involvement of the glutamate translocator [23]. Alternatively, glutamate formed from proline can leave the matrix space by efflux on the glutamate translocator. Since aspartate transport is strictly dependent on the presence of extramitochondrial glutamate [5–8], the formation of aspartate from proline requires the efflux of matrix glutamate [23]. In the experiment of fig.1, the effect of the transport inhibitor

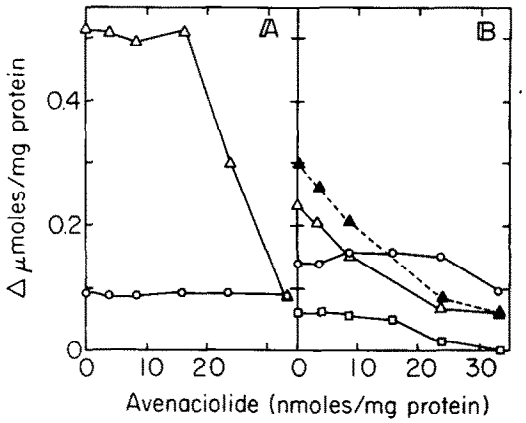

Fig.1. Effect of avenaciolide on the oxidation of glutamate and proline in rat liver mitochondria. Mitochondria (5.0 mg/ml) were incubated for 20 min under State 3 conditions, in the presence of 1 μM vitamin K_3, different concentrations of avenaciolide and with 10 mM glutamate (A) or 20 mM proline (B) as a substrate. Ammonia (○---○), aspartate (△---△) and glutamate (□---□) were measured enzymatically in deproteinized extracts. The sum of ΔGlu and ΔAsp (▲---▲) is shown as an estimate of total glutamate efflux during proline oxidation.

avenaciolide [24] on the oxidation of glutamate and proline was studied. Vitamin K_3 (2-methyl-1,4-naphtoquinone) was added to the incubation medium. This compound introduces an alternative pathway for the oxidation of NAD(P)H via mitochondrial NAD(P)H:(quinone-acceptor) oxidoreductase (EC 1.6.99.2) [3,25]. Under these conditions, ammonia is formed at a linear rate from either substrate, while vitamin K_3 has no effect on aspartate formation [3,23,25]. Avenaciolide, in concentrations up to 25 nmol/mg protein, had no effect on ammonia formation, whether glutamate was added to the incubation medium or was generated inside the mitochondria from proline. In contrast, the formation of glutamate and aspartate from proline was inhibited by low concentrations of avenaciolide, and the apparent rate of glutamate efflux (calculated as the sum of glutamate and aspartate formation; see [23]) was 50% inhibited by avenaciolide at a concentration of 15 nmol/mg protein. In other experiments (not shown) the inhibition of aspartate formation from proline by avenaciolide could be relieved by adding a low concentration of extramitochondrial glutamate, which activated

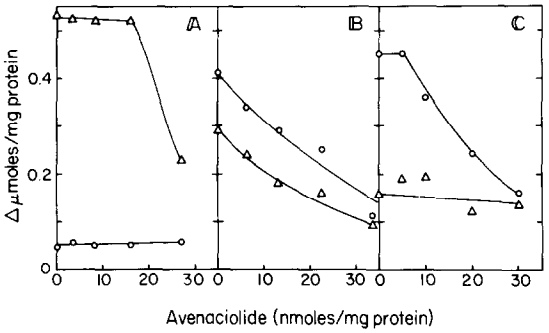

Fig.2. Effect of avenaciolide on glutamate oxidation under different reaction conditions. Mitochondria (6.4 mg/ml) were incubated for 20 min under State 3 conditions, with 10 mM glutamate as a substrate. (A) No further additions; (B) with FCCP (3 μM); (C) with vitamin K_3 (1 μM) and malonate (2 mM). (○---○) Ammonia formation; (△---△) Aspartate formation.

glutamate-aspartate exchange. In an earlier paper [23] we demonstrated that inhibitors of glutamate transport cause a dramatic accumulation of glutamate in the mitochondrial matrix during proline oxidation. These observations indicate that the effects of avenaciolide on proline oxidation can be ascribed to the inhibition of the glutamate translocator.

Concentrations of avenaciolide that were inhibitory to aspartate formation from proline had no effect on aspartate formation from added glutamate, despite the much higher flux rate through the transamination pathway. Thus, these results demonstrate that, under conditions where avenaciolide effectively blocked the glutamate translocator, ammonia formation from extramitochondrial glutamate was not affected.

In the experiments shown in fig.2, the oxidation of added glutamate was studied under conditions where the deamination pathway was maximally stimulated, either by the uncoupler FCCP (fig.2B) or by malonate plus vitamin K_3 (fig.2C). Again, in the control experiment in State 3 (fig.2A), avenaciolide had no effect on the rate of ammonia formation from glutamate, and inhibited aspartate formation only at high concentrations. However, when ammonia was stimulated to a rate of 20–25 $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$, avenaciolide significantly inhibited ammonia formation, causing a 50% inhibition at about 25 nmol/mg protein. Apparently, these reaction conditions stimulated ammonia formation to the extent that the glutamate translocator was operating close to its maximal capacity. Even at the highest concentration of avenaciolide studied, the rate of ammonia formation in the control experiment was less than half the rate of deamination in the presence of uncoupler or vitamin K_3 plus malonate. It can be concluded that the low rate of glutamate deamination under State 3 conditions is not due to a limited capacity of the mitochondrial glutamate transport system. Similar results (not shown) were obtained with bromcresolpurple, which inhibits glutamate transport by a mechanism that does not depend on interaction with thiol-groups on the translocator (*cf.* ref. [10,11]). As shown in fig.2 in uncoupled mitochondria the inhibition of glutamate deamination was evident at lower concentrations of avenaciolide than under energized conditions in the presence of vitamin K_3 plus malonate. This suggests that the maximal rate of the translocator is higher under the latter conditions. This difference is further illustrated in fig.3, where the reciprocal of the rate of deamination was plotted against the inhibitor concentration (*cf.* [26]). The data points shown were obtained from two in-

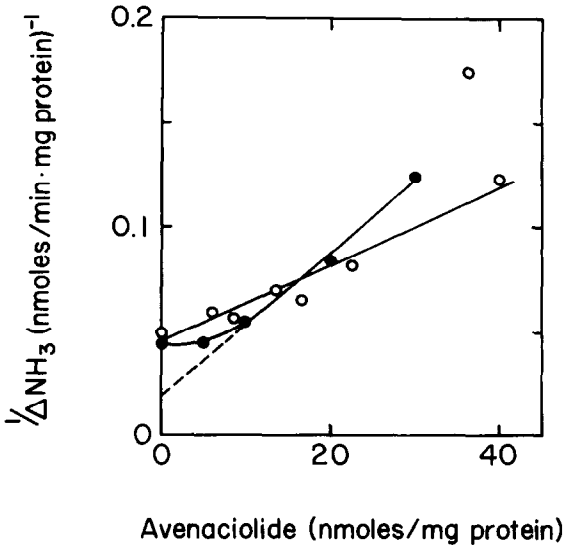

Fig.3. Effects of avenaciolide on ammonia formation from glutamate under energized and uncoupled conditions. Data were derived from two experiments, similar to those shown in fig.2B and 2C. (○---○) with FCCP (3 μM); (●---●) with vitamin K_3 (1 μM) and malonate (2 mM).

dependent experiments under similar conditions, and similar observations were obtained using bromcresolpurple as an inhibitor. Extrapolation of the linear portions of the Dixon-plots gave an apparent V_{max} of 23 nmol.min^{-1}.mg protein^{-1} (r = 0.976) in the presence of an uncoupler, and of 55 nmol.min^{-1}.mg protein^{-1} (r = 0.996) under energized conditions in the presence of vitamin K_3 plus malonate. Although the quantitative significance of these figures should be considered tentative since the interpretation of a non-linear Dixon-plot is not unequivocal, the apparent high rates of glutamate transport are supported by the kinetics of glutamate uptake reported earlier [11,19].

An unexpected observation in the experiment of fig.2 is the inhibition of aspartate formation by low concentrations of avenaciolide in the uncoupled state. In contrast to the situation in fully energized mitochondria, aspartate formation in uncoupled mitochondria is limited by the activity of the glutamate aspartate-translocator [6]. It appears from these results (and from similar observations with bromcresolpurple) that the glutamate aspartate translocator is sensitive to these inhibitors of the glutamate translocator, under the conditions used. This also explains the inhibition of aspartate formation under State 3 conditions by higher concentrations of avenaciolide (fig.1A and 2A). Effects of high concentrations of avenaciolide and bromcresolpurple on dicarboxylate transport have been reported by others [27,28].

4. DISCUSSION

The results presented here demonstrated that, under standard State 3 conditions, the glutamate translocator does not contribute significantly to the control of the rate of ammonia formation from extramitochondrial glutamate in rat-liver mitochondria. However, under conditions where the rate of deamination is maximally stimulated, the glutamate translocator activity can become an important rate controlling factor. The data show that the glutamate translocator can achieve a rate of 23 nmol.min^{-1}.mg protein^{-1} under uncoupled conditions, and probably significantly higher rates under energized conditions, at a pH of 7.4 and a glutamate concentration of 10 mM. These observations are in agreement with our earlier estimate of a high activity of glutamate transport from initial rate measurements [12,19]. The relatively lower activity of glutamate transport obtained in metabolic experiments under uncoupled conditions, probably does not reflect an effect of energization of the mitochondria. Direct measurements of both glutamate uptake [19] and efflux [13] in isolated mitochondria have demonstrated that the effects of uncouplers on this translocator are mediated by the decrease in the mitochondrial pH and not through a change in membrane potential. An inhibition of glutamate uptake by matrix acidification is also indicated by the observation that FCCP and nigericin decrease the rate of ammonium glutamate induced mitochondrial swelling (Hoek, J.B., unpublished; see also [29]). In contrast, an alkalinization of the extra-mitochondrial medium, inhibits glutamate uptake [19]. These properties of glutamate uptake mirror the effects of intra- and extra-mitochondrial pH on glutamate efflux, which is activated at higher external pH and lower matrix pH [13]. The experiments shown in fig.2 and 3 indicate that the effects of matrix pH on the activity of the glutamate translocator also operate under metabolic conditions and may become an important determinant of the rate of processes that depend on the transport of glutamate via the glutamate translocator; this includes not only glutamate deamination but also hydrogen shuttling via the malate-aspartate cycle or glutamine and proline oxidation (see [5] for a review). At the present time, the factors influencing the pH in cytosol and mitochondrial matrix in the liver are not well known. Further study is required to assess the regulatory significance of these parameters in the intact liver cell.

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