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Inhibition by glutamate of phosphate-dependent glutaminase of rat kidney

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A membrane-associated form of phosphate-dependent glutaminase was derived from sonicated mitochondria and purified essentially free of γ-glutamyl transpeptidase activity. Increasing concentrations of phosphate cause a sigmoidal activation of the membrane-bound glutaminase. Phosphate also causes a similar effect on the rate of glutaminase inactivation by the two affinity labels, L-2-amino-4-oxo-5-chloropentanoic acid and 6-diazo-5-oxo-L-norleucine, as observed previously for the solubilized and purified enzyme. Therefore the two forms of glutaminase undergo similar phosphate-induced changes in conformation. A sensitive radioactive assay was developed and used to determine the kinetics of glutamate inhibition of the membrane-associated glutaminase. The K_m for glutamine decreases from 36 to 4 mM when the phosphate concentration is increased from 5 to 100 mM. Glutamate is a competitive inhibitor with respect to glutamine at both high and low concentrations of phosphate. However, the K_I for glutamate is increased from 5 to 52 mM with increasing phosphate concentration. Therefore glutamine and glutamate interact with the same site on the glutaminase, but the specificity of the site is determined by the available phosphate concentration.

Rat renal phosphate-dependent glutaminase is associated with the mitochondrial inner membrane (Curthoys & Weiss, 1974; Kovacevic, 1976). The sensitivity of the glutaminase activity to changes in effector metabolite concentrations may be a key element in the regulation of renal ammonia synthesis. In addition to its activation by various polyvalent anions, the glutaminase is also inhibited by glutamate (Krebs, 1935; Goldstein, 1966). The rat renal concentration of glutamate is decreased 30% within 30 min after onset of acute acidosis (Vinay *et al.*, 1980). Similar changes have been observed with the isolated perfused rat kidney (Ross & Tannen, 1979). Since relief of end-product inhibition could increase glutaminase activity during acute acidosis, it is important to establish the kinetics of glutamate inhibition.

The rate of inactivation of purified phosphate-dependent glutaminase with L-2-amino-4-oxo-5-chloropentanoic acid decreases with increasing concentrations of phosphate (Shapiro *et al.*, 1978). In the absence of phosphate, inactivation by the chloroketone is inhibited by glutamate, but not by glutamine. In contrast, 6-diazo-5-oxo-L-norleucine inactivates only the catalytically active form of the purified glutaminase (Shapiro *et al.*, 1979). The glutaminase is completely resistant to the diazoketone in the absence of phosphate, and the increasing rate of inactivation observed with increasing concentrations of phosphate closely correlates with the phosphate activation profile. In addition, glutamate inhibition of inactivation by this affinity label is reversed by increasing concentrations of phosphate. The results of these studies were interpreted to indicate that glutamine and glutamate may interact with separate sites on the glutaminase. However, Chiu & Boeker (1979) pointed out that these observations are also consistent with the possibility that the substrate and inhibitor bind preferentially to two different conformations of the same site. In this study we demonstrate that a mitochondrial-membrane-bound preparation of the phosphate-dependent glutaminase exhibits similar kinetics of inhibition by the two affinity labels. In addition, we developed a sensitive radioactive assay and used it to characterize the kinetic properties of the membrane-bound glutaminase.

Experimental

Materials

White male Sprague–Dawley rats (200–300 g) were obtained from Zivic-Miller and were maintained on Purina Rat Chow. L-[U-^{14}C]Glutamine (290 Ci/mol) was purchased from New England Nuclear. L-2-Amino-4-oxo-5-chloropentanoic acid (Pinkus & Meister, 1972) and 6-diazo-5-oxo-L-

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norleucine (Holcenberg *et al.*, 1978) were synthesized by the methods reported in the references. All other biochemicals were obtained from Sigma Chemical Co.

Preparation of membrane-bound glutaminase

A rat was decapitated and its kidneys were removed and homogenized in 8 vol of 0.3 M-sucrose/1 mM-EDTA/5 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer, pH 7.4. Crude mitochondria were obtained by differential centrifugation (Curthoys & Weiss, 1974) and purified by velocity Ficoll step-gradient centrifugation (Curthoys & Shapiro, 1978). The purified mitochondria were subjected to swell–shrink sonication treatment (Curthoys & Weiss, 1974) and the resulting membrane fragments were pelleted by centrifugation at 12000 *g* for 10 min. The various fractions obtained during the isolation of the mitochondrial membrane preparation were assayed for phosphate-dependent glutaminase, by using glutamate dehydrogenase to quantify glutamate formation (Curthoys & Weiss, 1974). γ-Glutamyl transpeptidase activity was assayed with γ-glutamyl *p*-nitroanilide (Tate & Meister, 1974). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard. The specific activity of the phosphate-dependent glutaminase contained in the isolated membrane preparation decreased only slightly during storage for 1 week at 4°C. For the inactivation experiments, the membrane-bound glutaminase preparation was diluted to give an activity of 5 μmol/min per ml in 10 mM-Tris/acetate buffer, pH 8.6, containing the indicated concentrations of phosphate, L-2-amino-4-oxo-5-chloropentanoic acid or 6-diazo-5-oxo-L-norleucine. The samples were incubated at 20°C, and at various times samples were withdrawn and immediately assayed for glutaminase activity.

Glutaminase assay

This was developed by modifying the procedure of Prusiner & Milner (1970). A portion of L-[U-^{14}C]-glutamine was applied to a Dowex AG 1-X2 column (0.5 cm × 2 cm), pre-equilibrated with 10 mM-imidazole, pH 7.0, and eluted with 6× 250 μl of the imidazole buffer. The two fractions containing the maximum radioactivity were used as the source of labelled substrate for the glutaminase assay. The standard assay was done at 37°C with 60 μl of a solution containing 0.1 μCi of radioactivity, 20 mM-glutamine, 150 mM-potassium phosphate, 0.2 mM-EDTA and 20 mM-Tris/acetate, pH 8.6. The reaction was initiated by adding 1–3 μl of the mitochondrial membrane preparation. After 10 min, the samples were frozen in a solid-CO_2/acetone bath. The samples were later thawed by adding 1 ml of an ice-cold solution containing 30 mM-glutamine, 3 mM-glutamate and 20 mM-imidazole, pH 7.0, and applied to separate Dowex AG 1-X2 columns (0.5 cm × 2 cm), pre-equilibrated with imidazole buffer. The columns were then washed with 4 × 1 ml of 30 mM-glutamine/10 mM-imidazole, pH 7.0. The [^{14}C]-glutamate was then eluted with 2× 1 ml of 30 mM-HCl and collected directly in a scintillation vial. The radioactivity was determined after adding 15 ml of ACS scintillation solution (Amersham). The blanks contained less than 1% of the total radioactivity. For the various kinetic determinations reported, the concentrations of glutamine, phosphate and added inhibitors were varied as indicated in the Figure legends. All the data points represent means of duplicate determinations.

Results

Characterization of membrane-associated glutaminase

Rat kidney contains two distinct glutaminase activities: the mitochondrial phosphate-dependent glutaminase and a phosphate-independent, but maleate-stimulated, glutaminase (Katunuma *et al.*, 1967). The latter reaction is an activity of the brush-border membrane-associated γ-glutamyl transpeptidase (Curthoys & Kuhlenschmidt, 1975). Approx. 30% of the latter activity is recovered in a crude preparation of mitochondria obtained by differential centrifugation. As shown in Table 1, the transpeptidase activity is largely removed during purification of the mitochondria by Ficoll velocity gradient centrifugation. By isolating the large membrane fragments generated by sonication of the purified mitochondria, the specific activity of the transpeptidase was decreased to less than 3% of the value measured in crude mitochondria. The specific activity of the phosphate-dependent glutaminase was unaffected by this procedure.

When incubated with 3 mM-L-2-amino-4-oxo-5-chloropentanoic acid in the absence of phosphate, the glutaminase activity associated with the membrane preparation was inactivated with a $t_{\frac{1}{2}}$ (half-time) of 3 min (Fig. 1). The presence of increasing concentrations of phosphate resulted in a progressive decrease in the rate of inactivation. At a saturating phosphate concentration the $t_{\frac{1}{2}}$ for chloroketone inactivation was increased 6-fold. In contrast, the membrane-associated glutaminase activity was unaffected when incubated with 6 mM-6-diazo-5-oxo-L-norleucine in the absence of phosphate. However, in the presence of increasing concentrations of phosphate the glutaminase was inactivated at an increasing rate. In the presence of 100 mM-phosphate, the $t_{\frac{1}{2}}$ for inactivation was 8 min.

Mechanism of glutamate inhibition

The mitochondrial membrane preparation ex-

Table 1. *Removal of γ-glutamyl transpeptidase activity during the purification of rat renal mitochondrial membranes*
The kidneys from a single rat were homogenized and a crude preparation of mitochondria was obtained by differential centrifugation. The mitochondria were purified further by velocity Ficoll gradient centrifugation and then disrupted by sonication. About 10% of the glutaminase activity was released from the membrane preparation after storage for 1 week at 4°C. The solubilized glutaminase was removed by re-centrifugation at 12000 *g* for 10 min.

	Phosphate-dependent glutaminase activity		γ-Glutamyl transpeptidase activity	
Mitochondria	(μmol/min)	(μmol/min per mg of protein)	(μmol/min)	(μmol/min per mg of protein)
Crude	25.4	0.320	81.6	1.03
Purified	8.4	0.360	2.1	0.088
Sonicated	3.2	0.310	0.3	0.028

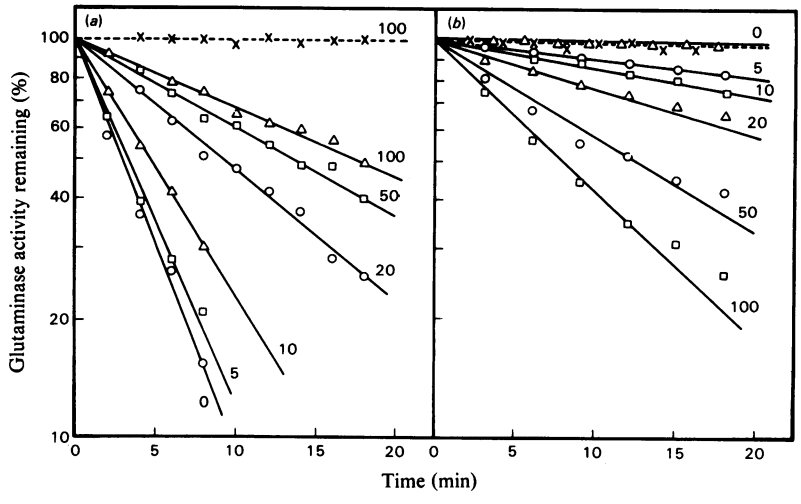

Fig. 1. *Effect of increasing phosphate concentration on the rate of inactivation of the membrane-associated phosphate-dependent glutaminase by* L-*2-amino-4-oxo-5-chloropentanoic acid and by 6-diazo-5-oxo-*L-*norleucine*
Mitochondrial membranes were incubated in the absence (——) or the presence of either 3 mM-chloroketone (*a*) or 6 mM-diazoketone (*b*) and the indicated concentration (mM) of phosphate.

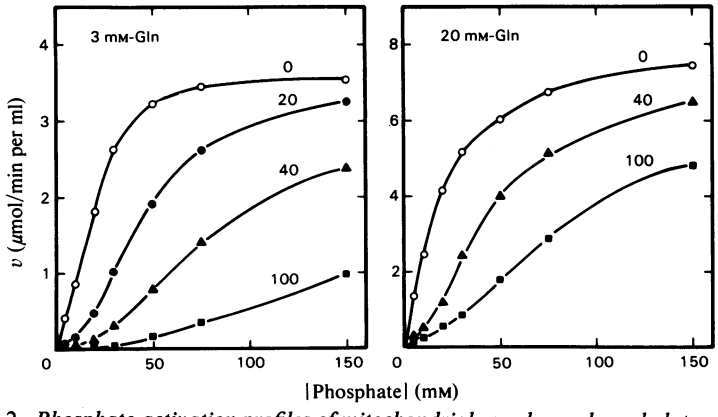

Fig. 2. *Phosphate-activation profiles of mitochondrial-membrane-bound glutaminase*
The data are presented as plots of activity versus the phosphate concentration determined in the presence of either 3 mM- or 20 mM-glutamine (Gln) and the indicated concentrations of glutamate: 0, ○; 20 mM, ●; 40 mM, ▲; 100 mM, ■.

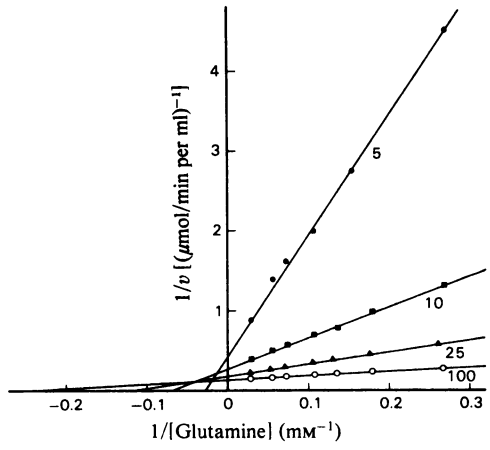

Fig. 3. *Double-reciprocal plots of mitochondrial-membrane-bound glutaminase activity versus the glutamine concentration determined in the presence of the indicated concentrations (mM) of phosphate*

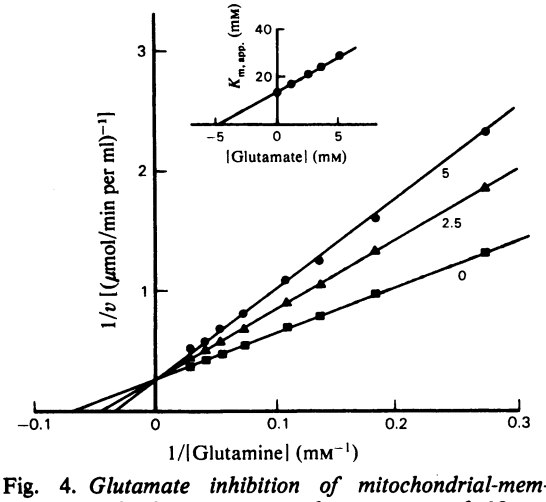

Fig. 4. *Glutamate inhibition of mitochondrial-membrane-bound glutaminase in the presence of 10 mM-phosphate*

The data are presented as double-reciprocal plots of activity versus the glutamine concentration determined in the presence of the indicated concentrations (mM) of glutamate. The insert is a replot of the apparent K_m for glutamine ($K_{m,app.}$) versus the corresponding concentration of glutamate.

hibits no glutaminase activity in the absence of a polyvalent anion (Fig. 2). The phosphate activation profile of the enzyme is sigmoidal, with half-maximal activation occurring at 20–25 mM-phosphate. The addition of increasing concentrations of glutamate causes a progressive shift in the phosphate activation profile to the right. The effect of glutamate as an inhibitor is less pronounced at high concentrations of glutamine.

In contrast, at all concentrations of phosphate the glutamine saturation profiles are hyperbolic (Fig. 3). Double-reciprocal plots of the data indicate that increasing concentrations of phosphate not only increase the glutaminase activity, but also decrease the apparent K_m of the enzyme for glutamine. As the phosphate concentration is increased from 5 to 100 mM, the K_m is decreased from 36 to 4 mM.

The effect of glutamate as an inhibitor with respect to glutamine at 10 mM-phosphate is illustrated in Fig. 4. When plotted as double-reciprocal plots, the data produce a series of straight lines which intercept on the $1/v$ axis. Therefore, glutamate is a competitive inhibitor with respect to glutamine. A replot of the apparent K_m for glutamine determined at various concentrations of glutamate indicates that under these conditions the K_i for glutamate is 5 mM. Glutamate was also found to be a competitive inhibitor with respect to glutamine when the phosphate concentration was maintained at 150 mM (Fig. 5). However, at the saturating concentration of phosphate the K_i for glutamate was increased to 60 mM. As shown in Fig. 6, the addition of increasing concentrations of phosphate causes a progressive decrease in the K_m for glutamine, while increasing the K_i for glutamate.

The normal concentrations of glutamine and glutamate in rat renal tissue are 1.5 and 2.4 μmol/g respectively (Vinay *et al.*, 1980). These concentrations are significantly less than the range of concentrations used to determine the kinetic con-

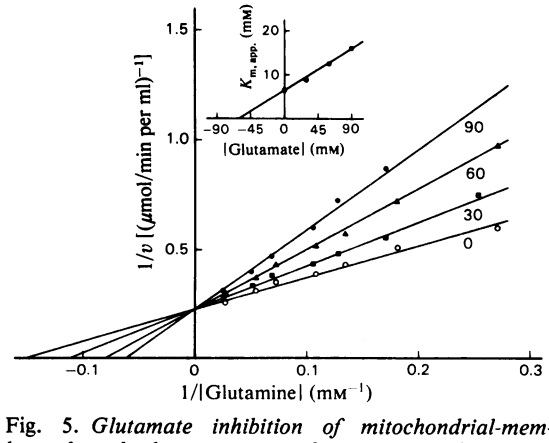

Fig. 5. *Glutamate inhibition of mitochondrial-membrane-bound glutaminase in the presence of 150 mM-phosphate*

The data are presented as described in the legend to Fig. 4.

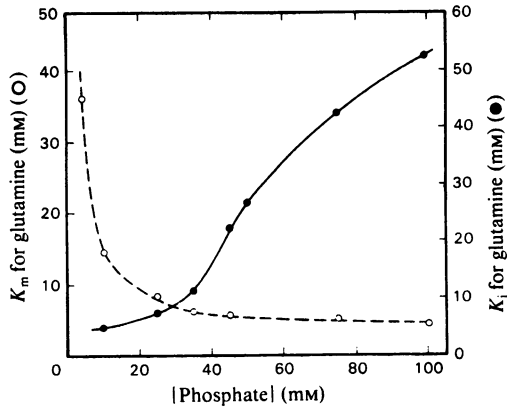

Fig. 6. *Effect of increasing phosphate concentration on the K_m for glutamine and the inhibition constant for glutamate of the mitochondrial-membrane-bound glutaminase*

The data were derived from plots similar to those presented in Figs. 3–5.

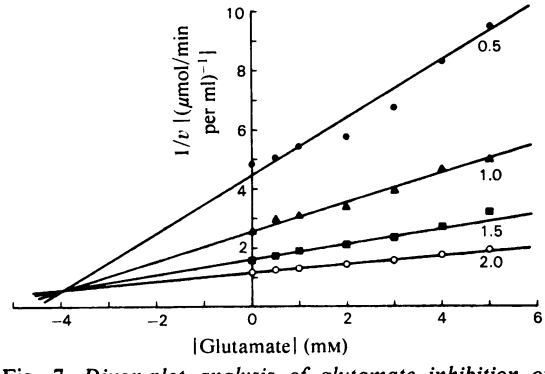

Fig. 7. *Dixon-plot analysis of glutamate inhibition of mitochondrial-membrane-bound glutaminase activity determined with 10 mM-phosphate and low concentrations of glutamine*

The data are presented as the reciprocal of the glutaminase activity versus the glutamate concentration determined at the indicated concentrations (mM) of glutamine.

stants for the phosphate-dependent glutaminase. Thus the kinetics of glutamate inhibition were redetermined by using a range of substrate and inhibitor concentrations which more closely approximate to those observed in renal tissue. As shown in Fig. 7, glutamate is a competitive inhibitor of the glutaminase activity even when assayed with 0.5–2.0 mM-glutamine. The K_i for glutamate observed under these conditions was 4 mM. This agrees well with the value of 5 mM determined with the same concentration of phosphate but higher concentrations of glutamine (3.5–40 mM).

Discussion

During solubilization from freeze-dried and resuspended mitochondria, the rat renal phosphate-dependent glutaminase undergoes partial proteolysis. As a result, the purified glutaminase preparation contains five to seven structurally related peptides, all of which are derived from a single type of subunit (Clark & Curthoys, 1979). The enzyme associated with the mitochondrial membrane retains its native structure. In addition, this form of the glutaminase is considerably more stable than the non-aggregated form of the purified glutaminase. As a result, the mitochondrial-membrane-bound form of the glutaminase was used to characterize the kinetics of glutamate inhibition.

In order to characterize the kinetic properties of the membrane-associated phosphate-dependent glutaminase, it was necessary to decrease the potential interference owing to the glutaminase activity of contaminating γ-glutamyl transpeptidase. The purification procedure used in these studies yields a preparation of mitochondrial membranes that are essentially free of transpeptidase activity. The maximum glutaminase activity of the transpeptidase in the absence of maleate is only 1% of the activity determined by using the γ-glutamyl *p*-nitroanilide assay (Tate & Meister, 1975). Therefore the residual phosphate-independent glutaminase associated with the membrane preparation could not have contributed to the observed results. The absolute dependence on phosphate observed for the membrane-associated glutaminase activity (Fig. 2) confirms this conclusion.

The effect of phosphate concentration on the rate of inactivation of purified glutaminase by the two affinity labels, L-2-amino-4-oxo-5-chloropentanoic acid and 6-diazo-5-oxo-L-norleucine, provides a sensitive probe for differences in conformation which can be readily applied to the glutaminase while still associated with the mitochondrial membrane. High concentrations (50 mM) of the two affinity labels are required to inactivate the glutaminase contained within intact mitochondria (R. A. Shapiro & N. P. Curthoys, unpublished work). In contrast, the kinetics of chloroketone inactivation of the glutaminase associated with a membrane preparation disrupted by sonication were nearly identical with those observed with the purified enzyme. Compared with the purified enzyme, slightly higher concentrations of the diazoketone were required to produce similar rates of inactivation of the membrane-associated glutaminase. However, the effect of phosphate on the kinetics observed by the two preparations are very similar. Therefore the membrane-associated glutaminase does undergo the same phosphate-induced conformational changes as observed with the purified enzyme.

Tveit *et al.* (1970) characterized the kinetic properties of a phosphate-dependent glutaminase purified from pig kidney. They observed that glutamate was a non-competitive inhibitor with respect to glutamine. In keeping with those data, we previously interpreted the observed effects of phosphate and glutamate on the inactivation of the rat renal glutaminase by various affinity labels (Shapiro *et al.*, 1978, 1979) as evidence that L-2-amino-4-oxo-5-chloropentanoic acid binds to a glutamate-binding site that is responsible for dead-end-complex formation. More recently Chiu & Boeker (1979), using a similar glutaminase purified from cow brain, reported that glutamate is a competitive inhibitor with respect to glutamine. They observed that increasing the phosphate concentration from 50 to 200mM had a slight effect on the K_m for glutamine and the K_I for glutamate. The data obtained with the affinity labels would be consistent with their kinetic data if, in the absence of phosphate, the active site of the glutaminase preferentially binds glutamate, and increasing concentrations of phosphate increase the specificity for glutamine. In order to distinguish between the two possible interpretations, which were based on kinetic data obtained for the enzyme derived from two different species, it was necessary to determine the kinetics of glutamate inhibition for the rat renal glutaminase.

The data presented in Figs. 4, 5 and 7 establish that glutamate is a competitive inhibitor with respect to glutamine at both high and low concentrations of phosphate. Thus glutamate inhibition of the rat renal glutaminase is caused by the binding of glutamate at the same site that binds glutamine. Increasing the phosphate concentration from 5 to 150mM resulted in a 9-fold decrease in the K_m for glutamine and a 12-fold increase in the K_I for glutamate. As suggested by Chiu & Boeker (1979), these data are consistent with the theory that in the absence of a bivalent anion the substrate-binding site of the catalytically inactive enzyme exists in a conformation that favours the binding of glutamate. Phosphate-induced activation of glutaminase apparently causes a change in conformation which increases the ability of glutamine to interact with the active site. As a result, the alteration in mitochondrial concentrations of phosphate, glutamine and glutamate during onset of acidosis could significantly alter the glutaminase activity.

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