GLUTAMINASE FROM PIG KIDNEY, AN ALLOSTERIC PROTEIN

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Sayre and Roberts (1958) purified glutaminase from dog kidney 2-400 fold and postulated that glutaminase has two cation binding sites and one sulfhydryl catalytic site. Glutamine was assumed to be attached to one of the cationic binding sites through its alpha carboxylate group, and its amino group was thought to be linked to phosphate which again was bound to the other cationic site. In this way the phosphate activation as well as the glutamate inhibition of glutaminase were explained.

We have obtained experimental evidence to show that glutaminase activity is greatly influenced by many metabolic intermediates and inorganic ions. We have also found that phosphate and other inorganic ions affect the state of dissociation of the protein, which together with kinetic data indicate allosteric interactions.

Methods

Pig kidneys kept deep frozen for several weeks are homogenized in phosphate buffer and fractionated with Na_2SO_4 according to Sayre and Roberts (1958). The enzyme preparation is further purified on Amberlite CG Type II, according to a modification of the method of Klingman and Handler (1958), and a final purification of 1000 - 2000 fold is obtained.

Glutaminase is assayed by two methods. By the first method, glutamate formed after 15 minutes incubation with glutamine and separated by paper chromatography, is eluated and the ninhydrine-copper compound measured colorimetrically. The spectrophotometric assay is based on the principle that the ammonia formed by hydrolysis of glutamine is determined by the glutamic dehydrogenase reaction, measuring the NADH oxidation at 340 mu. (Kvamme, Tveit and Reichelt, 1964). The two methods agree fairly well with each other.

The sucrose gradient technique is performed according to Martin and Ames (1961), and Stokes radii estimated as described by Ackers (1964) and Siegel and Monty (1965).

Results and discussion

Chloride in physiological concentrations (0.1-0.2 M) is found to be a powerful inhibitor of both purified and unpurified glutaminase (Table I). No particular difference could be obtained between the sodium and potassium salts of chloride. The inhibition is competitive with phosphate, non competitive with glutamine. Bicarbonate ions have also inhibitory action, and the same has Tris. In the absence of phosphate, members of the tricarboxylic acid cycle (e.g. citrate, alpha ketoglutarate, succinate, malate) and other metabolic intermediates (e.g. aspartate, alanine, acetyl-glutamate) exert a 2 - 4 fold stimulation, but no effect is seen in the presence of phosphate (Table II). It is thus assumed that the metabolic control executed on glutaminase by different ions and metabolic intermediates play an important physiological role under conditions where the phosphate concentration is low.

Table I. The effect of NaCl, p-chloromercuribenzoate and BTB

	umoles per minute				
0	NaCl	p-CMB	BTB	NaCl+BTB	p-CMB+BTB
3.1	1.6	1.4	4.8	4.8	3.7

The reaction mixture, 3 ml, contains (umoles): L-glutamine 20, alpha ketoglutarate 50, NADH 0.3, Na-phosphate pH 8, 20, EDTA 0.5, L-glutamic dehydrogenase (Sigma type II) 0.025 ml, and glutaminase 0.002 ml (0.001 mg protein, 1000 fold purified). Where indicated, are added (umoles): BTB 0.025, NaCl 500, and p-chloromercuribenzoate (p-CMB) 0.025. The NADH oxidation is followed at 340 mu.

Furthermore, glutaminase is strongly inhibited by Zn, Cu, Cd and Hg salts (10^{-3} M) and stimulated by the dye bromthymol blue (BTB) ($10^{-3} - 10^{-4}$ M). BTB was previously found to activate glutamine deamidation by intact Ehrlich ascites tumor cells (Kvamme and Svenneby, 1963). BTB counteracts the inhibitory effect of chloride, metals and p-chloromercuribenzoate (Table I). BTB

Table II. The effect of metabolic intermediates

	umoles per minute				
	0	alpha keto.	succinate	citrate	acetyl-glutamate
borate	0.8	3.1	4.7	3.3	5.1
borate + phosph.	6.7	6.1	6.5	6.0	6.5

The reaction mixture, 3 ml, contains (umoles): L-glutamine 100, Na-borate pH 8, 90, glutaminase 0.05 ml (1.2 mg protein, 150 fold purified), and where indicated Na-phosphate pH 8, 800, alpha ketoglutarate, succinate, citrate or N-acetyl-L-glutamate, 100. The mixtures are incubated for 15 minutes at 37°, deproteinized with ethanol, and glutamate isolated with paper chromatography (butanol, water, acetic acid 2:1:0.44).

decreases the Km values for phosphate as well as for glutamine, and the dye seems to act in cooperation with both substances (Kvamme et al. 1964).

Using a wide range of concentrations, a sigmoid curve is obtained when glutaminase activity is plotted versus chloride, Tris or sulfate concentration. In accordance with Monod's theory (Monod, Wyman and Changeux, 1965), the sigmoid shape of the curve with the inhibitor chloride does only appear when the activator phosphate is present. A sigmoid curve is also obtained when Tris or chloride is plotted against phosphate (Fig. 1) or glutamine concentration. Thus it is indicated that glutaminase is an allosteric protein which belongs to the K system (Monod et al. 1965).

BTB is also an allosteric effector because sigmoid curves are obtained when activity is plotted against BTB concentration. Furthermore, mild heat treatment or dilution abolish the BTB activation while the phosphate activation still can be demonstrated. In this way selectively desensitization of the protein towards the action of BTB seems to have taken place.

Using the technique of equilibrium dialysis the BTB binding power of glutaminase is increased by phosphate and sulfate which stimulate enzyme activity, and decreased by inhibitors as Tris and chloride (Table III).

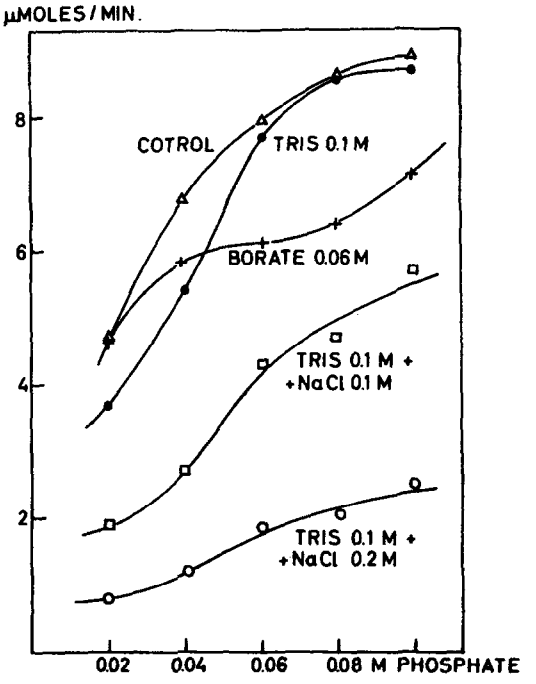

Fig. 1. The effect of glutaminase inhibitors on the shape of curves where rate is plotted versus phosphate concentration.

The experimental conditions are the same as described in Table II.

Table III. The binding of BTB and P^{32} to glutaminase

umoles BTB/mg protein					Counts/min/mg prot.	
Phosph.	Tris	Phosph. + Tris	Na_2SO_4 + Tris	NaCl + Tris	No BTB borate	BTB + borate
13.4	6.0	7.6	12.4	0.5	51	515

10 mg of protein and 1 umole of BTB in 3 ml of buffer (as indicated), are dialyzed for 17 hours in the cold against 500 ml of corresponding buffer solution (Na-phosphate 0.07 M, Tris 0.07 M, Na_2SO_4 0.3 M, Na-borate 0.01 M, all pH 8, NaCl 0.3 M, P^{32} 0.02 mC). The protein bound BTB is measured after adjustment to pH 10, at 620 mu. Tracerlab "1000 Scaler" is used for counting of P^{32}.

This is of interest in connection with the observation that the BTB binding power of phosphorylase b is stimulated by the

activator 5'AMP (Ullmann, Vagelos and Monod, 1964). Using dialysis it is also found that stimulatory concentrations of BTB increase P^{32} uptake into glutaminase, which is in accordance with the lowering effect of BTB on Km for phosphate. The BTB binding power of glutaminase is higher at pH 7 than at pH 8, but there is a qualitative difference in binding power depending upon the buffer used. Thus equilibration in borate buffer gives a lower protein binding pattern than equilibration in phosphate. The ionic strength of the buffer seems to be of minor importance in this connection.

Sucrose gradient studies revealed that the ionic composition of the buffer used for suspension of glutaminase, strongly affects the sedimentation constant of the enzyme (Table IV). It is of interest that combined sulfate + phosphate buffers give $S_{20,w}$ values which are insignificantly different from those of phosphate buffers alone, whereas sulfate + borate buffers give $S_{20,w}$ values close to those of borate + phosphate buffers, indicating that phosphate and sulfate ions can replace each other. BTB increases the $S_{20,w}$ of "Tris enzyme" (enzyme suspended in Tris buffer), as well as that of "phosphate enzyme" and "borate enzyme" markedly. When added to phosphate, glutamate, glutamine (0.01 M) or urea (1 M) lower the sedimentation constants so that they approach the constant of "Tris enzyme". Variations in ionic strength, at least over a 5 fold range, do not affect the $S_{20,w}$ values significantly.

Table IV. Sedimentation constants of glutaminase, suspended in different buffers

	Tris	phosph.	borate	borate + phosph.	borate + sulfate	phosph. + sulfate
No BTB	7.4	10.5	24.5	38.3	43.0	11.4
BTB	10.0	17.1	40.5			

2 mg of glutaminase is centrifuged in a linear sucrose gradient, 5-20% or 10-25%, 4½ hours, 39,000 rpm. To the 5-20% gradient is added 0.1 M Tris or 0.1 M Na-phosphate, to the 10-25% gradient is added 0.03 M Na-borate, 0.03 M Na-borate + 0.1 M Na-phosphate, 0.03 M Na borate + 0.1 M Na-sulfate, or 0.1 M Na-phosphate + 0.1 M Na-sulfate, as indicated. All buffers are adjusted to pH 8. When used, the concentration of BTB is 5×10^{-4} M. 0.3 ml fractions are assayed.

Using Martin and Ames' method (1961), the molecular weight of "Tris enzyme" is roughly estimated to be about 150,000, of "phosphate enzyme" about 245,000, of "borate enzyme" about 740,000 and of "borate-phosphate enzyme" about 1.450,000. However, molecular weight measurement of "borate-phosphate enzyme and to a certain extent also of "borate enzyme", is very inaccurate, due to technical difficulties in the determination of so heavy proteins with these methods. Some experiments gave also much higher values for the "borate-phosphate enzyme".

Stokes radii of glutaminase preparations are determined by measuring the elution patterns of the protein from Sephadex G 200 columns with the buffers mentioned (Ackers 1964). Assuming a partial specific volume of 0.73 cm^3 per g protein and combining the Stokes radii with the $S_{20,w}$ values according to Siegel and Monty (1965), molecular weights are calculated. The values are within the same range as those obtained with the Martin and Ames' method.

If the enzyme is assumed to be composed of identical protomers, the weight of the protomer may be guessed to be of the order of 70-80,000. In case this is true, the "Tris enzyme" contains 2 protomers, the "phosphate enzyme" 3, the "borate enzyme" 10, and the "borate-phosphate enzyme" 20 protomers, or more. Except the "Tris enzyme", the associations would be heterologous (Monod et al. 1965). It seems less likely that the "Tris enzyme" is the monomeric form, so that the "phosphate enzyme" contains 2 protomers in isologous association, the "borate enzyme" 5 protomers in heterologous association, and the "borate-phosphate enzyme" 10 or more protomers. The existence of a polymer with sedimentation constant of 17 (phosphate + BTB) which corresponds to a molecular weight of about 530,000, can also be explained from the assumption that the weight of the protomer is 70-80,000.

The results indicate that phosphate and sulfate are associated with the same allosteric site, whereas borate and possibly Tris and BTB are associated with different sites.

In conclusion, pig kidney glutaminase is considered to be an allosteric protein which is controlled by a great variety of inorganic ions (including phosphate) and metabolic intermediates. The allosteric transitions are shown to be accompanied by association into polymeric protein structures.

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