The Effect of Acetyl-Coenzyme A on Phosphate-Activated Glutaminase from Pig Kidney and Brain

By ELLING KVAMME and INGEBORG AASLAND TORGNER

Neurochemical Laboratory, The Oslo University Psychiatric Clinic,
Vinderen, Oslo 3, Norway

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Phosphate-activated glutaminase (EC 3.5.1.2; L-glutamine amidohydrolase) purified from pig kidney and brain is activated by CoA and short-chain acyl-CoA derivatives. Acetyl-CoA is the most powerful activator (K_A about 0.2 mM). Acetyl-CoA is maximally effective in the absence of other activating anions such as phosphate and citrate, and at low glutamine concentrations. The negative co-operative substrate activation observed at pH 7 becomes more pronounced in the presence of acetyl-CoA. Similarly to phosphate, acetyl-CoA produces at high protein concentrations a different type of activation, which is time-dependent, depends on protein concentration and is accompanied by an increase in the sedimentation coefficient. Acetyl-CoA, phosphate and citrate appear to have binding sites in common. No significant difference was observed between kidney and brain phosphate-activated glutaminase.

Phosphate-activated glutaminase (EC 3.5.1.2; L-glutamine amidohydrolase), a mitochondrial enzyme (Errera & Greenstein, 1949), has been purified to apparent homogeneity both from pig kidney (Kvamme *et al.*, 1970) and pig brain (Svenneby *et al.*, 1973). It has been shown that the regulation of this enzyme is very complex (Svenneby *et al.*, 1970; Svenneby, 1970, 1972): it is activated by phosphate and phosphorylated compounds, ATP being a more potent activator than P_i (Weil-Malherbe & Beall, 1970; Weil-Malherbe, 1972), and also by carboxylic acids and NH_3 (Svenneby *et al.*, 1970; Svenneby, 1971). Moreover, the extent of activation is dependent on whether the enzyme is in a low- or a high-molecular-weight form (Tveit *et al.*, 1970). A two- to three-fold time-dependent activation accompanied by conversion into higher-molecular forms, is observed on the addition of certain anions, e.g. phosphate or phosphate plus borate, to a high concentration of the low-molecular-weight form (Svenneby *et al.*, 1970; Svenneby, 1972; Olsen *et al.*, 1970, 1973).

The finding that the dye Bromothymol Blue, in lower concentrations than any known natural compound, is a powerful activator of phosphate-activated glutaminase (Kvamme *et al.*, 1965, 1970) started a search for physiological activators effective in the same concentration range. In the present paper an activation by acetyl-CoA is described.

Experimental

Materials

L-Glutamine, L-glutamate dehydrogenase (type II, in glycerol), yeast alcohol dehydrogenase (crystalline, salt-free), catalase (twice crystallized, from

jack beans), Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid], CoA (grade I, free acid), acetyl-CoA (grade II, sodium salt), acetoacetyl-CoA (grade II, sodium salt), propionyl-CoA (grade II, lithium salt) and malonyl-CoA (trilithium salt) were products of Sigma Chemical Co., St. Louis, Mo., U.S.A.

Methods

Assay of glutaminase. Glutaminase was measured (1) by spectrophotometric assay and (2) by chromatographic assay.

(1) Spectrophotometric assay. The rate of NH_3 formation was determined spectrophotometrically (Kvamme *et al.*, 1970) by using glutamate dehydrogenase as an auxiliary enzyme. The rate of NADH oxidation was followed at 340 nm and at 23°C. The reaction mixture (1 ml) contained 8 mM-L-glutamine, 5 mM-2-oxoglutarate, 0.1 mM-NADH, 40 mM-sodium phosphate, 0.2 mM-EDTA, 30 μ l (1–2 units) of L-glutamate dehydrogenase and glutaminase. The phosphate was in some experiments replaced by 15 mM-Hepes buffer. The pH of the reagents, except the L-glutamate dehydrogenase and NADH, were adjusted to 7.0. To remove free NH_3 , which contaminates the glutamine and glutamate dehydrogenase preparations, the reaction mixture was incubated for 5 min at 23°C before addition of glutaminase. The content of NH_3 and glutamate in the glutamine solutions was minimized by using either fresh preparations or solutions which were stored at –90°C in small vials after preparation, and only thawed once. The spontaneous hydrolytic cleavage of glutamine was negligible during the assay. If necessary, a correction was made for this amount of glutamate or NH_3 .

(2) Chromatographic assay. The amount of glutamate formed during a certain time (in most experiments for 5 min at 23°C) was measured as follows by a slight modification of the paper-chromatographic method of Kvamme *et al.* (1970). Glutaminase was incubated as described above (reaction volume usually 0.5 ml), and the enzymic reaction was stopped by the addition of 1 ml of cold (0°C) ethanol. Controls without enzyme were treated in the same manner for determination of non-enzymically hydrolysed glutamine. Corrections (usually negligible) were made on the basis of these data.

A portion of the ethanol extract, usually 100–250 μ l, was applied to paper (Whatman 3 MM) and chromatographed overnight in butanol–acetic acid–water (50:11:25, by vol.) (descending technique). After the paper had been dried, the chromatography overnight was repeated to obtain complete separation of the glutamate and glutamine spots ('drip and dry'). This was particularly important when the glutamine concentration greatly exceeded that of glutamate. The dry chromatograms were dipped in 0.25% ninhydrin in acetone, containing 5 ml of pyridine and 5 ml of acetic acid/100 ml of acetone. The chromatograms were developed overnight at room temperature. Equal-sized squares containing the glutamic acid spots were cut out, the ninhydrin colour was extracted with 5 ml of 50% (v/v) ethanol, and 25 μ l of 5% (w/v) $\text{Cu}(\text{NO}_3)_2$ was added. The E_{510} was read in a Beckman B spectrophotometer against a paper blank treated similarly.

As previously reported (Kvamme *et al.*, 1970) the rate was usually linear for at least 5 min, independent of the assay method, and proportional to the amount of glutaminase in the assay mixture.

Enzyme purification. Phosphate-activated glutaminase from pig kidney and pig brain was purified and solubilized as described previously (Kvamme *et al.*, 1970; Svenneby *et al.*, 1973). The second Tris–HCl-solubilized fraction [specific activity (μ mol

of NH_3 formed/min per mg of protein) about 40] was used in this work. This soluble enzyme form was previously called the Tris–HCl enzyme (Svenneby *et al.*, 1973) or T-form (Olsen *et al.*, 1973).

Sedimentation coefficients. Sedimentation coefficients were determined by the method of Martin & Ames (1961) by using sucrose-gradient centrifugation. Glutaminase was incubated for 10 min at 23°C in the presence of 5 mM-Tris–HCl buffer, 3 mM-EDTA and the other compounds to be tested at pH 7.0, and then applied to 5–20% (w/v) linear sucrose gradients containing the same concentrations of the compounds as the incubation mixture. The pH was 7.0 throughout. The centrifugation was performed at 4°C for 14 h at 130000g (Spinco ultracentrifuge, model L, rotor SW39). Fractions (0.1 ml) were collected and analysed for glutaminase activity by the spectrophotometric technique. Details of the procedure have been described (Svenneby *et al.*, 1970). Yeast alcohol dehydrogenase ($s_{20,w} = 7.4\text{S}$) and catalase ($s_{20,w} = 11.3\text{S}$) were used as markers, and were assayed by the methods of Racker (1957) and Chance & Maehly (1957) respectively.

Protein. Protein was determined by the method of Layne (1957).

Results

Activation by acyl-CoA derivatives

The soluble T-form glutaminase, purified from pig kidney or brain, is activated by CoA and short-chain acyl-CoA derivatives (Table 1). Acetate and acetyl-carnitine had negligible effect on the activity. Acetyl-CoA was the most powerful activator. Thus 0.1 mM-acetyl-CoA increased the activity about threefold, and the activator constant K_A was 0.2 mM. However, this K_A is six times the K_A for activation of pyruvate carboxylase by acetyl-CoA (Scrutton & Utter, 1967). The activation constant for the activation of kidney glutaminase by phosphate

Table 1. Activation of pig kidney glutaminase by short-chain acyl-CoA derivatives

Purified preparations of brain and kidney phosphate-activated glutaminase were incubated at 23°C and pH 7.0. The reaction mixture contained (sodium salts) 8 mM-L-glutamine, 15 mM-Hepes, 3 mM-EDTA and 0.01 mg of protein. The spectrophotometric assay was used. The results are expressed as the ratio of activity with acyl-CoA derivatives added to that without any addition. K_A is the concentration of activator required to give 50% maximal activation. The maximal activation was determined as described by Scrutton & Utter (1967).

Concn. (mM) ...	Kidney glutaminase activity						Brain glutaminase activity	
	0.05	0.10	0.25	0.50	1.00	K_A	0.10	0.50
CoA	1.08	1.22	1.63	1.85	2.22	1.5	1.46	2.50
Acetyl-CoA	1.91	2.61	3.77	4.77	5.88	0.2	2.94	8.05
Acetoacetyl-CoA	—	1.06	1.02	1.33	—	—	1.37	2.12
Propionyl-CoA	1.25	1.56	2.10	3.06	4.07	0.5	1.44	3.10
Malonyl-CoA	1.04	1.10	1.27	1.42	1.67	0.8	1.21	2.39

is 50mM (Klingman & Handler, 1958). It appears also from Table 1 that brain glutaminase is slightly more stimulated by acyl-CoA derivatives than kidney glutaminase, but the difference is not significant.

Acetyl-CoA prepared by reaction of acetyl anhydride with CoA (Ochoa, 1955) stimulated glutaminase similarly. The stimulation was lost when acetyl-CoA was destroyed by heating with NaOH.

Composite effect of acetyl-CoA and other activators

It was decided to compare the effect of acetyl-CoA with that of other known activators of glutaminase, such as phosphate, citrate and Bromothymol Blue. Kinetic studies have previously suggested that phosphate-activated glutaminase has at least two regulatory anionic binding sites, and that the binding of, for example, phosphate to one of these sites has a positive co-operative effect on the binding of this ligand to the other site(s) (Tveit *et al.*, 1970). Bromothymol Blue increases the affinity of the enzyme for phosphate, as demonstrated by the equilibrium-dialysis technique (Kvamme *et al.*, 1965). Moreover, phosphate also increases the Bromothymol Blue-binding power of glutaminase in a similar way to ligands affecting other allosteric proteins, such as haemoglobin (Antonini *et al.*, 1963) and phosphorylase b (Ullmann *et al.*, 1964).

Fig. 1 demonstrates that the activation of pig kidney and brain glutaminase by acetyl-CoA is

decreased by 5mM-phosphate. Double-reciprocal plots of velocity against acetyl-CoA concentration with and without phosphate show intersection

Fig. 1. Acetyl-CoA activation of pig brain and kidney glutaminase as affected by phosphate

Double-reciprocal plots of enzyme activity against concentration of acetyl-CoA are shown. The reaction mixture (0.5ml) contained (sodium salts), 40mM-L-glutamine, 15mM-Hepes, 3mM-EDTA and 0.01mg of protein. The glutamate formed was determined by the chromatographic technique after incubation for 5min at 23°C and pH7.0. ----, Brain glutaminase; —, kidney glutaminase; Δ , no phosphate added; $\circ$, 5mM-phosphate added.

Fig. 2. Phosphate (a) and citrate (b) activation of pig kidney glutaminase as affected by acetyl-CoA and Bromothymol Blue. The conditions were the same as in Fig. 1. Additions: Δ , no addition; $\circ$, 0.25mM-acetyl-CoA; $\square$, 0.02mM-Bromothymol Blue.

at one point on the ordinate, suggesting competition between phosphate and acetyl-CoA. This is further suggested by the results in Fig. 2(a), in which the phosphate concentration was varied. Similarly, competition between citrate and acetyl-CoA is suggested (Fig. 2b). When acetyl-CoA and either phosphate or citrate were both present the form of the double-reciprocal plots became convex-upwards, regardless of which activator was varied. Thus the fixed ligand causes a kinetic relationship of the varied ligand similar to that observed in the presence of compounds giving negative co-operative interactions. This combined effect of acetyl-CoA and phosphate is different from that of acetyl-CoA and Bromothymol Blue, in which case the form of the double-reciprocal plot became concave-upwards (positive co-operativity) (Fig. 2a).

The kidney and brain enzymes are activated by 2-oxoglutarate (by 50–60% over the range 5–20mM, with higher concentrations being slightly inhibitory). In the presence of 0.25mM-acetyl-CoA, 10mM-2-oxoglutarate activates by only about a further 30%, and higher concentrations are inhibitory, whereas in the presence of 25 μ M-Bromothymol Blue the activation by 2-oxoglutarate is much greater (three-fold at 20mM), and there is no inhibition at high concentrations.

Activation by glutamine as affected by acetyl-CoA

The activation of glutaminase by its substrate glutamine is dependent on pH. Thus at pH9 and

Fig. 3. Activation by glutamine of pig kidney glutaminase as affected by acetyl-CoA

The conditions were the same as in Fig. 1. Δ , No addition; $\square$, 0.05mM-acetyl-CoA added; $\circ$, 0.10mM-acetyl-CoA; $\bullet$, 0.25mM-acetyl-CoA.

Fig. 4. Time-dependent activation by acetyl-CoA of glutaminase from pig kidney

The conditions were the same as in Fig. 1 except that the enzyme was preincubated with acetyl-CoA for the time indicated at 23°C and pH7.0 in the presence of 5mM-Tris-HCl buffer and 3mM-EDTA before assay. The spectrophotometric assay of the rate of NH_3 formation was used, permitting the measurement of initial rates. The protein concentration in the preincubation mixture was 0.2mg/ml and in the assay 0.01mg/ml. Addition of acetyl-CoA to the preincubation mixture: Δ , no addition; $\blacktriangle$, 0.05mM added; $\circ$, 0.10mM added; $\bullet$, 0.25mM added. —, No acetyl-CoA added in the assay. The acetyl-CoA concentration in the assay was thus diluted 20-fold compared with that of the preincubation mixture. ----, 0.25mM-Acetyl-CoA added in the assay.

Fig. 5. Time-dependent activation of pig kidney glutaminase as affected by protein concentration

The conditions were the same as in Fig. 4 except that the enzyme was preincubated for 10min at 23°C before the addition of glutamine. The protein concentration in the preincubation mixture is indicated in the Figure. Δ , No acetyl-CoA added to the preincubation mixture; $\circ$, 0.10mM-acetyl-CoA added to the preincubation mixture. No acetyl-CoA was added in the assay.

Table 2. *Effect of acyl-CoA derivatives on the sedimentation coefficients of pig phosphate-activated glutaminases*

Purified phosphate-activated pig kidney and brain glutaminases were incubated for 10 min at 23°C and pH 7.0 in the presence of 5 mM-Tris-HCl buffer, 3 mM-EDTA and the acyl-CoA derivatives, or other compounds as indicated, before application to a sucrose density gradient. The protein concentration in the incubation mixture was 1 mg/ml. Protein (0.1 mg) was applied to linear 5–20% (w/v) sucrose gradients containing the compounds of the incubation mixture in the same concentrations. The pH was 7.0, and markers were alcohol dehydrogenase and catalase. The gradients were centrifuged for 14 h at 130000g in a Spinco preparative ultracentrifuge (model L, rotor SW39). Fractions (0.1 ml) were collected and assayed for enzyme activity by the spectrophotometric technique. h, heterogeneous; n, number of determinations.

Additions to the preincubation mixture	s values (S)					
	Kidney			Brain		
	Mean	Range	n	Mean	Range	n
None	6.36 ± 0.08	6.03–6.96	11	5.83	5.71–6.03	3
Acetyl-CoA (0.125 mM)	7.21 ± 0.09	6.73–7.40	8	6.43	6.37, 6.48	2
Acetyl-CoA (0.250 mM)	7.32 (h)	6.96–7.60 (h)	3	6.77 (h)		1
Acetyl-CoA (0.125 mM) + phosphate (50 mM)	7.07	6.96–7.18	2			
CoA (0.125 mM) + cysteine (0.250 mM)	6.54	6.37–6.77	3	6.26	6.20, 6.36	2
CoA (1.0 mM) + cysteine (2.0 mM)	7.60 (h)		1	7.78 (h)		1
Bromothymol Blue (0.125 mM)	9.67 (h)	9.46 (h), 9.87 (h)	2			

higher pH values, concave-upwards double-reciprocal plots (positive co-operativity) are obtained, whereas at pH 8 the activation obeys Michaelis-Menten kinetics (Svenneby, 1971), and at pH 7 convex-upwards double-reciprocal plots (negative co-operativity) are obtained. The curvatures of the plots at pH 7 are increased by acetyl-CoA, as demonstrated in Fig. 3.

Time-dependent activation by acetyl-CoA

The specific activity of soluble glutaminase from pig kidney (Svenneby *et al.*, 1970) and pig brain (Svenneby, 1972) increases two- to three-fold on addition of phosphate or phosphate-borate buffer, provided that the protein concentration is sufficiently high. This activation occurs in the course of 5–10 min at room temperature, and the enzyme undergoes polymerization. Time-dependent activation after the addition of acetyl-CoA has been demonstrated for both kidney (Fig. 4) and brain glutaminase. This activation is only slowly reversible, since initial rates were linear for several minutes in spite of the fact that the concentrations of acetyl-CoA were 20-fold diluted in the assay. When acetyl-CoA was added in the assay (one example shown in Fig. 4), the activities were about 30% higher and this increase in activity appeared to be virtually independent of the time of preincubation with acetyl-CoA. The dependence of time-dependent activation on protein concentration is shown for kidney glutaminase in Fig. 5. The activation by acetyl-CoA, like that by phosphate, is not time-dependent when the protein concentration is low, and we have previously only observed polymerization

at high protein concentration (Svenneby *et al.*, 1970). Therefore in the kinetic experiments described above it was important to maintain the protein concentration below that necessary for polymerization, thus making polymerization less likely as an explanation of the co-operativity phenomena. On addition of acetyl-CoA under the same conditions as reported in Fig. 4, at a high protein concentration, the sedimentation coefficient of glutaminase increased significantly (Table 2). Moreover, the addition of both 50 mM-phosphate and 0.125 mM-acetyl-CoA, or increasing the acetyl-CoA concentration from 0.125 to 0.250 mM, did not produce any further increase in the sedimentation coefficient compared with that produced by either phosphate or 0.125 mM-acetyl-CoA alone. Higher concentrations of CoA than of acetyl-CoA are required to produce the same increase in sedimentation coefficient. It is also noteworthy that Bromothymol Blue, in the same concentration as acetyl-CoA (0.125 mM), produces a much greater increase in the sedimentation coefficient. It appears from Table 2 that the sedimentation coefficient of brain glutaminase may be lower than that of the kidney glutaminase.

Discussion

This investigation started out as a search for a physiological activator for phosphate-activated glutaminase with a potency similar to that of Bromothymol Blue, which is effective at 30 μ M (Kvamme *et al.*, 1966, 1970). The effects of the dye and acetyl-CoA differ in that the former enhances the activating effect of phosphate, whereas acetyl-CoA decreases

this activation and may indeed bind at the same site(s) as phosphate.

Negative co-operative substrate interaction is rather unusual for mammalian enzymes. This effect by glutamine might be explained by isoenzymes with different kinetic properties, as discussed by Cook & Koshland (1970) for glutamate dehydrogenase, because phosphate-activated kidney glutaminase has been shown to contain isoenzymes composed of two subunit species (Torgner & Kvamme, 1973). However, this explanation is unlikely because purified brain glutaminase, which also shows negative co-operative substrate interaction, contains only one of these isoenzymes (Svenneby *et al.*, 1973), and the phenomenon is probably due to conformational changes in protein structure.

The time-dependent activation by acetyl-CoA is accompanied by a pronounced increase in the sedimentation coefficient similar to that produced by phosphate, but less than that produced by Bromothymol Blue. On the basis of electron-microscopic evidence, phosphate has been assumed to cause dimerization of glutaminase (called 'phosphate form' or 'P-form') (Olsen *et al.*, 1970), accompanied by a two-fold increase in specific activity; these effects are dependent on time and protein concentration. This time-dependent activation has also been observed on the addition of acetyl-CoA and supports the assumption that phosphate and acetyl-CoA have a similar mechanism of action.

The acetyl-CoA concentration of rat liver mitochondria has been estimated to be 0.5–0.6 nmol/mg of protein (Garland *et al.*, 1965). The local concentration of acetyl-CoA may therefore be about 1 mM if it is assumed to be confined (like the phosphate-activated glutaminase; Crompton *et al.*, 1973) to the mitochondrial matrix. It is known that the acetyl-CoA concentration may undergo considerable variations dependent on the metabolic state of the mitochondria; whether these changes regulate glutaminase activity is of course unknown.

Glutaminase has no absolute requirement for acetyl-CoA, in contrast with pyruvate carboxylase, which is also activated by much lower concentrations (K_A for acetyl-CoA 33 μ M; Scrutton & Utter, 1967). Other short-chain acyl-CoA derivatives, such as propionyl-CoA, may complement the effect of acetyl-CoA, if the metabolic conditions are such that the concentrations of these compounds increase sufficiently. However, the activation by acetyl-CoA is not evident at high concentrations of phosphate or of glutamine, so its physiological significance is difficult to assess. It seems unlikely that the intra-mitochondrial concentration of phosphate is ever less than 5 mM; it is, for example, known (Kovačević *et al.*, 1970) that kidney mitochondria

can concentrate phosphate from the surrounding medium. Nevertheless, there is clearly a possibility that changes in acetyl-CoA concentration may regulate glutaminase activity *in vivo*.

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