Inhibition of carbamoyl-phosphate synthase (ammonia) by Tris and Hepes

Effect on K_a for *N*-acetylglutamate

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The apparent K_a for *N*-acetylglutamate of rat liver carbamoyl-phosphate synthase is 11 μM in phosphate buffer, a value 10-fold lower than reported in other buffer systems. Tris and Hepes inhibit competitively with *N*-acetylglutamate. The proportion of carbamoyl-phosphate synthase in the active enzyme–acetylglutamate complex *in vivo* may be higher than previous calculations suggest, which re-opens the question of the involvement of *N*-acetylglutamate in the regulation of urea synthesis.

INTRODUCTION

In the course of validating a procedure for measuring *N*-acetylglutamate as glutamate, we came to the conclusion that the kinetic method, in which the amount of activator is estimated from the capacity of a liver extract to increase citrulline synthesis, is subject to errors arising from inhibition by deproteinizing agents and the fact that the liver itself contains an inhibitor (Lund & Wiggins, 1984). The inhibitor is detectable also in isolated mitochondria (Cohen, 1984). In our hands the inhibitor survived both deproteinization and dialysis, which led us to suspect that it might be an acid-stable carbamoyl-phosphate phosphatase, of low M_r and low K_i for P_i (see Ramponi, 1976). Consequently, we replaced Tris, the buffer commonly used in measuring citrulline synthesis, by phosphate, and found a large increase in response to low concentrations of added *N*-acetylglutamate, although the effect of the inhibitor was undiminished. Further details of the kinetics of citrulline synthesis are the subject of this paper. The most important observation is that K_a for *N*-acetylglutamate in phosphate buffer is about 10-fold lower (11 μM) than the values on which are based all hypotheses concerning the importance of *N*-acetylglutamate in the regulation of the activity of carbamoyl-phosphate synthase (EC 6.3.4.16).

MATERIALS AND METHODS

Isolation of mitochondria

Disrupted rat liver mitochondria provided the enzyme system for citrulline synthesis. Mitochondria were isolated from liver in 0.3 M-mannitol/5 mM-Hepes, pH 7.4, by the rapid method described by Kun *et al.* (1979). Suspensions were diluted to the required concentration (approx. 10 mg of protein/ml) with the mannitol medium and stored at -20°C , distributed in small stoppered tubes to avoid losses of activity that occur on repeated freezing and thawing. Mitochondrial protein was determined by the Lowry method, with bovine albumin as standard.

Assay of citrulline synthesis

The kinetic assay of *N*-acetylglutamate has been generally derived from the methods described by Charles *et al.* (1967) and McGivan *et al.* (1976). Therefore our assay contained, in 1 ml final volume: 10 mM-ornithine; 20 mM- NH_4Cl ; 5 mM-ATP; 15 mM- MgCl_2 ; 10 mM- KHCO_3 ; approx. 1.5 μCi of $\text{NaH}^{14}\text{CO}_3$. The buffer system and the amounts of *N*-acetylglutamate added are given in the text. Oligomycin was included as indicated. The tubes were equilibrated to 37°C , unless stated otherwise, before starting the reaction with mitochondria that had been stored at -20°C and thawed immediately before use. The amount of mitochondrial protein per assay was < 0.5 mg. For the experiments in Table 2, the freeze-thawed suspension was sonicated (3×10 s) to ensure complete disruption of the mitochondria, or was centrifuged (5 min at 32000 g) to remove particle-bound ATPase. Centrifugation decreased the amount of protein to < 0.3 mg per assay. Incubations were in stoppered tubes for 15 min unless stated otherwise. The reaction was stopped with 0.1 ml of 60% (w/v) HClO_4 . A portion of supernatant (0.5 ml) was transferred to a plastic scintillation vial, heated to 70°C for 60 min and dried in a fume cupboard before addition of 0.5 ml of water and 10 ml of scintillation fluid.

The radioactivity (d.p.m.) in all samples was corrected for radioactivity incorporated in the absence of *N*-acetylglutamate (in the 0.5 ml sample it was approx. 1×10^3 in Tris buffer, pH 7.2, and approx. 5×10^3 in phosphate; see below) before calculating kinetic constants. This 5-fold increase in incorporation in phosphate reflects either the increased sensitivity of the assay to endogenous *N*-acetylglutamate in the mitochondrial extract or the *N*-acetylglutamate-independent activity of carbamoyl-phosphate synthase (see Cohen, 1984). If the former, the incorporation represents the equivalent of about 1 nmol of *N*-acetylglutamate in both Tris and phosphate. When the assay contained 8 μM -*N*-acetylglutamate, in the absence of added ornithine, incorporation was 6×10^2 d.p.m., versus 8×10^4 d.p.m. in the complete system, showing conclusively that the acid-stable ^{14}C -labelled product formed was citrulline.

Materials

$\text{NaH}^{14}\text{CO}_3$ (50–60 mCi/mmol) was obtained from Amersham International. Scintillation fluid (Optiphase 'Safe'; LKB scintillation products) was from Fisons, Loughborough, Leics., U.K. Phosphocreatine and creatine kinase were from Boehringer Corp. (London), and oligomycin was from Sigma. Other chemicals were reagent grade.

Fig. 1. Comparison of potassium phosphate and Tris/HCl buffers on the activation of citrulline synthesis by *N*-acetylglutamate in freeze-thawed mitochondria

Incorporation of ^{14}C into citrulline was measured after 15 min at 37°C as described in the Materials and methods section: ●, 40 mM-potassium phosphate, pH 7.2; ■, 50 mM-Tris/HCl, pH 7.2. Reciprocal plots of these data give $0.5 V_{\text{max}}$ at $9.8 \mu\text{M}$ -*N*-acetylglutamate in phosphate and $98 \mu\text{M}$ in Tris.

RESULTS

Kinetics of citrulline synthesis in freeze-thawed mitochondria

As mentioned in the Introduction, Tris is the buffer most commonly used in measuring the synthesis of citrulline *in vitro*, and it was purely fortuitous that we replaced Tris by phosphate. The increased sensitivity of carbamoyl-phosphate synthase to low concentrations of *N*-acetylglutamate in phosphate buffer is illustrated in Fig. 1, in which Tris and phosphate buffers were compared in the same experiment. At concentrations of *N*-acetylglutamate below about $16 \mu\text{M}$, the incorporation of ^{14}C into citrulline is increased approx. 5-fold in phosphate buffer compared with Tris. In Tris, the reaction shows no sign of reaching V_{max} at $128 \mu\text{M}$ -*N*-acetylglutamate. The concentration-dependence in Tris is similar to that obtained by others, who found that the synthesis of citrulline is proportional to *N*-acetylglutamate concentration up to $40 \mu\text{M}$ (Meijer & van Woerkom, 1978) or $24 \mu\text{M}$ (Stewart & Walser, 1980). From the experiment in Fig. 1, double-reciprocal plots give $0.5 V_{\text{max}}$ at $98 \mu\text{M}$ -*N*-acetylglutamate in 50 mM-Tris at pH 7.2 and $9.8 \mu\text{M}$ in 40 mM-potassium phosphate at pH 7.2.

The inhibition by Tris was studied in more detail by comparison with Hepes, the other buffer widely used in measuring the kinetics of citrulline synthesis. Both Tris and Hepes show competitive inhibition with *N*-acetylglutamate (Figs. 2a and 2b), whereas the inhibition by phosphate, an end product of both carbamoyl-phosphate synthase and of ornithine carbamoyl-transferase, is non-competitive (Fig. 2c). Inhibition of citrulline synthesis by phosphate, assuming no inhibition in 20 mM, was 13% at 50 mM, 51% at 100 mM and 86% at 200 mM. The maximum rate of citrulline synthesis is obtained between pH 7.5 and 8.0 in phosphate buffer (results not shown), but K_a for *N*-acetylglutamate is not

Fig. 2. Kinetics of activation of citrulline synthesis by *N*-acetylglutamate in different buffer systems

For experimental details, see the Materials and methods section. (a) Tris, pH 7.2: ■, 25 mM (K_a 100 μM); ●, 50 mM (K_a 161 μM); ▲, 75 mM (K_a 256 μM). (b) Hepes, pH 7.2: ■, 25 mM (K_a 68 μM); ●, 50 mM (K_a 86 μM); ▲, 75 mM (K_a 102 μM). (c) Phosphate, pH 7.2: ■, 20 mM; ●, 40 mM; ▲, 100 mM (K_a 12.1 μM).

affected by pH over the range tested (12.3 μM at pH 7.0; 10.1 μM at pH 7.2; 9.8 μM at pH 7.5; 11.8 μM at pH 8.0). A low concentration of phosphate is commonly included in addition to the major buffer, especially when synthesis of citrulline depends on endogenously generated ATP. The presence of 10 mM-phosphate in 50 mM-Tris lowers the K_a for *N*-acetylglutamate to 67 μM .

A variety of other buffers were tested, at pH 7.2, over a range of added concentrations of *N*-acetylglutamate (0–60 μM): these were Mops (50 mM), glycylglycine (50 mM) and triethanolamine (50 mM). All gave lower rates of incorporation of $\text{H}^{14}\text{CO}_3^-$ at all concentrations of added *N*-acetylglutamate than did phosphate (results not shown).

Constancy of apparent K_a for *N*-acetylglutamate in phosphate buffer under different conditions

Although the activation of citrulline synthesis by *N*-acetylglutamate followed Michaelis–Menten kinetics in the crude mitochondrial preparation, the incubation time of 15 min had been arbitrarily chosen from information given by McGivan *et al.* (1976), and no precautions had been taken to inhibit ATPase. More detailed experiments showed that the shape of the progress curve was the same irrespective of whether the

buffer was Tris, Hepes or phosphate; synthesis was most rapid during the first 5 min, followed by a gradual slowing to 30 min without affecting K_a . Data for Tris and phosphate are compared in Table 1. It is impractical to measure kinetics at times shorter than 5 min because of limited incorporation of ^{14}C , especially in Tris. Oligomycin (10 $\mu\text{g}/\text{ml}$) increased V_{max} and improved linearity of the assay without affecting the apparent K_a . An ATP-regenerating system of phosphocreatine/creatine kinase (5 mM with 16 units of enzyme or 2.5 mM with 8 units) had a similar effect (results not shown).

Further confirmation of the low K_a in phosphate was obtained in the high-speed supernatant of freeze-thawed mitochondria, which contained negligible ATPase activity ($< 0.2 \mu\text{mol}$ of ATP hydrolysed under the assay conditions after 15 min at 37 °C) and in mitochondria disrupted by sonication. The data are summarized in Table 2. In phosphate, V_{max} of the high-speed supernatant is about 20% higher than in freeze-thawed mitochondria, even when no account is taken of the protein removed on centrifugation, which implies that the endogenous inhibitor of citrulline synthesis (Cohen, 1984; Lund & Wiggins, 1984) may be associated with the particulate fraction. Sonication, on the other hand, decreases V_{max} and should be avoided for studies of this enzyme system.

Table 1. Apparent K_a of *N*-acetylglutamate for carbamoyl-phosphate synthase (ammonia) in phosphate and Tris buffers: time-course and effect of oligomycin

Carbamoyl-phosphate synthase activity was measured in freeze-thawed mitochondria as incorporation of ^{14}C into citrulline as described in the Materials and methods section. V_{max} is expressed as nmol of citrulline formed/min per mg of mitochondrial protein.

Incubation time (min)	Phosphate buffer				Tris buffer			
	Control		+ Oligomycin (10 $\mu\text{g}/\text{ml}$)		Control		+ Oligomycin (10 $\mu\text{g}/\text{ml}$)	
	K_a (μM)	V_{max}	K_a (μM)	V_{max}	K_a (μM)	V_{max}	K_a (μM)	V_{max}
5	9.6	92	12.9	130	96	104	91	89
10	11.5	89	12.1	112	99	83	109	85
15	11.1	79	12.0	103	111	80	109	80

Table 2. Apparent K_a of *N*-acetylglutamate for carbamoyl-phosphate synthase in Tris and phosphate buffers in different preparations of liver mitochondria

Treatment of mitochondria was as described in the Materials and methods section. Buffers were 40 mM-potassium phosphate, pH 7.2, and 50 mM-Tris/HCl, pH 7.2. Incubation time was 15 min. V_{max} is expressed as nmol of citrulline formed/min per mg of mitochondrial protein, with no correction for removal of protein in the supernatant fraction. Values are means $\pm$ S.E.M. for the numbers of observations given in parentheses.

Mitochondrial preparation	Phosphate buffer		Tris buffer	
	K_a (μM)	V_{max}	K_a (μM)	V_{max}
Freeze-thawed	10.2 $\pm$ 0.5 (4)	82 $\pm$ 5 (4)	126 $\pm$ 18 (4)	92 $\pm$ 8 (4)
Supernatant	10.7 $\pm$ 0.3 (6)	106 $\pm$ 5 (6)	110 $\pm$ 16 (3)	94 $\pm$ 12 (3)
Sonicated	10.7 $\pm$ 0.7 (3)	60 $\pm$ 10 (3)	100 $\pm$ 8.0 (3)	50 $\pm$ 8 (3)

DISCUSSION

Activation of carbamoyl-phosphate synthase (ammonia) by *N*-acetylglutamate

Our experiments show that the choice of buffer system, and the concentration of buffer in the assay, may explain some of the discrepancies between K_a values for *N*-acetylglutamate reported in the literature. For the purified rat enzyme the value of 100 μM was obtained by Lusty (1978) in Bistris buffer, whereas the lower value (40 μM) reported by Kerson & Appel (1968) was obtained in phosphate. Values of about 100 μM were also found for the bovine enzyme in glycylglycine (Elliott & Tipton, 1974), frog enzyme in Tris (Marshall *et al.*, 1961) and human enzyme in triethanolamine (Pierson & Brien, 1980). In disrupted mitochondria, the *N*-acetylglutamate-dependence curves obtained in Tris gave 0.5 V_{max} at 0.18 mM (McGivan *et al.*, 1976). Interestingly, a low value was found by Cohen (1984), who found $K_a = 16.8 \mu\text{M}$ in HEPES buffer when Mg^{2+} was replaced by Mn^{2+} in the assay. The apparent K_a of 11 μM that we find in phosphate, as opposed to the values of at least 100 μM from which all speculation as to its physiological importance has been based, must warrant a reassessment of the arguments (see, e.g., Lund & Wiggins, 1984; Meijer & Verhoeven, 1984). Although our kinetic constant has been obtained in a relatively crude enzyme system, it gives the same value in Tris as is reported for the isolated enzyme. The differences cannot be explained by different concentrations of K^+ in the two buffer systems: activity is not affected by varying K^+ between 40 and 100 mM (Nuzum & Snodgrass, 1976). The susceptibility of carbamoyl-phosphate synthase to inhibition by such a wide range of compounds makes it unwise to speculate on the significance of alterations in the liver content of *N*-acetylglutamate *in vivo*. Until the distribution of *N*-acetylglutamate synthase (EC 2.3.1.1), which might be expected to parallel that of carbamoyl-phosphate synthase in the periportal cells (Gaasbeek-Janzen *et al.*, 1981), and the mitochondrial concentration

of *N*-acetylglutamate are known with certainty, it is too early to reach any conclusions. Our data merely indicate that the proportion of carbamoyl-phosphate synthase in the active enzyme-acetylglutamate complex in rat liver may be higher than previous calculations suggest.

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