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Purification and properties of acetyl-CoA:L-glutamate *N*-acetyltransferase from human liver

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Acetyl-CoA:L-glutamate *N*-acetyltransferase (amino acid acetyltransferase, EC 2.3.1.1) was isolated from human liver mitochondria by precipitation with $(NH_4)_2SO_4$ and chromatography on hydroxyapatite, DEAE-cellulose and Sephacryl 300. This gave a 360-fold purification. The molecular weight was estimated to be approx. 190000. The kinetic properties in the absence of arginine are compatible with a rapid-equilibrium random Bi Bi mechanism. The estimated constants are: for the substrates $K_{m,\text{acetyl-CoA}}$ 4.4mM, $K_{i,\text{acetyl-CoA}}$ 4.7mM, $K_{m,\text{glutamate}}$ 8.1mM, $K_{i,\text{glutamate}}$ 8.8mM; for the products, $K_{i,\text{acetylglutamate}}$ 0.28mM, $K_{i,\text{CoA}}$ 5.6mM. The rate constant for the forward direction is $1.24\,s^{-1}$. If *in vivo* the constants are of the same order of magnitude as *in vitro*, the synthesis of *N*-acetylglutamate, an obligate activator of the first step of urea synthesis, can be expected to occur in the mitochondrion under conditions where the amino acid acetyltransferase is not saturated by its substrates. The regulation of the first step of urea synthesis could thus depend mainly on the intramitochondrial substrate and perhaps product concentrations of amino acid acetyltransferase.

The mitochondrial acetyl-CoA:L-glutamate *N*-acetyltransferase (amino acid acetyltransferase, EC 2.3.1.1) catalyses the formation of *N*-acetylglutamate and CoA from glutamate and acetyl-CoA. *N*-Acetylglutamate is an obligate activator of the first enzyme of urea synthesis, the mitochondrial carbamoyl phosphate synthetase (EC 6.3.4.16). The amino acid acetyltransferase of rodent liver and duodenal mucosa has been purified and characterized by Shigesada & Tatibana (1971*a*,*b*) and Ushiyama *et al.* (1981). They have also shown that it is activated by arginine, thus disclosing a positive feedback system for urea synthesis. Since the enzyme also plays a role in man, as exemplified by our discovery of a hyperammonaemic patient lacking amino acid acetyltransferase activity (Bachmann *et al.*, 1981), we have purified the human enzyme and investigated some of its properties.

Materials and methods

Materials

L-[U-^{14}C]Glutamic acid was obtained from New England Nuclear Chemical (Dreieich, Germany), and impregnated silica-gel thin-layer sheets were from Gelman Instrument Co. (Ann Arbor, MI, U.S.A.). Otherwise all reagents were of analytical grade.

Purification of amino acid acetyltransferase

The enzyme was purified from a human liver obtained 4h *post mortem*. The whole procedure was performed at 4°C. The liver was minced, washed with 0.9% NaCl and homogenized in 4vol. of pH7.5 buffer, containing mannitol (0.21M), sucrose (0.07M), Tris (5mM) and EGTA (1mM).

Mitochondria were prepared as described elsewhere (Hogeboom, 1955; Myers & Slater, 1957). All of the following buffers contained dithioerythritol (0.5mM). The mitochondrial pellet was suspended in 200ml of potassium phosphate buffer (pH7.5, 50mM) and sonicated intermittently (total time 5min, 40W) with efficient cooling. The disrupted mitochondria were centrifuged (60min, 105000*g*) and $(NH_4)_2SO_4$ (243g/l) was slowly added, with stirring, to the supernatant. After centrifugation for 30min at 15000*g*, the precipitate was taken up in 20ml of the potassium phosphate buffer and centrifuged (30min, 105000*g*).

Chromatography on hydroxyapatite. The supernatant was applied to a hydroxyapatite column (2.5cm × 25cm), previously equilibrated with potassium phosphate buffer (10mM, pH7.0), and eluted at 20ml/h with potassium phosphate buffer, pH7.0, first with 150ml of 10mM, then with 150ml of 70mM, and finally with 300ml of 250mM; 10ml fractions were collected. The fractions eluted

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between 420 and 460ml were pooled, dialysed against potassium phosphate buffer (10mM, pH7.5) and concentrated to 8ml with an Amicon filtration system.

DEAE-cellulose chromatography. The concentrate was applied on a DEAE-cellulose column (1.6cm × 25cm) equilibrated with potassium phosphate buffer (10mM, pH7.5). A linear gradient (total volume 400ml) of increasing phosphate (pH7.5) concentration was applied, from 10mM up to 250mM (flow rate 20ml/h); 60 fractions each of 6ml were collected, and the fractions eluted between 60 and 96ml were pooled and dialysed against potassium phosphate buffer, pH8.0 (100mM). They were concentrated as described above to 8ml.

Gel-permeation chromatography. The concentrate was used for chromatography on a Sephacryl-300 column (1.6cm × 75cm) equilibrated with potassium phosphate (100mM, pH8.0), and eluted at 15ml/h with the equilibration buffer. The fractions (4ml each) eluted from 76 to 92ml were dialysed against potassium phosphate (100mM, pH8.0) containing L-arginine (1mM), and meanwhile the same column was equilibrated with this latter buffer. The non-diffusible material was concentrated to 8ml and the chromatography was repeated with the arginine-containing buffer. The fractions (each 4ml) eluted between 68 and 92ml were dialysed against potassium phosphate buffer (10mM, pH8.0) without arginine and stored at −20°C in small portions.

During chromatography, protein was monitored at 280nm and the relevant fractions were analysed for protein by the method of Lowry *et al.* (1951). The polyacrylamide-gel electrophoresis was performed in Tris/glycine buffer containing dithioerythritol (0.1mM) as described by Berüter *et al.* (1978).

Molecular-weight determination

The Sephacryl-300 column described above (total volume 150ml, void volume determined with Blue Dextran 48ml) was used, eluted with potassium phosphate buffer (100mM, pH8.0) at 15ml/h. Reference proteins (2mg of each) were aldolase (M_r 158000), catalase (M_r 210000), ferritin (M_r 440000) and thyroglobulin (M_r 669000). Calculations were done as described by Laurent & Killander (1964).

Assay methods

The amino acid acetyltransferase activity was assayed at 37°C in Tris buffer (pH8.7, 0.125M) containing 0.11% EDTA, acetyl-CoA and [^{14}C]-glutamic acid purified beforehand on Dowex 50 (X8; H^+ form). Acetyl-CoA was used at 2mM and glutamate at 4mM for assaying the activity during purification, and otherwise as indicated. The final incubation volume of 0.1ml contained approx. 1×10^6 d.p.m. of [U-^{14}C]glutamate.

Chromatography of the deproteinized incubation mixture with added carrier *N*-acetylglutamate was done on impregnated silica-gel thin-layer sheets with chloroform/methanol/formic acid (160:5:2, by vol.) and light petroleum (b.p. 40–60°C)/diethyl ether/formic acid (50:30:2, by vol.); the *N*-acetylglutamate spot revealed by Bromophenol Blue was cut out and its radioactivity determined. Details of the assay are given elsewhere (Colombo *et al.*, 1982).

The kinetic data of the primary plots were computed by the method of Wilkinson (1961), and unweighted regression was used for the secondary plots and the calculation of the molecular weight.

Results

Purification

The purification resulting from each step is shown in Table 1. The whole procedure leads to a 360-fold purification with respect to the mitochondrial activity, with a recovery of 67%.

General properties of the purified enzyme

The optimum pH (with 3.5mM-acetyl-CoA and 7mM-glutamate) with or without arginine (1mM) at 37°C was 8.5, but was not critical between 8.25 and 8.75. The purified enzyme was stable over 2 months if kept frozen at −20°C.

The molecular weight calculated from the elution volume of the amino acid acetyltransferase (81ml) is approx. 190000 [188000 ± 11000 (S.D.)]. The elution volumes of the reference proteins were: aldolase 83ml, catalase 80ml, ferritin 69ml and thyroglobulin 66ml.

As judged from polyacrylamide-gel electrophoresis (Fig. 1), the final product still contains some minor impurities. Gels run in parallel were tested for enzyme activity. In the final product amino acid acetyltransferase activity was only found associated with the main protein band. On two-dimensional t.l.c. only one product was detected, in a position identical with *N*-acetylglutamate (by radioautography), when the final enzyme of the purification was used for the assay. In contrast, enzyme from the previous step still also gave a product co-chromatographing with malate (2% of total product activity).

Kinetic data

The protocol used was as proposed by Rudolph & Fromm (1979). We used in duplicate assays five concentrations of acetyl-CoA (0.7, 0.875, 1.17, 1.75, 3.5mM) each with five concentrations of glutamate (4.2, 5.25, 7.0, 10.5, 21mM).

Table 1. *Purification of amino acid acetyltransferase from human liver*
Details of each step are described in the text.

	Volume (ml)	Total activity (nmol/min)	Protein (mg)	Specific activity (nmol/min per mg of protein)
Disrupted mitochondria (supernatant)	78	11.9	1543	0.008
$(NH_4)_2SO_4$ precipitate	22	23.2	458	0.051
Hydroxyapatite	8	16	105	0.153
DEAE-cellulose	8	9.5	27	0.349
Sephacryl without arginine	8	8.4	6.5	1.29
Sephacryl (arginine-containing buffer)	20	8.0	2.8	2.86

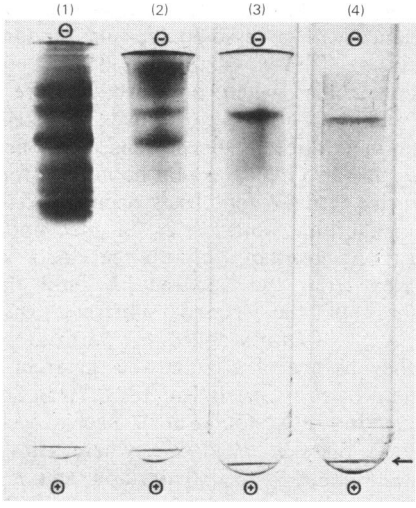

Fig. 1. *Polyacrylamide-gel electrophoresis of the product obtained (1) after hydroxyapatite column chromatography, (2) after DEAE-cellulose column, (3) after first Sephacryl column, and (4) after final purification*
For full details see the text. The arrow indicates the end of the gel.

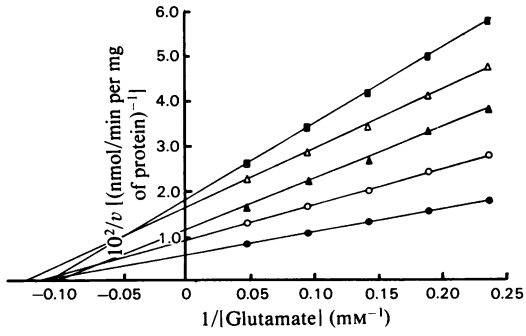

Fig. 2. *Double-reciprocal plot of dependence on glutamate concentration of amino acid acetyltransferase activity at five concentrations of acetyl-CoA:* ●, *3.5 mM*; ○, *1.75 mM*; ▲, *1.17 mM*; △, *0.875 mM*; ■, *0.7 mM*
The points are means of duplicate determinations.

Fig. 2 shows a Lineweaver–Burk plot of the data. Slopes and intercepts calculated as described by Wilkinson (1961) were used for secondary plots, from which Dalziel (1957) parameters were obtained. These are shown in Table 2 as well as derived kinetic constants. Assuming a molecular weight of 190000, a rate constant of $1.24\,s^{-1}$ is obtained for product formation under maximum rate conditions.

Since we were interested in the enzyme mechanism, product inhibition was also assayed by using various concentrations of the tested product and by varying one substrate while keeping the other constant at a non-saturating concentration. As shown in Figs. 3 and 4, competitive inhibition by *N*-acetylglutamate (glutamate variable) and CoA (acetyl-CoA variable) was found. Replots of the slopes are linear and allow calculation of the apparent inhibition constants. These are $K_{i,\text{acetylglutamate}}$ 0.49 mM with acetyl-CoA at 3.5 mM, and $K_{i,\text{CoA}}$ 10.1 mM with glutamate constant at 7 mM.

The experiment shown in Fig. 2 was repeated in the presence of 0.36 mM-*N*-acetylglutamate; the resulting Dalziel parameters were ϕ_0 2.45 ± 0.12, ϕ_A 22.1 ± 0.85, ϕ_B 11.5 ± 0.12 and ϕ_{AB} 213.6 ± 0.89 (means ± S.D., $n = 5$; units as in Table 2). Only the ϕ_{AB} term is affected.

Activation of amino acid acetyltransferase by L-arginine was tested at fixed concentrations of glutamate (7 mM) and acetyl-CoA (3.5 mM) with increasing arginine concentrations up to 4 mM. We measured an increase from 75 nmol/min per mg of protein at zero arginine to 145 nmol/min per mg with arginine above 0.5 mM. The half-maximal activation was found at 30 μM-arginine.

Table 2. *Dalziel (1957) parameters and derived kinetic constants of human amino acid acetyltransferase*
Key: A, glutamate; B, acetyl-CoA. Mean values ± S.D. are given ($n = 5$).

ϕ_0	2.58 ± 0.84 min·g of protein·mmol^{-1}	$K_{glutamate}$	8.1 mM
ϕ_A	21.0 ± 5.6 min·g of protein·litre^{-1}	K_{AcCoA}	4.4 mM
ϕ_B	11.2 ± 0.89 min·g of protein·litre^{-1}	$K_{i,glutamate}$	8.8 mM
ϕ_{AB}	98.3 ± 5.9 mmol·min·g of protein·litre^{-2}	$K_{i,AcCoA}$	4.7 mM

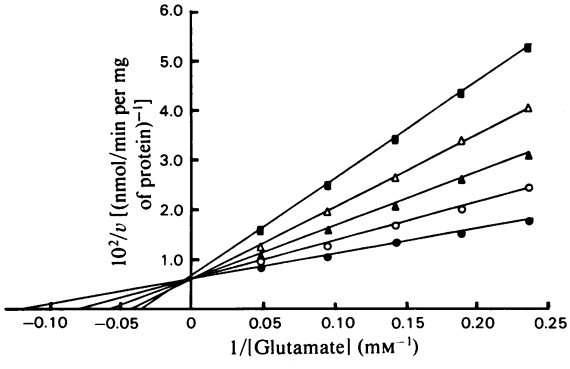

Fig. 3. *Competitive product inhibition: double-reciprocal plot of dependence on glutamate concentration of amino acid acetyltransferase activity assayed in the presence of five concentrations of N-acetylglutamate:* ■, *1.25 mM*; △, *0.83 mM*; ▲, *0.5 mM*; ○, *0.28 mM*; ●, *zero*
Acetyl-CoA was constant at 3.5 mM. The values shown are means of duplicate determinations.

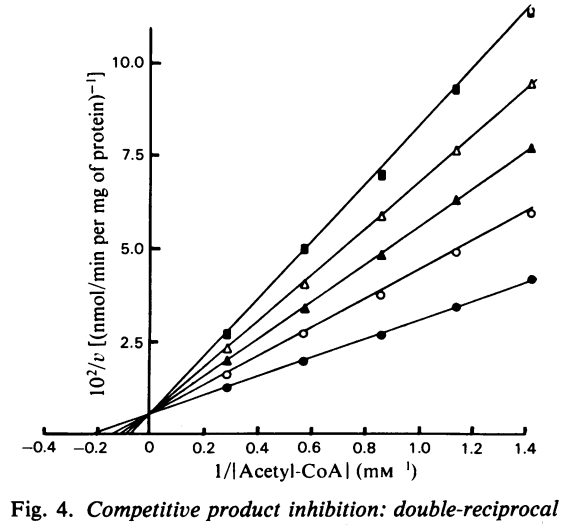

Fig. 4. *Competitive product inhibition: double-reciprocal plot of amino acid acetyltransferase activity versus acetyl-CoA concentration measured at five CoA concentrations:* □, *20 mM*; △, *15 mM*; ▲, *10 mM*; ○, *5 mM*; ●, *none*
Glutamate was constant at 7 mM. The values shown are means of duplicate determinations.

Discussion

N-Acetylglutamate is an obligate activator of carbamoyl phosphate synthetase and plays a crucial role in the short-term regulation of urea synthesis in man (Bachmann *et al.*, 1981). The kinetic data are in accordance with a rapid-equilibrium random Bi Bi mechanism. This is supported by the product-inhibition studies, where both products are competitive and only the ϕ_{AB} term is affected when incubation is done in the presence of *N*-acetylglutamate; this also indicates that no notable formation of abortive ternary complexes occurs. Assuming a rapid-equilibrium random Bi Bi mechanism, the dissociation constants of the products can be calculated from the apparent K_i and the concentration of the second substrate; these are $K_{i,acetylglutamate}$ 0.28 mM and $K_{i,CoA}$ 5.6 mM.

In the absence of arginine the apparent K_m for glutamate (acetyl-CoA 0.7 mM) is 8.7 mM, i.e. in the concentration range found in rat liver mitochondria (3–15 mM; Siess *et al.*, 1977), whereas for acetyl-CoA (glutamate 3–15 mM) the apparent K_m (4.5–4.6 mM) is higher than the intramitochondrial acetyl-CoA concentration (0.6–0.8 mM). Provided that at physiological pH the rate constants are not grossly altered, the initial velocity of amino acid acetyltransferase will thus be markedly affected by small changes in substrate concentrations. In addition, the low product-inhibition constant of *N*-acetylglutamate is of interest. The degradation of *N*-acetylglutamate is negligible in mitochondria, whereas an acylase activity is present in the cytosol (Reglero *et al.*, 1977). Thus Meijer & Van Woerkom (1979) have postulated that transport of *N*-acetylglutamate across the mitochondrial membrane is needed for preventing its accumulation in the mitochondria.

The dissociation constant of the amino acid acetyltransferase – *N* - acetylglutamate complex (0.28 mM) is of the order of magnitude of the intramitochondrial-matrix concentration of *N*-acetylglutamate found in rats (0.33 mM or higher; Cheung & Raijman, 1980). With 0.33 mM-*N*-acetylglutamate and 0.7 mM-acetyl-CoA the initial velocity would thus be decreased by 55% with 3 mM-glutamate, or by 28% with 15 mM-glutamate, as compared with the activity without *N*-acetylglu-

tamate binding to the enzyme, if the data *in vitro* could be applied to the situation *in vivo*. It should, however, be recognized that our measurements were done at pH 8.7 in an artificial buffer and that any *N*-acetylglutamate produced will also bind to the mitochondrial carbamoyl phosphate synthetase ($K_{acetylglutamate}$ 0.1 mM), which is present at much higher concentration (approx. 0.4 mM; Cheung & Raijman, 1980) than amino acid acetyltransferase (0.1 μM) as calculated from our results of the purification. More data on the reverse reaction and kinetic studies in the presence of arginine are needed to assess the respective roles of synthesis and transport of *N*-acetylglutamate out of the mitochondrion in order to understand better the regulation of the early steps of urea synthesis.

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