Subcellular Localization and Ionic Properties of Acetyl-Coenzyme A synthetase in the Electric Organ of *Torpedo marmorata*

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Acetyl-CoA synthetase activity was shown to be present in pure cholinergic synaptosomes from electric organ of *Torpedo marmorata*. After osmotic disruption of synaptosomes a substantial part of the activity was recovered in the soluble fraction. The effects of varying pH and increasing K^+ concentrations on the synaptosomal enzyme activity were shown to differ from those observed with the mitochondrial enzyme. Whereas this latter enzyme showed optimal activity above pH 8.5, and a maximal activation in the presence of 120 mM- K^+ , the synaptosomal enzyme exhibited an optimal activity at pH 7.9 and a moderate K^+ stimulatory effect with an optimal concentration of 30 mM.

The origin of the acetyl-CoA used in the cytoplasm as substrate for choline acetyltransferase is yet unclear. The demonstration that acetate is a main precursor of acetylcholine in the lobster nerve (Cheng & Nakamura, 1970) at the neuromuscular junction of the rat (Dreyfus, 1975) and in the electric organ of *Torpedo* (Israël & Tuček, 1974) raises interest in the enzyme associated with the activation of acetate into acetyl-CoA, i.e. acetyl-CoA synthetase (EC 6.2.1.1). No study of this enzyme is yet available on tissues where activated acetate is used for the synthesis of acetylcholine. In fact, attention has only been given to the acetyl-CoA synthetase of the brain where no acetate is used for the synthesis of acetylcholine; in this tissue, acetyl-CoA synthetase was mainly mitochondrial (Schuberth, 1965; Tuček, 1970; Tencati & Rosenberg, 1973; Reijnierse *et al.*, 1975).

In previous work (Diebler & Morot-Gaudry, 1977) we indeed showed that acetyl-CoA synthetase activity was present in a homogenate of *Torpedo* electric organ. The present study aimed to investigate the subcellular localization of acetyl-CoA synthetase in the same tissue; in addition we assessed the effects of Na^+ and K^+ ions and of pH on both synaptosomal and mitochondrial enzymic activity.

Experimental

Torpedo marmorata was purchased from the Marine Station, Arcachon, France.

Subcellular fractionation

Synaptosomes of the electric organ were prepared as follows. Slices of electric organ (about 20 g) were chopped with a scalpel blade and the resulting 'hash' was suspended in 200 ml of *Torpedo* physiological medium (sucrose/urea/saline solution; Israël *et al.*, 1976). After stirring at 4°C, the suspension was

filtered through three stainless-steel grids (mesh size, 1000, 500 and 200 μ m) and a nylon gauze (mesh size, 50 μ m). The filtrate was then centrifuged for 20 min at 6000g. The supernatant was discarded. The pellet (P) resuspended in 40–45 ml of physiological medium was carefully layered (6 ml) on a discontinuous gradient of sucrose/saline media (for details of the method, see Israël *et al.*, 1976; Morel *et al.*, 1977). After centrifugation at 63900g (r_{av} , 11.8 cm) for 40 min at 3°C the following fractions were separately collected (from top to bottom): supernatant (fraction A), three bands at the interfaces of the 0.1 M/0.3 M (fraction B), 0.3 M/0.5 M (fraction C), 0.5 M/0.7 M (fraction D) sucrose solutions and a pellet E in the 0.7 M-sucrose layer. It was shown that this procedure yielded a highly pure synaptosomal fraction (C) (Israël *et al.*, 1976; Morel *et al.*, 1977), whereas mitochondria are recovered in pellet E (the present paper).

Hypo-osmotic shock of the synaptosomes

Fraction C was slowly diluted 3-fold with physiological medium in the cold-room and the resulting suspension was centrifuged for 30 min at 12000g. The pelleted synaptosomes (C_{1c}) were then subjected to mild hypo-osmotic shock by suspension with 0.2 M-sucrose buffered with 0.01 M-sodium phosphate, pH 7.4 (final tissue concentration about 1.5 g/ml). This suspension was centrifuged for 30 min at 12000g. The resulting supernatant was referred to as S_{2c} ; the pellet (C_{2c}) was resuspended in buffered 0.2 M-sucrose (about 1.5 g/ml).

Enzyme assays

Except for succinate dehydrogenase and monoamine oxidase, enzyme assays were performed on tissue preparation pretreated with Triton X-100 (final

concentration 0.5%, v/v) at 4°C for 30 min. Lactate dehydrogenase was assayed as described by Johnson & Whittaker (1963). Fumarase was determined as described by Bergmeyer (1974). Succinate dehydrogenase was measured by the method of Arrigoni & Singer (1962). Monoamine oxidase was determined using the method of Kralj (1965). Choline acetyltransferase was assayed by the method of Fonnum (1975). Acetyl-CoA synthetase was measured as previously described (Diebler & Morot-Gaudry, 1977) by the method of Wieland & Weiss (1963). To minimize the inhibitory effects of high sucrose molarity (above 0.5M) on the enzyme activity (Reijnierse *et al.*, 1976), it was necessary to adjust the enzyme fraction to a low sucrose molarity. Hence, fraction E was diluted with physiological medium to the sucrose concentration of fraction C (0.4M), whereas the other fractions (P, A, B, C and D) were kept and assayed unaltered.

For demonstrating the effect of Na⁺ and K⁺ ions, these were removed from the reaction components (including the enzyme preparations). The CoA used was a trilithium salt neutralized with Tris buffer before use. The Tris salt of ATP was prepared from the dipotassium salt by passage through a Dowex 50 (H⁺ form) column by the method of Von Korff (1953). Enzymic fractions C and E were centrifuged for 30 min at 12000g and the pelleted synaptosomes or mitochondria were resuspended in 0.4M-sucrose.

To study the pH effect all substrates of the reaction were adjusted to the desired pH with 1M-Tris buffer and then diluted to the convenient concentration. The final pH of the incubation mixture was carefully checked.

In standard conditions and unless otherwise stated acetyl-CoA synthetase activity was assayed at 38°C in presence of 35mM-K⁺, no added Na⁺ and at pH7.9.

Protein assay

Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Chemicals

Citrate synthase, malate dehydrogenase, CoA, and acetyl-CoA were obtained from Boehringer, Mannheim, Germany; NAD⁺ and ATP were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Results

Subcellular distribution of acetyl-CoA synthetase activity

We have previously detected acetyl-CoA synthetase activity in a *Torpedo* electric-organ homogenate (Diebler & Morot-Gaudry, 1977). About 30% of the activity is recovered in a crude synaptosomal

Table 1. Subcellular distribution of acetyl-CoA synthetase, choline acetyltransferase and succinate dehydrogenase

Electric-organ synaptosomes were prepared as described in the Experimental section. Fraction P is the parent fraction; fractions A, B, C, D and E are the sub-fractions of fraction P. The pure synaptosomal fraction is fraction C. Enzyme activities were measured as described in the Experimental section. Results are means $\pm$ s.e.m. for the number of experiments indicated. Rg represents the percentage recovery from the parent fraction P.

	Number of experiments	Enzyme activity						Rg
		Fraction	P	A	B	C	D	E
Acetyl-CoA synthetase (nmol/h per mg of protein)	4		111 $\pm$ 23	113 $\pm$ 21	82 $\pm$ 18	198 $\pm$ 33	116 $\pm$ 30	86 $\pm$ 18
Choline acetyltransferase (nmol/h per mg of protein)	7		150 $\pm$ 25	89 $\pm$ 23	251 $\pm$ 61	526 $\pm$ 50	322 $\pm$ 67	57 $\pm$ 12
Succinate dehydrogenase (Δ O.D/h per mg of protein)	5		2.03 $\pm$ 0.54	0.40 $\pm$ 0.21	0.27 $\pm$ 0.13	0.37 $\pm$ 0.15	0.16 $\pm$ 0.10	3.07 $\pm$ 0.51
								89 $\pm$ 10
								95 $\pm$ 9
								98 $\pm$ 6.0

preparation (fraction P). Table 1 shows that the highest acetyl-CoA synthetase activity is found in the pure synaptosomal fraction C, but activity is also detected in the other fractions. The synaptosomal marker choline acetyltransferase is recovered with fraction C, as described previously by Israël *et al.* (1976) and the mitochondrial marker succinate dehydrogenase is associated with the pellet E. In standard conditions of assay the specific activity of acetyl-CoA synthetase is 198 ± 34 (mean $\pm$ S.E.M.; $n = 4$) nmol/h per mg of protein in the highly pure synaptosomal fraction and 86 ± 18 (mean $\pm$ S.E.M.; $n = 4$) nmol/h per mg of protein in the mitochondrial fraction E. The gradient recovery of the enzyme was 89%.

After mild hypo-osmotic shock of the synaptosomes (fraction C) about 45% of the acetyl-CoA synthetase activity is recovered in the soluble sub-fraction S_{2c} (Table 2). This relative distribution is

Table 2. Relative distribution of acetyl-CoA synthetase, lactate dehydrogenase and monoamine oxidase after osmotic disruption of synaptosomes

Disrupted synaptosomes were centrifuged at 12000g for 30min. Supernatant S_{2c} and pellet C_{2c} were collected. Acetyl-CoA synthetase activity was measured at pH7.9 in the presence of 35mM- K^+ and 30mM- Na^+ . For details, see the Experimental section. Results are expressed as the percentage of the recovered activity (mean $\pm$ S.E.M. for four experiments).

Fraction	Enzyme activity (%)	
	S_{2c}	C_{2c}
Acetyl-CoA synthetase	45.5 ± 1.2	55.6 ± 1.8
Lactate dehydrogenase	44.8 ± 3.1	55.2 ± 2.2
Monoamine oxidase	9.6 ± 4.5	90.4 ± 4.5

Table 3. Subsynaptosomal distribution of acetyl-CoA synthetase, lactate dehydrogenase and fumarase

Synaptosomes were submitted to a mild osmotic lysis and further frozen and thawed. After centrifugation at 12000g for 30min, supernatant S_{2c} and pellet C_{2c} were collected. For details, see the Experimental section. Results are expressed as the percentage of the recovered activity (for two experiments).

Fraction	Enzyme activity (%)	
	S_{2c}	C_{2c}
Acetyl-CoA synthetase	87	13
	83	17
Lactate dehydrogenase	65	35
	64	36
Fumarase	26	74
	27	73

Fig. 1. Effect of K^+ on acetyl-CoA synthetase activity. The assay was performed at pH7.9 in the presence of various K^+ concentrations. For the method of removal of ions from the reaction components, see the Experimental section. Enzymes were prepared from the pure synaptosomal fraction C (●) and the mitochondrial fraction E (○). Results are expressed as a percentage of the activity measured in the presence of 20mM- K^+ .

Fig. 2. Effect of Na^+ on acetyl-CoA synthetase. The assay was performed in the presence of 35mM- K^+ at pH7.9 and at various $NaCl$ concentrations. Ions were removed from the reaction mixture components as described in the Experimental section. Enzymes were prepared from the synaptosomal fraction C (●) and the mitochondrial fraction E (○). Results are expressed as a percentage of the activity measured without added Na^+ .

similar to that of the cytoplasmic marker lactate dehydrogenase and contrasts with that of the mitochondrial marker monoamine oxidase mainly

Fig. 3. *Effect of pH on acetyl-CoA synthetase activity* The assay was performed at various pH values in the presence of 35 mM-K⁺ (a), 120 mM-K⁺ (b), and 200 mM-K⁺ (c) and no added sodium. All reaction components were adjusted to the desired pH and the final pH of the incubation medium was carefully checked. Enzymes were prepared from the synaptosomal fraction C (●) and the mitochondrial fraction E (○). For details, see the Experimental section. Results are expressed as a percentage of the maximal activity at each K⁺ concentration.

recovered (90%) with the pellet C_{2c} (Table 2). Moreover, when the osmotically lysed synaptosomes are submitted to a freezing and thawing cycle (Table 3), the acetyl-CoA synthetase activity recovered in the supernatant increases to 87% of the total, whereas

lactate dehydrogenase recovered in this last fraction is only 65% of the total. As indicated by the distribution of fumarase activity mainly in the pellet (74%), the more severe condition of synaptosomal disruption only leads to a small release of the matrix contents in the supernatant.

Effect of K⁺ and Na⁺ on acetyl-CoA synthetase

Fig. 1 shows the stimulatory effect of increasing K⁺ concentrations on synaptosomal (C) and mitochondrial (E) enzyme fractions. It can be observed that fraction E activity is significantly stimulated (1.5-fold) by the presence of K⁺ with an optimal concentration of about 120 mM. In contrast, the synaptosomal activity is moderately stimulated by K⁺, the maximal effect being observed at 30 mM-K⁺.

Fig. 2 shows that the addition of Na⁺ has a similar marked inhibitory effect on the enzymes of both fractions C and E.

Effect of pH on acetyl-CoA synthetase

The effect of varying pH on the synaptosomal and mitochondrial enzymes is shown in Figs. 3(a)–3(c). In standard ionic assay conditions, i.e. in the presence of 35 mM-K⁺ and no added Na⁺, the synaptosomal activity has a distinct optimal pH of 7.9, whereas mitochondrial activity shows an optimal pH above 8.5 (Fig. 3a). When K⁺ concentration is increased to 200 mM, the optimal pH of the synaptosomal enzyme does not change, whereas that of the mitochondrial enzyme broadens at pH values (Figs. 3b and 3c).

Acetyl-CoA is unstable at high pH values (Decker, 1965). To test the stability of acetyl-CoA above pH 8, we assayed in routine conditions known amounts of acetyl-CoA; it was found that no more than 10% of the acetyl-CoA was hydrolysed when at pH values between 8 and 8.5.

Discussion

The preparation of pure cholinergic synaptosomes from electric organ of *Torpedo* permits us to demonstrate the presence of acetyl-CoA synthetase activity in these nerve endings. These results differ from those on brain synaptosomes, where Reijnierse *et al.* (1975) have shown that no significant activity could be demonstrated. This is consistent with the fact that acetate is an acetylcholine precursor in synapses of the neuromuscular type, such as the electric organ, but not in the brain. In our material, activity is also recovered in fraction E. This last fraction is heterogeneous (Israël *et al.*, 1976; Morel *et al.*, 1977), but the distribution of the characteristic marker succinate dehydrogenase indicates that it contains most of the mitochondria. In contrast, fraction C has a low succinate dehydrogenase activity and indeed very few mitochondria are found in electric-organ nerve endings, as shown by electron-microscopic

examination (Israël *et al.*, 1976; Morel *et al.*, 1977). It is therefore likely that the high acetyl-CoA synthetase activity observed in pure synaptosome preparation is not wholly located in synaptic mitochondria.

The present results indicate that after mild osmotic disruption of synaptosomes 45% of acetyl-CoA synthetase activity is recovered in the soluble subfraction, as is the cytoplasmic marker lactate dehydrogenase; in contrast, the relative distribution of monoamine oxidase indicates that most of the synaptosomal mitochondria are present in pellet C_{2c}. In spite of the low mitochondrial content of the *Torpedo* nerve endings, this last enzyme marker was detected, since the assay is very sensitive. In more severe conditions of synaptosomal disruption (osmotic lysis plus freezing and thawing), 87% of acetyl-CoA synthetase activity is present in the soluble subfraction, whereas 65% of lactate dehydrogenase is associated with this last fraction. The presence of fumarase in the supernatant (26%) indicates mitochondrial damage and we can therefore infer that at the most 26% of the acetyl-CoA synthetase present in the soluble subfraction originates from the matrix. Acetyl-CoA synthetase and lactate dehydrogenase have thus a similar subsynaptosomal distribution (about 60% in supernatant S_{2c}). These results strongly suggest that a substantial part of the acetyl-CoA synthetase activity measured in intact synaptosomes is located in the cytosol.

The present results on the effects of pH and K⁺ ions clearly indicate that acetyl-CoA synthetase recovered with the pure synaptosomal fraction C exhibits a sensitivity different from that observed for the enzyme associated with fraction E. In this latter fraction the optimum activity at pH greater than 8.5 and the pattern of K⁺ stimulatory effect with a peak at 120mM-K⁺ are identical with the properties described for the mitochondrial enzyme of rat brain (Von Korff, 1953; Tencati & Rosenberg, 1973; Banns *et al.*, 1978), or of ox heart (Webster, 1966). On the other hand, synaptosomal enzyme of the electric organ shows a distinct optimal activity at pH 7.9, and the K⁺ concentration required for its maximal stimulation is about 30mM. The inhibitory effect of added Na⁺ on the rat brain enzyme (Von Korff, 1953; Tencati & Rosenberg, 1973) was also observed in our material with both synaptosomal and mitochondrial fractions.

The present data indicate that in synapses able to efficiently incorporate acetate into acetylcholine,

there exists a high acetyl-CoA synthetase activity, which is mainly located in the cytosol. Moreover, the synaptosomal acetyl-CoA synthetase activity shows characteristics different from those of the non-synaptic mitochondrial enzyme.

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