Acetaldehyde formation by mitochondria from the free-living nematode *Panagrellus redivivus*

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The mitochondrial fraction from the free-living nematode *Panagrellus redivivus* decarboxylates pyruvate to produce significant amounts of acetaldehyde. Acetaldehyde formation is stimulated by thiamin pyrophosphate, shows a sharp optimum at pH 6.8 and is greater under anaerobic than aerobic conditions. The pyruvate decarboxylase activity cofractionates with, and is probably a partial reaction of, the pyruvate dehydrogenase complex. Acetaldehyde production is modulated by NAD⁺, ATP and acetyl-CoA and is greatly stimulated by lipoic acid. The pyruvate decarboxylase system is extremely sensitive to thiol-group inhibitors and is inhibited by oxygen in the presence of pyruvate.

Under anaerobic conditions, invertebrates characteristically break down carbohydrate into a range of reduced organic end products, including organic acids such as lactate, alanine, acetate, succinate, propionate, 2-methylbutyrate and 2-methylvalerate. The production of alcohols by invertebrates is much less common. Ethanol production has, however, been described in acanthocephalans (Körting & Fairbairn, 1972), cestodes (Agosin, 1957; von Brand & Bowman, 1961) nematodes (Barrett, 1981) and insect larvae (Wilps & Schöttler, 1980). Recently, ethanol production under anaerobic conditions has been reported in vertebrates, in cyprinid fish (Shoubridge & Hochachka, 1980; Johnston & Bernard, 1983). None of these animals has been shown to possess a soluble pyruvate decarboxylase. Mourik *et al.* (1982) showed that under anaerobic conditions mitochondrial preparations from goldfish muscle would decarboxylate pyruvate to acetaldehyde and suggested that this was catalysed by the pyruvate dehydrogenase complex. When kept anaerobically the free-living nematode *Panagrellus redivivus* excretes large quantities of ethanol and this has proved a useful model system in which to study the mechanism of ethanol production in metazoa.

Materials and methods

Panagrellus redivivus were maintained in culture on autoclaved Quaker Oats. Worms were washed

from the surface of the cultures with distilled water and cleaned by sedimentation followed by migration through several layers of filter paper. Microscopic examination of the cleaned worms showed them free from contaminating yeast cells.

Mitochondrial fraction

The worms were washed twice in extraction buffer {210 mM-mannitol/120 mM-sucrose/50 mM-triethanolamine/HCl/50 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/2 mM-EDTA containing 0.3% bovine serum albumin, adjusted to pH 7.2}, then homogenized in a power-driven glass homogenizer at 5°C. The homogenate was centrifuged at 125g for 10 min at 5°C, the precipitate discarded and the supernatant centrifuged for a further 5 min at 14000g. The mitochondrial pellet was washed twice by resuspending in extraction buffer and recentrifuging at 14000g for 5 min. The pellet was finally suspended in extraction buffer to give a concentration of approx. 20 mg of mitochondrial protein/ml.

A mitochondrial membrane fraction was prepared by disrupting mitochondria suspended in extraction buffer with 3 × 10 s sonication, alternating with 2 min cooling periods, in an MSE 150 W ultrasonic disintegrator fitted with a microtip, at a power setting of 50 W. The membranes were washed twice by suspending in extraction buffer and recentrifuging at 100000g for 15 min.

Rat liver mitochondria were prepared as described by Johnson & Lardy (1967).

Enzyme assays

All enzymes were assayed at 30°C, with a Gilford model 240 recording spectrophotometer. Succinate dehydrogenase (EC 1.3.99.1), 2-oxoglutarate dehydrogenase (EC 1.2.4.2), lactate dehydrogenase (EC 1.1.1.27) and alcohol dehydrogenase (EC 1.1.1.1) were assayed as previously described (Körting & Barrett, 1977). In crude preparations pyruvate dehydrogenase (EC 1.2.4.1) was assayed by measuring acetyl-CoA production with arylamine acetyltransferase (EC 2.3.1.5) and *p*-nitroaniline as described by Coore *et al.* (1971). Arylamine acetyltransferase was prepared from pigeon liver by the method of Wieland *et al.* (1972). In soluble preparations pyruvate dehydrogenase was assayed by following NADH production; the reaction mixture (1 ml) contained 50 mM-potassium phosphate buffer, pH 8.0, 2 mM-MgCl₂, 10 mM-pyruvate, 0.1 mM-thiamin pyrophosphate, 0.5 mM-CoA, 0.3 mM-NAD⁺ and 1 mM-L-cysteine. Acid phosphatase (EC 3.1.3.2) and glucose-6-phosphatase (EC 3.1.3.9) were assayed by the methods of Hübscher & West (1965) and de Duve *et al.* (1955), respectively.

Nucleotides, cofactors and potential modulators were all neutralized before use, where necessary.

Acetaldehyde production

Approx. 0.5 mg of mitochondrial protein was incubated in snap-cap vials at 30°C; the reaction mixture (0.5 ml) contained 105 mM-mannitol, 60 mM-sucrose, 25 mM-triethanolamine/HCl, 10 mM-Hepes, 1 mM-EDTA, 5 mM-KH₂PO₄, 1 mM-CaCl₂, 0.1 mM-thiamin pyrophosphate and 10 mM-pyruvate, adjusted to pH 6.8. For anaerobic incubations the assay mixture was pregassed with O₂-free N₂. Alternatively, O₂ was removed biochemically by the addition of 10 µmol of ascorbate followed by 2 units of ascorbate oxidase. Incubations were terminated by the addition of 0.1 ml of 0.6 M-HClO₄, the tubes were stood on ice for 10 min, neutralized with 2 M-KHCO₃ and allowed to stand at 0°C for a further 15 min. The KClO₄ precipitate was spun down at 2000g for 10 min and aliquots of the supernatant were taken for acetaldehyde determination. The latter were measured enzymically; the assay mixture (1 ml) contained 100 mM-triethanolamine/HCl, pH 7.6, 0.3 mM-NADH, 0.1–0.2 ml of supernatant and 15 µg of alcohol dehydrogenase. Acetoin and 2-acetolactate were determined in the HClO₄ supernatant by the method of Saz *et al.* (1958).

Results and discussion

When incubated under anaerobic conditions, the mitochondrial fraction of *P. redivivus* produces significant amounts of acetaldehyde

(3.3 ± 0.2 nmol of acetaldehyde/min per mg of mitochondrial protein at 30°C; mean $\pm$ S.E.M., $n = 20$). Acetaldehyde production was protein-dependent, but decreased with time, probably as a result of product inhibition (Fig. 1). Thiamin pyrophosphate is required for maximum activity, but the addition of Mn²⁺ ions to the reaction mixture had no effect on the rate of acetaldehyde formation (Table 1). Rat liver mitochondria incubated under the same conditions produced no detectable acetaldehyde.

The mitochondrial fraction of *P. redivivus* decarboxylated 2-oxoisobutyrate at about 40% of the rate of pyruvate. Pyruvate decarboxylation was inhibited by 2-oxoisovalerate (Table 2) and showed

Fig. 1. Effect of protein concentration (a) and the time course (b) of acetaldehyde production by the mitochondrial fraction of *P. redivivus* incubated under anaerobic conditions

product inhibition, the I_{50} for acetaldehyde being 200 μ M. Plant pyruvate decarboxylases also show product inhibition, but the I_{50} for plant enzymes is in the range of 20 mM.

The mitochondrial membrane marker enzymes succinate dehydrogenase and pyruvate dehydrogenase show a parallel distribution, with a 12-fold enhancement in the mitochondrial fraction and a 25-fold enhancement in the mitochondrial membrane fraction (Table 2). Approx. 56% of the total mitochondrial marker enzyme content was recovered in the mitochondrial fraction, but less than 1% of the cytoplasmic marker (lactate dehydro-

genase) and 1.5% of the lysosomal marker (acid phosphatase). Microsomal contamination, as judged by recovery of the microsomal marker enzyme (glucose-6-phosphatase) was approx. 4%. Pyruvate decarboxylase activity in *P. redivivus* tissues was associated with the mitochondrial membrane fraction (Table 3). The enzyme system was readily solubilized by sonication or freeze-thawing and could be partially purified by isoelectric precipitation at pH 5.5. Insufficient material was available for further purification. The partially purified preparation migrated as one major and four minor bands on sodium dodecyl sulphate/polyacrylamide disc gel electrophoresis. Acetaldehyde production and pyruvate dehydrogenase activity have a similar subcellular distribution and maintained a constant activity ratio in the different preparations, showing that they are probably due to the same enzyme. The partially purified enzyme showed no succinate dehydrogenase activity, but still had appreciable 2-oxoglutarate dehydrogenase activity.

Pyruvate decarboxylation by the mitochondrial fraction from *P. redivivus* had a sharp pH optimum at 6.5 (Fig. 2). If incubations were carried out in air, acetaldehyde production was decreased, but the pH optimum was unchanged. The effect of different gas phases on acetaldehyde formation (Table 4) suggests that this effect is due to O_2 rather than N_2 . The inhibition of acetaldehyde production in the presence of O_2 does not involve the cytochrome chain, since acetaldehyde production is unaffected by cyanide, ferricyanide or ascorbate (Table 2).

The enzyme system is only inhibited by O_2 when pyruvate is present; prolonged incubation (up to

Table 1. *Effects of cofactors and modulators on acetaldehyde production by the mitochondrial fraction of P. redivivus*

Incubations were anaerobic; for details see the Materials and methods section. Acetaldehyde production is the mean of five determinations; the range is given in parentheses.

Additions	Final concentration (mM)	Acetaldehyde production (% of control)
Without thiamin pyrophosphate	—	41 (28–56)
Mn^{2+}	1	94 (90–101)
ATP	10	31 (17–50)
ADP	4	85 (82–90)
AMP	2	97 (90–100)
Cyclic AMP	1	101 (98–103)
NAD^+	1.5	71 (57–86)
$NADH$	1.5	100 (89–126)
$NADP^+$	1.5	92 (75–100)
$NADPH$	1.5	97 (90–100)
CoA	1	116 (107–126)
Acetyl-CoA	1	59 (51–66)

Table 2. *Effect of inhibitors and thiol agents on acetaldehyde production by the mitochondrial fraction of P. redivivus*

For details see the Materials and methods section. Acetaldehyde production is the mean of five determinations, with the range in parentheses.

Additions	Final concentration (mM)	Acetaldehyde production (% of control)	
		Anaerobic incubations	Aerobic incubations
Oxoisovalerate	5	96 (90–100)	—
	50	27 (8–47)	—
CN^-	1	100 (70–102)	100 (93–102)
Arsenite	10	0	0
4-Chloromercuribenzoic acid	1	0	0
Iodoacetamide	1	65 (40–72)	55 (45–73)
Cystamine	10	54 (40–65)	46 (34–53)
Cysteine	10	28 (23–34)	20 (0–40)
Dithiothreitol	10	100 (98–101)	100 (96–103)
Glutathione			
Oxidized	2	102 (97–107)	100 (99–102)
Reduced	10	87 (80–100)	100 (100–101)
Mercaptoethanol	10	104 (95–113)	101 (98–107)
Ascorbate	15	100 (98–103)	100 (90–102)
Ferricyanide	1.5	100 (95–106)	98 (87–100)

Table 3. *Partial purification of pyruvate decarboxylase activity from P. redivivus*
Cell fractions were prepared as described in the Materials and methods section.

Enzyme system	Fraction ... homogenate	Specific activity (nmol/min per mg of protein at 30°C)					Solubilized enzyme	pH 5.5 precipitate
		Supernatant fraction	Mitochondrial fraction	Mitochondrial membranes	Mitochondrial sap			
Lactate dehydrogenase	4.8	15.7	0.85	0	2.3	—	—	
Alcohol dehydrogenase	7.7	22.4	3.28	0	8.0	—	—	
Acid phosphatase	0.04	0.139	0.013	0.04	0	—	—	
Glucose-6-phosphatase	0.4	0.153	0.35	0.14	0.02	—	—	
Succinate dehydrogenase	0.5	0.14	6.05	13.8	0.24	0.13	0	
Pyruvate dehydrogenase	0.06	0.01	0.73	1.32	0.03	1.6	1.7	
Acetaldehyde production	0.15	0.04	1.3	2.05	0.1	2.36	2.34	
Acetaldehyde production/pyruvate dehydrogenase	2.5	—	1.78	1.56	—	1.47	1.38	
Total protein (mg)	2030	599.5	93.8	22.4	30.4	11.7	5.8

Fig. 2. Effect of pH on acetaldehyde production by the mitochondrial fraction of *P. redivivus* incubated under anaerobic (●) and aerobic (▲) conditions

The effect of pH was determined with acetate/HCl buffer (pH 4.0–5.5), potassium phosphate buffer (pH 5.5–7.5), Hepes buffer (pH 7.0–8.0) and triethanolamine/HCl buffer (pH 7.0–9.0) (all at 100 mM final concentration). No significant differences were found in acetaldehyde production with the different buffer systems in the regions of overlap.

Table 4. Effect of gas phase on acetaldehyde production by the mitochondrial fraction of *P. redivivus*

Incubation conditions are described in the Materials and methods section. Acetaldehyde production is the mean of five determinations, with the range in parentheses.

Gas phase	Acetaldehyde production (% of N ₂ control)
N ₂	100
Air	46 (30–76)
O ₂	30 (28–25)
H ₂	82 (70–94)
Ar	93 (79–100)

60 min) under 100% O₂ in the absence of pyruvate did not effect subsequent acetaldehyde production. Neither reduced glutathione, mercaptoethanol nor dithiothreitol protected the enzyme against inhibition by O₂ in the presence of pyruvate (Table 2). These three thiol-group reagents also had no effect on acetaldehyde production during anaerobic incubations, although cysteine and cystamine were inhibitory under both aerobic and anaerobic conditions (Table 2). Acetaldehyde production was

again strongly inhibited by arsenite, iodoacetamide and 4-chloromercuribenzoate, reagents all known to react with thiol groups (Table 2). The inhibition by cysteine and cystamine may be due to the ability of these compounds to form covalent links with free thiol groups.

The inhibition of acetaldehyde production by O₂ in the presence of pyruvate may be due to the formation of a stable intermediate with lipoic acid. Incubation of the mitochondrial fraction of *P. redivivus* in the presence of 1 mM-lipoic acid (DL-6,8-thio-octic acid amide) caused a 16% increase in anaerobic acetaldehyde production, but an 80% increase in aerobic acetaldehyde production. The addition of FAD or FMN to the incubation mixture had no effect (1 mM final concentrations).

Acetaldehyde formation by the mitochondrial fraction of *P. redivivus* is modulated by a number of physiological effectors (Table 1). The production of acetaldehyde was strongly inhibited by ATP, NAD⁺ and acetyl-CoA, but ADP was slightly inhibitory; AMP, 3',5'-cyclic AMP and NADP⁺ were without effect and CoA caused slight activation. In the last case, CoA may be acting as a thiol reagent. When the tissues of the nematode become anaerobic the fall in the [ATP]/[ADP] ratio and in the free [NAD⁺]/[NADH] ratio would stimulate acetaldehyde production. The accumulation of acidic anaerobic end products in *P. redivivus* would cause a drop in tissue pH and again acetaldehyde production would be favoured (Fig. 2).

Acetaldehyde is not the only product formed during incubations of the mitochondrial fraction; almost as much acetoin is produced as acetaldehyde (2.3 ± 0.9 nmol/min per mg of mitochondrial protein at 30°C; mean ± S.E.M., n = 5), but no 2-acetolactate formation could be detected. In contrast with acetaldehyde production, acetoin formation is the same under aerobic and anaerobic conditions.

In prokaryotes the reduction of acetaldehyde to ethanol during anaerobiosis maintains the cytoplasmic redox balance and these organisms possess a soluble cytoplasmic pyruvate decarboxylase. In *P. redivivus* acetaldehyde is probably produced within the mitochondrion, but ethanol dehydrogenase activity is largely, but not totally, in the cytosol (Table 3). In these nematodes ethanol formation may again be involved in maintaining the cytoplasmic redox balance, but it could also act as a mitochondrial electron sink. In *P. redivivus*, ethanol is not the only anaerobic end product and, in common with other nematodes, they produce a range of organic acids as well (Barrett, 1981). Ethanol is a neutral end product which can readily diffuse across membranes. The main advantage to the nematode in ethanol production is that, in contrast with organic acid production, it

effectively removes a proton and so helps to suppress tissue acidification.

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