Regulation of Citrate Synthase Activity in Methylotrophs by Reduced Nicotinamide–Adenine Dinucleotide, Adenine Nucleotides and 2-Oxoglutarate

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Citrate synthase from two typical facultative methylotrophs, but not from four obligate methylotrophs or from two restricted facultative methylotrophs, is inhibited by 0.1 mM-NADH. ATP or ADP (both at 10 mM) inhibits all the citrate synthases, whereas 2-oxoglutarate (10 mM) inhibits those from only three of the eight methylotrophs.

The citrate synthases of Gram-negative bacteria are inhibited by low concentrations of NADH, whereas those of Gram-positive bacteria, yeast, animals and plants are not (Weitzman & Jones, 1968; Borris & Ohman, 1972; Eidels & Preiss, 1970). Weitzman & Jones (1968) considered this to be feedback inhibition when NADH is regarded as an end product of the catabolic function of the tricarboxylic acid cycle. Such a control mechanism would not be expected to operate in obligate methylotrophs and restricted facultative methylotrophs, because the low or zero specific activities of some tricarboxylic acid-cycle enzymes found under all growth conditions (Colby & Zatman, 1972, 1975; Dahl *et al.*, 1972; Davey *et al.*, 1972; Patel *et al.*, 1969) suggests that the cycle plays an anabolic rather than an 'amphibolic' role in these organisms. Feedback inhibition of citrate synthase by NADH would, however, be expected in Gram-negative typical facultative methylotrophs such as bacterium 5B1 and *Pseudomonas* 3A2, which contain high activities of all the tricarboxylic acid-cycle enzymes when growing on some non- C_1 compounds (Colby & Zatman, 1972, 1975); under the latter conditions the cycle is presumably important for NADH generation.

The effect of NADH and of 15 other potential inhibitors on citrate synthases from four obligate methylotrophs, two restricted facultative methylotrophs and two typical facultative methylotrophs has been investigated.

Materials and methods

CoA (Li^+ salt), ATP, ADP, AMP, NAD^+ , $NADP^+$, NADH, NADPH, D(-)-3-phosphoglyceric acid (Na^+ salt), phosphoenolpyruvic acid (trisodium salt), sodium pyruvate, oxaloacetic acid, 2-oxoglutaric acid, glyoxylic acid, sodium glutamate, sodium fumarate, pig heart citrate synthase (EC 4.1.3.7) and 5,5'-dithiobis-(2-nitrobenzoic acid) were obtained from Sigma (London) Chemical Co. Ltd., Kingston-

upon-Thames, Surrey, U.K. Sodium hydrogen malate and sodium succinate were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Acetyl-CoA was prepared from CoA and acetic anhydride as described by Stadtman (1957), and solutions were standardized by using commercial citrate synthase and the assay method described below.

All cultures were incubated at 30°C. Organisms 4B6, C2A1, W3A1, 5B1, *Pseudomonas* 3A2 and *Bacillus* PM6 were grown in 8-litre batches of mineral base E (Owens & Keddie, 1969), containing 0.2% trimethylamine hydrochloride and vitamins where required (Colby & Zatman, 1973, 1975). The culture vessels were as described by Colby & Zatman (1974), except that additional aeration was achieved by bubbling filtered air through the cultures by means of a glass tube immersed in the culture medium. *Methylobacterium albus* (BG8) was similarly grown in 8-litre batches of nitrate-mineral-salts (NMS) medium (Whittenbury *et al.*, 1970) containing 0.4% methanol. *Methylosinus trichosporium* (OB3b) was grown in shaken 2-litre conical flasks, each containing 750 ml of nitrate-mineral-salts medium and each connected to a football bladder containing about 1500 ml of methane. Organisms were harvested in mid-exponential phase, washed twice with ice-cold 50 mM-sodium phosphate buffer, pH 7.5, and resuspended in the same buffer at a concentration of about 200 mg wet wt. of organisms/ml. All further procedures were done at 0–4°C. Crude sonic homogenates of organisms 4B6, C2A1, W3A1, 5B1, *Pseudomonas* 3A2 and *Bacillus* PM6 were prepared as described by Colby & Zatman (1973). Crude homogenates of *Methylobacterium albus* (BG8) and *Methylosinus trichosporium* (OB3b) were obtained by one passage through a French pressure cell. Extracts were obtained from the crude homogenates by centrifugation at 35 000g for 30 min. Extracts of organisms 4B6, C2A1 and W3A1, which contained little or no malate dehydrogenase activity (Colby & Zatman, 1972, 1975), were dialysed for 3 h against 1000 vol. of 20 mM-sodium phosphate buffer, pH 7.5, and then used as sources of citrate synthase without further treatment. Extracts of *Pseudomonas*

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3A2, 5B1, *Bacillus* PM6, *Methylomonas albus* (BG8) and *Methylosinus trichosporium* (OB3b) had appreciable malate dehydrogenase activity, which made a study of the effects of NADH on citrate synthase activity impossible. The citrate synthases in these extracts were partially purified as follows. Salmine sulphate solution (15 mg/ml) was added to the extracts (about 20 mg of protein/ml) until 2.5 mg had been added per mg of protein and then the precipitate removed by centrifugation. The supernatants were fractionated by adding solid $(\text{NH}_4)_2\text{SO}_4$. With extracts of *Methylomonas albus* (BG8) citrate synthase was precipitated between 30 and 50% saturation, whereas with the other extracts citrate synthase was precipitated between 50 and 70% saturation. The active precipitates were resuspended in 4 ml of 20 mM-sodium phosphate buffer, pH 7.5, dialysed for 2h against 1000 vol. of the same buffer and then applied to the top of columns (1.5 cm $\times$ 25 cm) of DEAE-cellulose (Cellex D, standard capacity; Bio-Rad Laboratories, Richmond, Calif., U.S.A.) equilibrated with the same buffer. The columns were eluted with linear NaCl gradients of 0–0.5 M in 20 mM-sodium phosphate buffer, pH 7.5. Citrate synthase was always eluted after malate dehydrogenase. The active fractions were combined and dialysed against 1000 vol. of 20 mM-sodium phosphate buffer, pH 7.5, for 2h. The citrate synthase activity in the final preparations was always at least five times the malate dehydrogenase activity.

Enzyme assays were done at 30°C. Malate dehydrogenase (EC 1.1.1.37) was assayed as described by Sottacasa *et al.* (1967). Citrate synthase was assayed by the method of Srere *et al.* (1963). Assay mixtures

contained, in 1 ml: 100 μmol of Tris-HCl buffer, pH 8.0; 0.15 μmol of acetyl-CoA; 0.1 μmol of 5,5'-dithiobis-(2-nitrobenzoic acid); extract; 0.2 μmol of oxaloacetic acid. Reactions were started by the addition of oxaloacetic acid and the increase in E_{412} was measured. Potential inhibitors, when present, were incubated with the assay mixture for 2 min before the reaction was started with oxaloacetic acid. Protein concentrations were determined with the Folin phenol reagent (Kennedy & Fewson, 1968) with crystalline bovine plasma albumin as standard.

Results and discussion

With the exception of *Bacillus* PM6, the methylo-trophs studied in the present paper are Gram-negative bacteria and, on this basis, it would be expected that their citrate synthase activity would be inhibited by NADH (Weitzman & Jones, 1968). The results given in Table 1, however, show that only the citrate synthases isolated from *Pseudomonas* 3A2 and bacterium 5B1, both typical facultative methylo-trophs, are inhibited by NADH. The absence of this feedback-control mechanism in the obligate methylo-trophs and in the restricted facultative methylo-trophs is consistent with the absence of the catabolic function of the tricarboxylic acid cycle in these organisms.

NADH is a potent inhibitor of the citrate synthases from bacterium 5B1 and *Pseudomonas* 3A2, and the inhibition is relieved by AMP (Fig. 1). Weitzman & Jones (1968) found that Gram-negative bacteria could be divided into two groups depending on whether or not AMP relieved the NADH-mediated

Table 1. *Effect of various potential inhibitors on citrate synthase activity in methylo-trophs*

All values are percentage inhibitions of citrate synthase activity compared with a control containing no inhibitor. For definitions of the terms obligate methylo-troph, restricted facultative methylo-troph and typical facultative methylo-troph see Colby & Zatman (1972, 1975). The following compounds had no effect on the citrate synthases from any of the methylo-trophs when tested at 10 mM and 2 mM: NAD⁺, NADP⁺, NADPH, AMP, 3-phosphoglycerate, phosphoenolpyruvate, pyruvate, glutamate, fumarate, succinate, malate, glyoxylate. NT, not tested.

Inhibitor	Concentration (mM)	Methylo-troph ...	Inhibition of citrate synthase (%)							
			Obligate				Restricted facultative		Typical facultative	
			4B6	C2A1	BG8	OB3b	W3A1	PM6	5B1	3A2
NADH	10		4	0	0	4	5	3	100	100
	0.1		NT	NT	NT	NT	NT	NT	100	80
ATP	10		57	58	64	65	50	65	55	52
	2		23	18	21	25	9	27	31	18
ADP	10		35	31	27	34	27	47	29	32
	2		5	10	6	8	3	11	8	11
2-Oxoglutarate	10		44	49	0	0	64	0	0	0
	2		1	11	0	0	20	0	0	0

Fig. 1. Inhibition of the citrate synthase of bacterium 5B1 by NADH and its reversal by AMP

The cuvette contained initially, in 0.9 ml: 100 μmol of Tris-HCl buffer, pH 8.0; 0.15 μmol of acetyl-CoA; 0.1 μmol of 5,5'-dithiobis-(2-nitrobenzoic acid); enzyme. Additions (each 50 μl) were made as indicated: A, 0.2 μmol of oxaloacetic acid; B, 0.5 μmol of NADH; C, 2.0 μmol of AMP. Similar results were obtained with the citrate synthase purified from *Pseudomonas* 3A2.

inhibition of their citrate synthase, and that this division followed established taxonomic divisions. In this respect, bacterium 5B1 and *Pseudomonas* 3A2 resemble other bacteria of the Pseudomonadaceae, Azotobacteriaceae and Neisseriaceae but differ from bacteria of the Enterobacteriaceae. This is in keeping with other morphological and physiological properties of the organisms (Colby & Zatman, 1973).

The citrate synthases from all the methylotrophs were inhibited by ATP and to a lesser extent by ADP (Table 1). Inhibition of bacterial citrate synthase activity by ATP, and in most cases also by ADP, has been reported in *Escherichia coli* (Weitzman, 1966; Jangaard *et al.*, 1968), *Rhodopseudomonas spheroides* (Borriss & Ohman, 1972) and two thiobacilli (Taylor, 1970). However, the high concentrations of ATP required, and the fact that ADP also inhibits, makes it unlikely that this inhibition is physiologically important in bacteria (cf. Borriss & Ohman, 1972). Inhibition of citrate synthase activity by lower concentrations of ATP has been observed in yeast (Hathaway & Atkinson, 1965), animals (Hathaway & Atkinson, 1965; Jangaard *et al.*, 1968; Shepherd & Garland, 1966; Kosicki & Lee, 1966) and plants (Bogin & Wallace, 1966). This could represent a feedback control mechanism, as ATP can be considered another end product of the catabolic function of the tricarboxylic acid cycle.

The citrate synthases from organisms 4B6, C2A1 and W3A1, but not those from the other methylo-

trophs, were inhibited by high concentrations (10 mM for 44–64% inhibition) of 2-oxoglutarate (Table 1). Inhibition of citrate synthase activity by 2-oxoglutarate (1 mM) has been reported in Gram-negative bacteria of the Enterobacteriaceae (Weitzman & Dunmore, 1969). A physiological role for this control mechanism has been postulated (Weitzman & Dunmore, 1969), as 2-oxoglutarate is an anabolic end product of the incomplete tricarboxylic acid cycle (lacking 2-oxoglutarate dehydrogenase) that is present in these organisms during fermentative growth (Amarasingham & Davis, 1965). The citrate synthases from two obligate chemolithotrophs are also inhibited by 2-oxoglutarate (Taylor, 1970); these organisms also lack 2-oxoglutarate dehydrogenase. The tricarboxylic acid cycle plays only an anabolic role during methylotrophic growth (Colby & Zatman, 1972, 1975), and it might be expected that citrate synthase activity in methylotrophs would be regulated by 2-oxoglutarate. However, in view of the high concentration of this inhibitor that is required, it is doubtful that inhibition by 2-oxoglutarate is of any physiological importance in organisms 4B6, C2A1 and W3A1, and it is not observed with citrate synthases from the other methylotrophs. In addition, the low specific activities of citrate synthase, aconitate hydratase and isocitrate dehydrogenase found in organisms 4B6, C2A1 and W3A1, and the higher activities of glutamate dehydrogenase found in organisms 4B6 and C2A1 (W3A1 does not contain glutamate dehydrogenase), make it unlikely that 2-oxoglutarate would accumulate during the growth of these organisms (Colby & Zatman, 1972, 1975).

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