The Tricarboxylic Acid Cycle in *Thiobacillus denitrificans* and *Thiobacillus-A2*

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(Accepted for publication 9 September 1970)

SUMMARY

The enzymes involved in the tricarboxylic acid cycle and some other related reactions were measured quantitatively in cell-free extracts from the obligate autotroph *Thiobacillus denitrificans* grown anaerobically, and in the facultative autotroph *Thiobacillus-A2* grown under various conditions. The presence of an incomplete tricarboxylic acid cycle in *T. denitrificans* and in *Thiobacillus-A2* (when grown autotrophically on thiosulphate) is described. The cell-free extracts from these two organisms lacked α -ketoglutarate dehydrogenase and succinyl CoA synthetase. The data from *Thiobacillus-A2* grown on succinate, aerobically or anaerobically, and on glutamate aerobically suggest that both the enzymes are repressed during autotrophic growth. Results are further discussed in relation to possible reasons for autotrophy.

INTRODUCTION

It is generally assumed that the pathways of intermediary metabolism in autotrophic organisms are essentially the same as those in heterotrophic organisms with the exception of the unique ability of the autotrophs to use inorganic electron donors as the source of energy. However, the lack of one or more enzymes required for the metabolism of organic compounds has been proposed as a hypothesis to explain the biochemical basis of autotrophy.

In order to reach a conclusion about the relationship, if any, between enzyme deficiency and autotrophy, it was necessary to conduct detailed enzymic analyses. The present report presents a comprehensive study of the enzymes of the tricarboxylic acid cycle and the glyoxylate pathway (and two related enzymes, glutamate dehydrogenase and 3-ketoacyl CoA transferase) in *Thiobacillus denitrificans* and *Thiobacillus-A2*. Certain enzymes of the tricarboxylic acid cycle have previously been measured in *T. thioparus* by Cooper (1964), in *T. thioparus* and *T. thiooxidans* by Smith, London & Stanier (1967) and in *T. thioparus* and *T. neapolitanus* by Johnson & Abraham (1969 *a, b*).

METHODS

Growth of bacteria and preparation of cell-free extracts. *Thiobacillus denitrificans* was grown on thiosulphate under anaerobic conditions with nitrate as the final electron acceptor in an inorganic medium described by Sargeant, Buck, Ford & Yeo (1966).

Thiobacillus-A2 was grown according to the method described by Taylor & Hoare (1969) using thiosulphate, succinate or glutamate as individual substrates for autotrophic or heterotrophic growth. With succinate as the carbon and energy source, the bacteria were grown aerobically as well as anaerobically and in the latter case nitrate served as the terminal oxidant.

The organisms were harvested during the early log phase using a DeLaval continuous flow centrifuge maintained at 6°. In the case of *Thiobacillus-A2* the bacterial paste was washed three times with 0.05 M-tris-HCl buffer pH 8.0. The wet packed organisms were suspended in the same buffer (30 %, w/v) containing 0.3 M-sucrose, 5 mM-MgCl₂, 0.5 mM-Na₄EDTA and 0.5 mM-glutathione (reduced form). Organisms were broken by sonic disruption for 3 min. in a Bronson Sonifier (Heat Systems Ultrasonics, Inc.) operated at full power; the temperature was maintained at 5°. The suspension was centrifuged first at 30,000 g for 30 min. and further at 144,000 g for 60 min. yielding the appropriate cell-free preparations. The cell-free extracts from *T. denitrificans* were prepared as described previously (Peeters & Aleem, 1970).

Enzyme assays. All determinations, unless otherwise indicated, were made with the 144,000 g supernatant and the various enzymes were measured using well-established methods. The optical procedure described by Ochoa (1955) was used for the assay of citrate synthetase (EC 4.1.3.7). Isocitrate dehydrogenase (EC 1.1.1.42), malate dehydrogenase (EC 1.1.1.37) and glutamate dehydrogenase (EC 1.4.1.4) were assayed according to the method described by Sottocasa, Kuylenskierna, Ernster & Bergstrand (1967); succinyl CoA synthetase (EC 6.2.1.5) was determined as described by Bridger, Ramaley & Boyer (1969). For succinate dehydrogenase (1.3.99.1) the spectrophotometric adaptation of the manometric method with phenazine methosulphate was used (Veeger, DerVartanian & Zeylemaker, 1969). The decline in fumarate concentration was followed at 300 nm. as described by Hill & Bradshaw (1969) for the assay of fumarase (EC 4.2.1.2). The α -ketoglutarate dehydrogenase was measured by the method of Kaufman (1955) and also by the method of Amarasingham & Davies (1965) using 3-acetylpyridine-NAD. Other enzymes assayed were: aconitase (EC 4.2.1.3), Afinsen (1955); malate synthetase (EC 4.1.3.2), Dixon & Kornberg (1965); isocitrate lyase (EC 4.1.3.1), Olson (1959) and 3-ketoacyl CoA transferase (EC 2.8.3.5), Pearce, Leach & Carr (1969). As the cell-free preparations from the two organisms showed a powerful NADH oxidase activity, those determinations which involved NADH oxidation or NAD⁺ reduction were performed anaerobically or in the presence of 1 mM-potassium cyanide; the latter completely blocked the NADH oxidase activity.

Specific activities are reported as nanomoles substrate converted/min./mg. protein. The following millimolar extinction coefficients were used in the calculations: reduced NADP and NAD (340 nm.), 6.2; *cis*-aconitic acid (240 nm.), 4.88; thio-ester bond in succinyl CoA and acetyl CoA (230 nm.), 4.5; 2,6-dichlorophenol indophenol (600 nm.), 20.6; glyoxylic semicarbazone (252 nm.), 12.4. For fumarate the millimolar extinction coefficient at 240 nm. is 2.11. We have observed that the ratio between the extinction of fumarate at 240 and 200 nm. was 63; and, since fumarate measurements were made at 300 nm., we used a millimolar extinction coefficient of 0.0335 in our calculations. Finally, a value of 11.9 was used for the magnesium complex of acetoacetyl CoA (Stern, Coon, DelCampillo & Scheider, 1956). Protein was determined by the method of Gornall, Bardawill & David (1949).

Chemicals. All chemicals were obtained from commercial sources and were of the highest purity available. The ATP, CoA, acetyl CoA, acetoacetyl CoA, NADP⁺, NADPH, NAD⁺, NADH and 3-acetylpyridin-NAD were purchased from Sigma Chemical Co., St Louis, Missouri, U.S.A.

RESULTS

The cell-free extracts from *Thiobacillus denitrificans* contained most of the tricarboxylic acid cycle enzymes and their activities were comparable with those found by Smith *et al.* (1967) and Johnson & Abraham (1969*a*) in *T. thioparus* (Table 1).

Table 1. *Tricarboxylic acid cycle and some related enzymes in cell-free extracts from Thiobacillus denitrificans as compared to Thiobacillus thioparus*

Activities are expressed as nanomoles/min./mg. protein.

Enzyme	<i>T. denitrificans</i>	<i>T. thioparus</i>
Citrate synthase	30.1	40.7*
Aconitase (isocitrate as substrate)	23.1	12.0*
Isocitrate dehydrogenase (NADP-specific)	45.0	204.4*
		74.0†
α -Ketoglutarate dehydrogenase	0.0	0.0*
Succinyl CoA synthetase	0.0	—
Succinate dehydrogenase	0.5	7.5†
Fumarase (fumarate as substrate)	215.0	79.6*
Malate dehydrogenase (oxaloacetate as substrate)	32.3	223.5*
		28.0†
Isocitrate lyase	91.5	—
Malate synthetase	12.0	—
3-Ketoacyl CoA transferase	16.3	—
Glutamate dehydrogenase	2.6	—

* Smith *et al.* (1967). † Johnson & Abraham (1969*a*).

Assays for α -ketoglutarate dehydrogenase were repeated several times using both the methods described by Kaufman (1955) and Amarasingham & Davis (1965), but without success. In addition, we failed to demonstrate the presence of succinyl CoA synthetase in *Thiobacillus denitrificans*. The latter enzyme was also absent in the autotrophically grown blue-green algae *Anabaena variabilis* and *Anacystis nidulans* (Pearce *et al.* 1969). Succinate dehydrogenase activity was quite low, but was increased by a factor of 1.5 when pellet was added back to the cell-free extract. It should be noted that, while the apparently higher succinate dehydrogenase activity in *T. thioparus* was based on the rate of reduction of cytochrome *c*, we have expressed the specific activity of this enzyme on the basis of the rate of reduction of phenazine methosulphate. Among the other enzymes assayed, isocitrate lyase was present at a high level, while the activity of glutamate dehydrogenase was quite low.

The same enzymes were measured in extracts of *Thiobacillus-A2* grown under various conditions (Table 2). Cell-free extracts from the organism grown autotrophically showed some distinct but important similarities with the *T. denitrificans* enzyme pattern: citrate synthase, isocitrate dehydrogenase, fumarase and malate synthetase were present at about the same level of activity while α -ketoglutarate dehydrogenase and succinyl CoA synthetase could not be detected. Succinate dehydro-

genase and glutamate dehydrogenase, however, were present at about the same level in both autotrophically and heterotrophically grown organisms.

Among the aerobic and anaerobic heterotrophic growth conditions examined significant differences in the levels of various enzymes were observed in the case of organisms grown anaerobically on succinate. These organisms contained comparatively greater aconitase, succinate dehydrogenase, fumarase and malate synthetase activities. Under all of the heterotrophic growth conditions both the α -ketoglutarate dehydrogenase and the succinyl CoA synthetase were easily detected.

Table 2. *The tricarboxylic acid cycle and some related enzymes in cell-free extracts from Thiobacillus-A2*

Activities are expressed as nanomoles/min./mg. protein.

Enzyme	Organism grown			
	Aerobically with			Anaerobically with succinate
	thiosulphate	glutamate	succinate	
Citrate synthase	23.2	10.2	6.0	NT
Aconitase	35.4	NT	21.5	513.0
Isocitrate dehydrogenase	34.8	140.8	258.0	270.0
α -Ketoglutarate dehydrogenase	0.0	95.1	10.6	35.3
Succinyl CoA synthetase	0.0	NT	267.0	300.0
Succinate dehydrogenase	7.5	6.4	8.7	42.5
Fumarase	195.0	174.0	211.0	1065.0
Malate dehydrogenase	213.0	85.5	196.3	195.9
Isocitrate lyase	16.2	17.3	9.7	8.7
Malate synthetase	10.3	NT	13.2	144.9
3-Ketoacyl transferase	50.2	31.3	37.6	42.1
Glutamate dehydrogenase	22.0	21.6	20.0	30.0

NT = Not tested.

DISCUSSION

Smith *et al.* (1967) advanced the hypothesis that obligate autotrophs lack both NADH oxidase and α -ketoglutarate dehydrogenase activities and proposed that the absence of these enzymes could provide a biochemical explanation for the inability of strict autotrophs to use organic compounds as sources of energy.

However, NADH oxidase has been demonstrated in many obligate autotrophs. Its presence in substantially high levels has been reported in *Nitrobacter agilis* (Aleem, 1968; Smith & Hoare, 1968), in *Nitrosomonas europaea* (Hooper, 1969), in *Thiobacillus neapolitanus* (Hempfling & Vishniac, 1965; Aleem, 1969), in *Thiobacillus* strain c (Trudinger & Kelly, 1968), in *T. thioparus* and *T. thiooxidans* (Johnson & Abraham, 1969a), and in *T. denitrificans* (Peeters & Aleem, 1970a). Although the role of NADH oxidase in the obligate autotrophs has not yet been well established, the enzyme has been shown to catalyse the process of oxidative phosphorylation with remarkable efficiency in *Nitrobacter wynogradskii* (Kiesow, 1964) and in *N. agilis* (Aleem, 1968).

Based upon direct enzymic analyses, and the study of the incorporation of labelled acetate, the existence of an incomplete tricarboxylic acid cycle, lacking α -ketoglutarate dehydrogenase, has been fairly well established in several of the thiobacilli (Smith *et al.* 1967; Kelly, 1967; Johnson & Abraham, 1969b), blue-green algae (Hoare & Moore,

1965; Smith *et al.* 1967; Pearce *et al.* 1969), and also in two photosynthetic bacteria (Callely, Rigopoulos & Fuller, 1968; Fuller, Smillie, Sisler & Kornberg, 1961).

Our results further substantiate the hypothesis that in most of the autotrophic organisms the tricarboxylic acid cycle is incomplete. In agreement with Taylor, Hoare & Hoare (1969) we were unable to detect the α -ketoglutarate dehydrogenase in *Thiobacillus denitrificans*. We also failed to demonstrate this enzyme in cell-free extracts from *Thiobacillus-A2* when grown autotrophically. Our results further indicate that the absence of α -ketoglutarate dehydrogenase cannot be considered as a causative feature of obligate autotrophy. Indeed, the fact that this enzyme is present in *Thiobacillus-A2* when grown heterotrophically suggests that it is repressed under autotrophic growth conditions. As already suggested by Smith *et al.* (1967), such a repression might be expected, because in autotrophic organisms the Krebs cycle appears to lose its energetic function and may thus fulfil only biosynthetic needs for which the presence of α -ketoglutarate dehydrogenase is not necessary. The same authors could not find such a repression in the facultative autotroph *Hydrogenomonas eutropha*, but it is interesting to note that Amarisingham & Davis (1965) reported a repression of α -ketoglutarate dehydrogenase in *Escherichia coli* when this organism was grown on carbon sources that do not require terminal respiration. Although the mechanism of repression under autotrophic growth conditions is not clearly understood at present, one might still expect to find a low α -ketoglutarate dehydrogenase activity even in obligate autotrophs. Smith & Hoare (1968) already reported low levels in *Nitrobacter agilis*, although Aleem (1970) failed to observe any activity of α -ketoglutarate dehydrogenase in pure cultures of this organism. Quite puzzling is the finding of Butler & Umbreit (1969), who reported that the α -ketoglutarate dehydrogenase activities in *T. thiooxidans* were twice the highest figures published for *E. coli*; while Smith *et al.* (1967), working with the same organism, could not detect any activity.

One might further expect that, if the Krebs cycle serves only the biosynthetic needs during chemosynthesis (or autotrophy), the levels of all the enzymes involved would decrease; this was indeed the case in *Thiobacillus denitrificans* and for most of the enzymes in *Thiobacillus-A2* grown autotrophically. Surprising is the absence of succinyl CoA synthetase in both organisms, under autotrophic growth conditions especially, in view of the important biosynthetic role of this enzyme. Pearce *et al.* (1969) did not find this enzyme in *Anacystis nidulans* and *Anabaena variabilis*, and suggested that its function was taken over by the enzyme 3-ketoacyl CoA transferase. Although the latter enzyme was present under both autotrophic and heterotrophic growth conditions, it is not clear why this should be so. It is apparent, therefore, that the control exerted by autotrophic conditions on the heterotrophic cellular metabolism warrants further investigations.

The glyoxylate pathway is present in these organisms, but no evidence can be deduced from our results that this pathway is more important in autotrophic organisms.

This work was supported in part by National Science Foundation grant GB-14661, the Department of the Interior OWRR grant A-027 KY, the University of Kentucky Research Foundation, and the U.S. PHS Biomedical Research support no. 5S-05-FRO7114-02. T.L.P. was a U.S. National Science Foundation Research Associate and an 'Aspirant' of the Nationaal Fonds voor Wetenschappelijk Onderzoek of the Belgian National Science Foundation.

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