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Oxalate metabolism by the acetogenic bacterium *Moorella thermoacetica*

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Abstract

Whole-cell and cell-extract experiments were performed to study the mechanism of oxalate metabolism in the acetogenic bacterium Moorella thermoacetica. In short-term, whole-cell assays, oxalate consumption was low unless cell suspensions were supplemented with CO2, KNO3, or Na2S2O3. Cell extracts catalyzed the oxalate-dependent reduction of benzyl viologen. Oxalate consumption occurred concomitant to benzyl viologen reduction; when benzyl viologen was omitted, oxalate was not appreciably consumed. Based on benzyl viologen reduction, specific activities of extracts averaged 0.6 μmol oxalate oxidized min−1 mg protein−1. Extracts also catalyzed the formate-dependent reduction of NADP+; however, oxalate-dependent reduction of NADP+ was negligible. Oxalate- or formate-dependent reduction of NAD+ was not observed. Addition of coenzyme A (CoA), acetyl-CoA, or succinyl-CoA to the assay had a minimal effect on the oxalate-dependent reduction of benzyl viologen. These results suggest that oxalate metabolism by M. thermoacetica requires a utilizable electron acceptor and that CoA-level intermediates are not involved.

Introduction

Information on the bacterial metabolism of oxalate ($^{-}$OOC-COO^{-}) has been obtained mostly through studies with *Ralstonia oxalatica*[1–6] and *Oxalobacter formigenes*[7–11]. With both of these bacteria, oxalate is first activated to oxalyl-coenzyme A (CoA) and then decarboxylated by oxalyl-CoA decarboxylase to CO_2 and formyl-CoA; the CoA of formyl-CoA is transferred by formyl-CoA transferase to a new molecule of oxalate, and formate is produced [4–6,8–10]. With *R. oxalatica*, formate is oxidized by a NAD-dependent formate dehydrogenase, and this oxidation is coupled to ATP synthesis via electron transport phosphorylation with O_2 as the terminal electron acceptor [5,12–14]. With *O. formigenes*, the formate derived from oxalyl-CoA decarboxylation is not metabolized and is released as an end product [7,8]. Furthermore, *O. formigenes* lacks the ability to form ATP by substrate-level or electron transport phosphorylation. It is now known that a membrane-bound, oxalate–formate antiporter and a cytoplasmic oxalyl-CoA decarboxylase work together in *O. formigenes* to create a proton gradient (for ATP synthesis) by coupling the electrogenic exchange of oxalate and formate across the membrane with a proton-consuming decarboxylation reaction [7,15,16]. Lastly, in contrast to *R.*

oxalatica and *O. formigenes*, *Pseudomonas* sp. OX-53 engages yet another mechanism for oxalate metabolism. This aerobic bacterium possesses an oxalate oxidase (oxalate:oxygen oxidoreductase) that directly couples oxalate oxidation to the reduction of oxygen, yielding CO_2 and hydrogen peroxide [17].

Moorella thermoacetica[18,19] is a thermophilic acetogenic bacterium that uses CO_2 as a terminal electron acceptor and concomitantly synthesizes biomass and acetate via the acetyl-CoA or Wood–Ljungdahl pathway [20–24]. This anaerobe is the most metabolically diverse acetogen characterized to date [22,24,25] and has spores that are extremely resistant to high temperatures [26]. Furthermore, oxalate can be used by this thermophile for growth and acetate synthesis [27]. However, the mechanism by which *M. thermoacetica* catabolizes oxalate has yet to be resolved. If formate is formed during oxalate catabolism (e.g., in a manner similar to that found in *R. oxalatica* and *O. formigenes*), it would be oxidized to CO_2 and reductant by a formate dehydrogenase present in *M. thermoacetica*[28] and the reductant used in the acetyl-CoA pathway for ATP, acetate, and biomass synthesis [22,24]. The purpose of this study was to examine, through whole-cell and enzymatic studies, the nature of the oxalate-degrading activity in *M. thermoacetica*.

Materials and methods

2.1Culture conditions and cell preparation

M. thermoacetica ATCC 39073 was grown at 55°C in rubber-stoppered, screw-capped infusion flasks (1200 ml) containing 500 ml of a CO_2-enriched, undefined culture medium [27]. The undefined culture medium (pH 6.8) was supplemented with 30 mM oxalate. Growth was initiated by injecting 50 ml of inoculum, and culture flasks were incubated without shaking. Following incubation (3–5 days), cells were harvested by centrifugation, and the pelleted cells were washed twice in anoxic 50 mM sodium phosphate buffer (pH 7) and used immediately. All cell manipulations, including the preparation of cell extracts for enzyme assays, were carried out in an anaerobic chamber (100% N_2 atmosphere), and all whole-cell and enzyme assays were carried out at 55°C under anaerobic conditions (100% N_2) in butyl rubber-stoppered, aluminum crimp-sealed assay tubes (18×150 mm).

2.2Whole-cell assays

Standard assay conditions consisted of washed cells resuspended to an optical density (660 nm) of 9–10 (4.0–4.5 mg [dry weight] cells ml^{-1}) in anoxic 50 mM sodium phosphate buffer (pH 7; this pH was used since it is similar to the pH of the culture medium used to grow *M. thermoacetica*[27]) and dispensed (5 ml) into tubes. Assay tubes were pre-incubated for 30 min at 55°C, and assays were started by injecting oxalate to a final concentration of 2 mM. When necessary, at the beginning of the pre-incubation period, additional substrates (methanol, CO_2, potassium nitrate [KNO_3], or sodium thiosulfate [$Na_2S_2O_3$]) were added at the concentrations indicated. After the addition of oxalate, aliquots were removed at

various time intervals and analyzed for oxalate consumption and for the formation of oxalate-derived products.

2.3Enzyme assays

Washed cells were resuspended in a buffered lysozyme solution (lysozyme, 5 mg ml^{-1}; phenylmethylsulfonyl fluoride, 0.1 mM; DNase I, 1 μg ml^{-1}; dithioerythritol, 10 mM; and sodium phosphate buffer, 50 mM, pH 7) and incubated for 2 h at 37°C [29]. Following incubation, cell debris was removed by centrifugation in a microcentrifuge (14 000 rpm for 5 min), and the supernatant fluid (i.e., cell extract) was transferred to crimp-sealed serum vials and stored anaerobically (100% N_2) at 4°C until analyzed.

Reactions were initiated by adding 5–20 μl of cell extract (15–30 mg protein ml^{-1}). Unless noted otherwise, activities for an oxalate-catabolizing enzyme system and for formate dehydrogenase were determined by following the oxalate- and formate-dependent reduction of the artificial electron acceptor benzyl viologen at 578 nm ($?_{578}$=8.65 mM^{-1} cm^{-1}), respectively [30]. The standard assay mixture (5 ml) consisted of 50 mM Tris–HCl (pH 8.5; this buffer system and pH have been used in a previous study to measure hydrogenase activities in cell extracts of *M. thermoacetica*[30]), 1 mM benzyl viologen, and 10 mM oxalate or formate for the respective enzyme assay. Specific activities were calculated from initial velocities of benzyl viologen reduction, and one unit (U) of activity was equivalent to 2 μmol benzyl viologen reduced (1 μmol of oxalate or formate oxidized) min^{-1}. When oxalate or formate consumption was measured, assays contained 5 mM benzyl viologen and 1 mM oxalate or 2 mM formate. Aliquots were removed at various time intervals and analyzed for oxalate or formate consumption and for the formation of oxalate-derived products.

2.4Analytical methods

The optical density of cell suspensions was measured at 660 nm with a Spectronic 501 spectrophotometer (Bausch and Lomb, Rochester, NY, USA); the optical path width (inner diameter) of assay tubes was 1.5 cm. Cell dry weights were calculated as previously described [29]. Protein in cell extracts was measured by the Bradford method [31]. Oxalate and formate in whole-cell and enzyme assay fluids were quantitated by high-performance liquid chromatographic (HPLC) analysis [27].

Results and discussion

3.1Oxalate metabolism by whole cells

Concentrated cell suspensions of oxalate-grown *M. thermoacetica* were examined for their ability to metabolize oxalate in the absence of an external electron acceptor (i.e., standard, whole-cell assay conditions). Under these assay conditions, oxalate consumption by *M. thermoacetica* was minimal (data not shown and Table 1).

During the 1-h assay period, less than 10% of the oxalate was consumed. However, when assay mixtures were supplemented with an external electron acceptor (CO_2, nitrate, or thiosulfate; all are utilizable electron acceptors for *M. thermoacetica*[25,32,33]), oxalate consumption by cell suspensions was stimulated (Table 1). In contrast, the addition of methanol (a utilizable electron donor for *M. thermoacetica* as well as preformed methyl-level carbon for acetate synthesis [24,25]) did not result in the apparent stimulation of oxalate consumption (Table 1). Only when an electron acceptor (CO_2) was added along with methanol was oxalate consumption by cell suspensions stimulated.

Addition[a]	% Oxalate consumed[b]
None (control)	7.6
CO_2	14.7
$Na_2S_2O_3$	18.8
KNO_3	28.4
Methanol	5.7
Methanol+CO_2	19.5

[a]Standard assay conditions (control) consisted of concentrated cell suspensions in 50 mM sodium phosphate buffer (pH 7) containing 2 mM oxalate under anaerobic conditions (100% N_2). Assay mixtures (5 ml) were modified as indicated: CO_2, 22.4 mM (112 μmol $tube^{-1}$); $Na_2S_2O_3$, 5 mM; KNO_3, 5 mM; and methanol, 10 mM.
[b]Percentage of the 2 mM oxalate consumed by cells after 1 h of incubation.

Table 1. Oxalate consumption by *M. thermoacetica* in whole-cell assays

When oxalate was consumed in whole-cell assays, oxalate-derived intermediates (e.g., formate) or end products (e.g., acetate) were not detected (data not shown). Interestingly, of the three electron acceptors tested, nitrate increased oxalate consumption the most (Table 1). Recent studies with *M. thermoacetica* have shown that oxalate-grown cells (unlike cells grown at the expense of other substrates) actually prefer CO_2, rather than nitrate, as their terminal electron acceptor and only engage in nitrate dissimilation during the stationary growth phase [34].

3.2Oxalate metabolism by cell extracts

Cell extracts of *M. thermoacetica* catalyzed the oxalate-dependent reduction of benzyl viologen (Fig. 1; Table 2, Expt. A). In the absence of oxalate, the reduction of benzyl viologen by cell extracts was negligible (Fig. 1; specific activities approximated 0.02 μmol of oxalate oxidized min^{-1} mg $protein^{-1}$). Cell extracts also catalyzed the formate-dependent reduction of benzyl viologen, and these activities were nearly 20-fold higher than oxalate-dependent activities (Table 2, Expt. A).

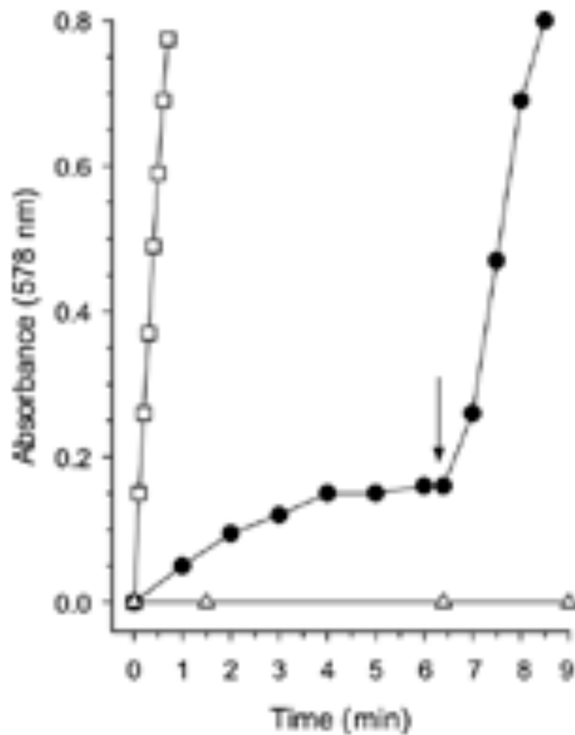

Figure 1. Oxalate-dependent reduction of benzyl viologen by cell extracts of *M. thermoacetica*. Symbols and conditions: □, cell extract added to the standard assay mixture (50 mM Tris–HCl [pH 8.5], 1 mM benzyl viologen, and 10 mM oxalate); ?, cell extract added to the standard assay mixture lacking oxalate (the arrow indicates when oxalate was added to assay mixture); and ▵, buffered lysozyme solution (without cell extract) added to the standard assay mixture.

Expt.	Addition	Specific activity (U mg protein^{-1})[a]	
		OCES	FDH
A[b]	None	0.63	12.77
B[c]	None	0.34	7.68
	Acetyl-CoA	0.46	9.14
	Succinyl-CoA	0.43	8.47
	CoA	0.61	6.56
C[d]	Benzyl viologen	0.30	8.22 (0.04)[e]
	$NADP^+$	0.01	2.11 (0.005)[e]
	NAD^+	0	0

[a]For the oxalate-catabolizing enzyme system (OCES) and formate dehydrogenase (FDH), μmol of oxalate and formate oxidized min^{-1} mg protein^{-1}, respectively. Values are the means of duplicate or triplicate measurements.
[b]Standard assay mixture (50 mM Tris–HCl [pH 8.5], 1 mM benzyl viologen, and 10 mM oxalate or formate for OCES and FDH assays, respectively).
[c]Standard assay mixture supplemented with 5 mM $MgCl_2$. Assay mixtures (5 ml) were modified as indicated: acetyl-CoA, 0.1 mM; succinyl-CoA, 0.1 mM; and CoA, 0.1 mM. Regarding the latter, in addition to 0.1 mM CoA, this assay mixture was also supplemented with ATP (5 mM), succinate (10 mM), acetate (10 mM), and cocarboxylase (0.056 mM).
[d]Assay reaction mixtures consisted of 50 mM Tris–HCl (pH 8.5) and 10 mM oxalate or formate for OCES and FDH assays, respectively. Assay mixtures (5 ml) were modified as indicated: benzyl viologen, 1 mM; $NADP^+$, 1 mM; and NAD^+, 1 mM. The reduction of $NADP^+$ or NAD^+ was measured at 340 nm ($?_{578}$=6.2 mM^{-1} cm^{-1}) [12].
[e]Parenthetical value is the OCES-to-FDH activity ratio (OCES activity divided by FDH activity).

Table 2. Effects of CoA and electron acceptors on oxalate-catabolizing enzyme system and formate dehydrogenase activities in extracts of oxalate-grown cells of *M. thermoacetica*

HPLC analysis of assay mixtures showed that with cell extracts: (i) oxalate and formate were not consumed in the absence of benzyl viologen; (ii) oxalate and formate were consumed during benzyl viologen reduction; and (iii) formate or other oxalate-derived intermediates and end products were not detected during oxalate consumption in the presence of benzyl viologen. The inability to detect formate, if it was formed, is not surprising given the high level of formate dehydrogenase activity in cell extracts. Therefore, one possible explanation for the nature of the oxalate-dependent activity is that the observed (i.e., measured) activity was actually the result of formate dehydrogenase and its oxidation of the formate generated by the oxalate-catabolizing enzyme system (e.g., via a non-oxidative decarboxylation reaction [$^-OOC\text{-}COO^- + H^+ \rightarrow HCOO^- + CO_2$] as observed in *R. oxalatica*[3] and *O. formigenes*[7]). Another possibility is that the oxalate-catabolizing enzyme system is equivalent to an oxalate dehydrogenase (oxalate:benzyl viologen oxidoreductase), and this enzyme coupled the direct oxidation of oxalate (e.g., via an oxidative decarboxylation reaction [$^-OOC\text{-}COO^- + 2H^+ \rightarrow 2CO_2 + 2H$]) to the reduction of benzyl viologen.

To further address these possibilities, experiments were performed to determine the effects of CoA on oxalate- and formate-dependent reduction of benzyl viologen by cell extracts of *M. thermoacetica*. The addition of CoA, acetyl-CoA, or succinyl-CoA

to assay mixtures had only minor effects on oxalate- or formate-dependent activities (Table 2, Expt. B). These findings suggest that CoA-level intermediates are not involved in oxalate catabolism by *M. thermoacetica* and are in contrast to *R. oxalatica*[4–6] and *O. formigenes*[8,9], both of which require CoA for oxalate decarboxylation. The ability of cell extracts of *M. thermoacetica* to catalyze oxalate- or formate-dependent reduction of different electron acceptors was examined (Table 2, Expt. C). Cell extracts catalyzed the formate-dependent reduction of $NADP^+$ whereas oxalate-dependent reduction of $NADP^+$ was essentially absent in cell extracts. Oxalate- or formate-dependent reduction of NAD^+ was also not observed. In this regard, *M. thermoacetica* is known to contain a $NADP^+$-dependent formate dehydrogenase [28]. Furthermore, the activity ratio for oxalate- and formate-dependent reduction of $NADP^+$ was nearly 10-fold less than the activity ratio observed with benzyl viologen, suggesting that formate dehydrogenase and the oxalate-catabolizing enzyme system have differential electron acceptor specificities.

3.3Expression of an oxalate-catabolizing enzyme system

Cells grown at the expense of oxalate had higher levels of activity of the oxalate-catabolizing enzyme system and formate dehydrogenase than cells grown at the expense of glucose (Table 3). These observations are in agreement with a previous study [27] that showed oxalate induces the differential expression of proteins in *M. thermoacetica*. In fact, in oxalate-grown cells (unlike in cells grown at the expense of formate, CO, or glyoxylate), the majority of the soluble protein consists of two proteins with approximate molecular masses of 33 000 and 42 000 [27]. Based on these collective observations, it is tempting to speculate that one or both of these proteins play an integral role in the oxalate-catabolizing enzyme system.

Growth substrate[a]	Specific activity (U mg protein^{-1})[b]	
	OCES	FDH
Oxalate	0.30	9.78
Glucose	0.01	1.63

[a]Cells were grown in the undefined medium with oxalate (30 mM) or glucose (10 mM) [27].
[b]For the oxalate-catabolizing enzyme system (OCES) and formate dehydrogenase (FDH), μmol of oxalate and formate oxidized min^{-1} mg protein^{-1} (benzyl viologen as electron acceptor), respectively. Values are the means of duplicate or triplicate measurements. Assay reaction mixtures (5 ml) consisted of 50 mM Tris–HCl (pH 8.5), 1 mM benzyl viologen, and 10 mM oxalate or formate for OCES and FDH assays, respectively.

Table 3. Oxalate-catabolizing enzyme system and formate dehydrogenase activities in extracts of oxalate- and glucose-grown cells of *M. thermoacetica*

3.4Summary

The results of the present study suggest that the enzyme system for the catabolism of oxalate in *M. thermoacetica* was inducible and was obligately coupled to an utilizable electron acceptor. Furthermore, oxalate- and formate-dependent catabolic

activities displayed differential electron acceptor specificities, and CoA-level intermediates were apparently not involved in oxalate catabolism. Thus, it appears that oxalate metabolism by *M. thermoacetica* occurs via a novel reaction sequence where oxalate, not formate, is subject to oxidative decarboxylation, presumably by an oxalate dehydrogenase, resulting in the formation of CO_2 and reductant. To our knowledge, this is the first report of such a mechanism for the direct oxidation of oxalate by an obligate anaerobe. It will be interesting to see if *Moorella thermoautotrophica*[18,35], a thermophilic acetogenic bacterium which also utilizes oxalate as a growth-supportive substrate [27], possesses a similar reaction mechanism for the metabolism of oxalate.

With *M. thermoacetica*, the reductant generated from the direct oxidation of oxalate would ultimately be used for the reduction of CO_2 via the acetyl-CoA pathway and the conservation of oxalate-derived energy and biomass synthesis [20–24]. However, additional studies will be required to determine: (i) the nature of the catalyst(s) and native electron acceptor(s) involved in the oxidative decarboxylation of oxalate; (ii) how these components of the oxalate-catabolizing enzyme system interface with the acetyl-CoA pathway; and (iii) whether oxalate decarboxylation itself is coupled to an energy-conserving process in *M. thermoacetica* as is observed with fermenting bacteria that decarboxylate succinate, malonate, malate, and glutarate [36].

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