

Biosynthetic and Bioenergetic Functions of Citric Acid Cycle Reactions in *Rhodopseudomonas capsulata*

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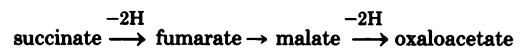
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Rhodopseudomonas capsulata can grow in a number of alternative modes, including (i) photosynthetic, defined here as anaerobic growth with light as the energy source, and (ii) heterotrophic, referring to aerobic heterotrophic growth in darkness. The functions of citric acid cycle sequences in these growth modes were investigated using wild-type and appropriate mutant strains. Results of growth tests and O₂ utilization experiments showed that in the heterotrophic mode, energy conversion is dependent on operation of the classical citric acid cycle. Alpha-ketoglutarate dehydrogenase (KGD) activity in wild-type strain B10 is substantially higher in cells grown heterotrophically than in cells grown photosynthetically. Molecular oxygen, even at low concentration, appears to be important in regulation of KGD synthesis and, thus, in expression of citric acid cycle activity. Extracts of (photosynthetically grown) mutant strain KGD11 lack demonstrable KGD activity, and in contrast to the wild type, KGD11 is unable to grow heterotrophically on succinate, malate, or pyruvate owing to failure of the energy conversion function of the citric acid cycle. KGD11, however, grows well photosynthetically on malate or on CO₂ + H₂. The KGD activity level required to support the bioenergetic function of the citric acid cycle is evidently much higher than that necessary to satisfy biosynthetic demands; thus, a very low rate of succinyl-coenzyme A formation (needed for biosynthesis) in the mutant would suffice for growth under photosynthetic conditions. In wild-type *R. capsulata*, the α -ketoglutarate required for glutamate synthesis is ordinarily generated via citric acid cycle reactions, which include the conversions catalyzed by citrate synthase and isocitrate dehydrogenase. Mutants blocked in the former or both of these enzymes can grow photosynthetically if provided with an exogenous source of α -ketoglutarate or glutamate, but grow very poorly (if at all) as heterotrophs since the energy supply under these conditions depends on operation of the complete citric acid cycle.

The citric acid cycle effects the catabolism of acetyl-coenzyme A (CoA) to provide the reducing power that drives the oxidative phosphorylation machinery of aerobic cells. Citric acid cycle reaction sequences, however, are also important for providing biosynthetic intermediates in most, if not all, types of cells. Several cycle intermediates (α -ketoglutarate [KG], oxaloacetate, and succinyl-CoA) are used for net synthesis of various essential cell constituents. In certain kinds of bacteria, segments of the cycle function only for biosynthetic purposes, for example, in strict anaerobes such as *Clostridium kluveri* (35) and in so-called "obligate" chemoautotrophs (32). In facultative heterotrophic aerobes-anaerobes, the relative commitments of citric acid cycle sequences to biosynthetic and bioenergetic func-

tions may be markedly affected by nutritional circumstances. Thus, when *Escherichia coli* is grown fermentatively on sugars, the cells lack KG dehydrogenase (KGD), and under these conditions, succinate required for formation of the biosynthetic precursor succinyl-CoA is produced reductively (2) by reversal of the sequence:



This illustrates the special importance of KGD as a "regulatory catalyst" in overall operation of the citric acid cycle.

Little is known of the controls that govern "opening" (for biosynthesis) and "closing" (for energy conversion) of the citric acid cycle, and these are of particular significance for organisms that can grow either as anaerobes or aerobes. The photosynthetic bacterium *Rhodopseudo-*

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monas capsulata presents unusual opportunities for investigation of this and related problems because it is capable of growing in five distinctly different ways (24): (i) anaerobically as a photoautotroph on H_2 plus CO_2 with light as the energy source, (ii) anaerobically as a photoheterotroph on various organic carbon sources with light as the energy source, (iii) as a fermentative anaerobe in darkness on sugars as sole carbon and energy sources, (iv) aerobically as an ordinary chemoheterotroph in darkness, and (v) aerobically as a chemoautotroph in darkness with H_2 as the source of energy and reducing power. In this communication, we present observations on operation of the citric acid cycle and component sequences in *R. capsulata* mainly in connection with two particularly prominent growth modes, namely, photoheterotrophic (anaerobic) and aerobic (dark) chemoheterotrophic.

MATERIALS AND METHODS

Bacterial strains. The general characteristics of *R. capsulata* wild-type strain B10 are typical for the species (36). Mutant WE29 is a derivative of B10, and lacks both citrate synthase (CS) and isocitrate dehydrogenase (ICD) activities (7). Although this strain was originally thought to be a single-site mutant, more detailed genetic analysis (J. T. Beatty, M.A. thesis, Indiana University, Bloomington, 1978) has shown that the enzymatic deficiencies noted result from two separate mutations; similar mutants of *E. coli* have been described by others (19). Mutant T500, deficient in CS activity, was derived from B10 as detailed below. Strain KGD11 was also obtained from B10, after ethyl methane sulfonate mutagenesis, and is virtually devoid of KGD activity (6a).

Culture media and growth conditions. Unless otherwise noted, the bacteria were grown in a minimal synthetic medium, designated RCVB, which contains 30 mM DL-malate and 7.5 mM $(NH_4)_2SO_4$ as the C and N sources, respectively (6a). When organic carbon sources other than malate were used, these and the potassium phosphate component were added aseptically to the remainder of the sterile medium. In all instances, organic carbon sources were added so as to provide an initial C concentration equivalent to that of 30 mM malate.

For photoautotrophic growth, RCVB medium was modified by omission of malate and increase of phosphate buffer concentration from 10 mM to 20 mM (note that phosphate concentration was also doubled for cultures grown photoheterotrophically on succinate and for cultures grown aerobically in darkness). Erlenmeyer flasks of 1-liter capacity, containing 50 ml of inoculated medium under a gas phase of 5% CO_2 in H_2 , were sealed with rubber stoppers.

For routine maintenance, stock cultures were grown photosynthetically in completely filled 17-ml screw-cap tubes: strain B10 in RCVB medium, mutants WE29 and T500 in RCVB medium supplemented with 10 mM L-glutamate, and strain KGD11 in RCVB

medium modified to contain succinate in place of malate. Media used for growth of mutants were supplemented with 0.05% L-ascorbate, freshly made and added at the time of culture inoculation. Cultures for enzyme assays were grown photosynthetically in full screw-cap tubes or square "milk dilution" bottles of 165-ml capacity.

Photosynthetically grown cultures were illuminated (at 32°C) with ca. 600 ft-c (ca. 6,400 lx) intensity of incandescent light, provided by Lumiline lamps. For aerobic dark growth, Erlenmeyer flasks containing 10% of their nominal capacity of growth medium were incubated (32°C) on a rotary shaker at 250 rpm.

Fluoroacetate selection for CS mutants. The procedure for selection of CS mutants (such as T500) was devised to obtain strains with the same phenotype as WE29 and was based on the observation that growth of the latter mutant is resistant to fluoroacetate. After ethyl methane sulfonate mutagenesis (26) of a B10 culture, the cells were grown photosynthetically (to the stationary phase) in RCVB medium supplemented with 10 mM L-glutamate and 100 μ M fluoroacetate. After three consecutive subcultures in the same medium, plates of solid RCVB medium (1.5% [wt/vol] Difco agar) containing 65 μ M L-glutamate were spread with culture dilutions. Potential mutants of interest were identified as small colonies after incubation of plates in GasPak anaerobic jars (BBL Microbiology Systems, Cockeysville, Md.) under photosynthetic conditions for 3 days. Putative mutants were picked and tested for photosynthetic growth on RCVB medium and on RCVB medium supplemented with either 10 mM L-glutamate or 10 mM KG. Isolates that grew in the presence of L-glutamate or KG, but not on unsupplemented DL-malate plates, were purified by streaking on RCVB plates containing 10 mM L-glutamate (plates were incubated photosynthetically). A similar procedure was independently devised and used for isolation of CS mutants of *Acinetobacter lwoffii* by Harford and Weitzman (14).

Photometric measurement of cell concentrations. Culture densities were measured with a Klett-Summerson photometer fitted with a no. 66 filter. The photometer was modified so as to accept 17-ml-capacity screw-cap culture tubes (completely filled for experiments in which anaerobic [photosynthetic] growth was followed). For aerobic (dark) growth kinetics, the Erlenmeyer flasks used were fitted with side-arm tubes to facilitate photometer measurements.

Preparation of cell extracts. Extracts were ordinarily made of cells grown to the late logarithmic phase (in a few instances, stationary-phase cultures [density, >350 photometer units] were used).

For experiments concerned with assay of CS, aconitate hydratase, and ICD, cells were harvested by centrifugation, washed, and resuspended in 50 mM Tris-hydrochloride buffer (pH 7.5) containing 2 mM $MnCl_2$ and 0.1 mM sodium isocitrate. (The latter supplements stabilize ICD activity.) Cell suspensions were disrupted by passage through a French pressure cell (operated at 16,000 to 20,000 lb/in²). The crude extracts were clarified by centrifugation at 140,000 $\times g$ for 90 min, and the supernatant fluids were retained for enzyme assays.

When KGD activity or O_2 uptake was to be mea-

sured, cells were washed and resuspended in 50 mM potassium phosphate buffer (pH 7.0). Suspensions of 5 ml or greater in volume were disrupted with a French pressure cell as described above; with smaller volumes, extracts were made by sonication as detailed by Beatty and Gest (6a). The crude extracts were not centrifuged, and enzymatic activities were assayed immediately. Essentially the same activity levels were found with both cell breakage procedures.

Enzyme assays and measurement of O₂ uptake. CS (EC 4.1.3.7) activity was determined by colorimetric measurement of oxaloacetate-dependent reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) by reduced CoA (8). ICD (EC 1.1.1.42) was assayed by measurement of DL-isocitrate-dependent reduction of NADP⁺ (7). The activity of KGD (EC 1.2.4.2) was determined by measurement of KG-dependent reduction of NAD⁺ in the presence of 10⁻⁵ M rotenone (6a). Aconitate hydratase (EC 4.2.1.3) activity was estimated by measurement of increase in absorbance at 250 nm due to formation of *cis*-aconitate from isocitrate (31).

Uptake of O₂ was measured polarographically with a Clark electrode. The latter was polarized at -0.8 V and connected to a chart recorder on which 1.0 mV registered as the maximum deflection. The glass reaction chamber contained 3.0 ml of reaction mixture and a small magnetic "flea" and was closed off from the atmosphere by means of a Teflon stopper which had a 0.75-mm hole drilled through it. The hole permitted additions to be made to the reaction mixture through a syringe needle, while the concentration of O₂ dissolved in the medium was continuously monitored. The entire assembly was water-jacketed and maintained at 30°C over a magnetic stirrer.

It was observed that intact cells showed little or no substrate-dependent O₂ uptake when suspended in phosphate buffer. When cells were suspended in RCVB medium from which DL-malate had been omitted, however, significant respiratory activity was observed upon addition of substrates. Consequently, cells were suspended in this way, and O₂ utilization was measured with 40 mM DL-malate as the substrate. The concentration of O₂ dissolved in air-saturated RCVB minus malate medium at 30°C was found to be ca. 253 nmol·ml⁻¹ (estimated from oxidation of known amounts of NADH by cell extracts). This value was used for all assays, regardless of atmospheric pressure. Oxygen uptake with 5 mM NADH as substrate (NADH oxidase activity) was measured using crude unfractionated extracts.

The quantities of extracts or cell suspensions used for assays were in the range where activity was proportional to amount of preparation used.

Other procedures. Light intensity was measured with a Weston illumination meter (model 756), calibrated in foot-candles. Gases were mixed in volume-to-volume ratios with a Matheson rotameter, using commercially available (Matheson) calibration curves of flow rate versus milliliters of gas per minute. The bacteriochlorophyll (BChl) content of intact cells was determined as described by Sojka et al. (33), and protein was estimated by the method of Lowry et al. (22) with crystalline bovine serum albumin as the standard.

RESULTS

Citric acid cycle activity in *R. capsulata* as measured by O₂ consumption. The potential capacity of *R. capsulata* cells to catalyze aerobic (dark) citric acid cycle activity was assessed from O₂ utilization experiments. Table 1 shows the results of measurements using cells (or derived extracts) grown photosynthetically (anaerobically) or aerobically in darkness with malate as the carbon source (RCVB medium). Cells grown aerobically, and extracts therefrom, showed activities ca. two- to threefold higher than those grown photosynthetically. This difference presumably indicates elevated synthesis of the respiratory system when growing cells obtain energy by oxidative phosphorylation associated with dark aerobic electron flow.

To validate the assumption that aerobic oxidation of malate (and related substrates) by *R. capsulata* occurs via the citric acid cycle, experiments were performed with appropriate inhibitors and with two mutants blocked at different steps of the cycle. The inhibitors tested were fluoroacetate, which gives rise to fluorocitrate, a potent inhibitor of aconitase (27), and arsenite, which inhibits ketoacid dehydrogenase complexes through reaction with their lipoic acid moieties (10). Both inhibitors caused striking inhibition of malate-dependent O₂ utilization by intact cells (Fig. 1). Depression of respiratory rate to 50% of normal occurred with ca. 0.5 μM fluoroacetate or 0.5 mM arsenite. The latter compound at 1 mM concentration completely inhibits KGD activity in crude extracts of *R. capsulata* (6a). These results support the conclusion that the citric acid cycle is the major mechanism used by *R. capsulata* for the aerobic oxidation of malate. Further evidence was obtained by using mutants KGD11 (blocked in KGD activity; 6a) and T500 (blocked in CS). Cultures of the mutants were grown photosynthetically in malate medium supplemented with either 5 mM succinate (KGD11) or 5 mM L-glutamate (T500). Cells of T500 and KGD11 were found to consume O₂ with malate as sub-

TABLE 1. Utilization of O₂ by intact cells and extracts of *R. capsulata* B10

Growth conditions	Rate of O ₂ consumption ^a (nmol·min ⁻¹ ·mg protein ⁻¹)	
	Intact cells	Extracts
Aerobic/dark	104 ± 11	82 ± 25
Anaerobic/light	38 ± 11	40 ± 12

^a In darkness. Mean values and standard deviations are given for replicate experiments (four experiments with intact cells and seven with extracts). Substrates were DL-malate for intact cells and NADH for extracts.

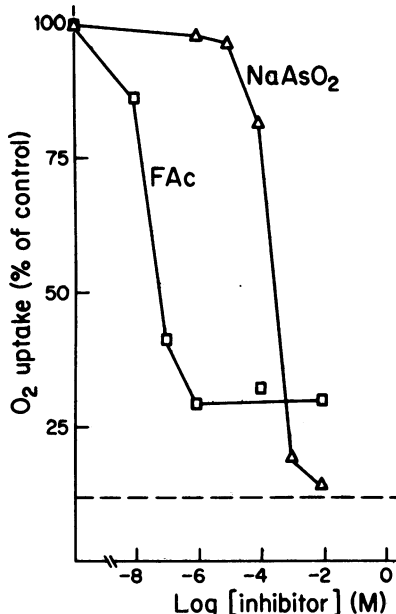


FIG. 1. Effects of fluoroacetate (FAc) and arsenite (NaAsO₂) on the respiratory activity of *R. capsulata* cells. Strain B10 was grown aerobically in darkness in RCVB medium, and the harvested cells were washed and resuspended in 50 mM potassium phosphate buffer (pH 7.0). Inhibitors (adjusted to pH 6.8) were added to ca. 2.9 ml of RCVB minus malate medium in the oxygen electrode chamber, followed by the addition of ca. 100 μ l of cell suspension and then DL-malate (40 mM). The O₂ uptake rate observed in the control (no inhibitor) was 96 nmol \cdot min⁻¹ \cdot mg cell protein⁻¹. The dashed line shows the O₂ uptake rate in the absence of cells.

strate at 12 and 4%, respectively, of the rate observed with the wild type (B10 strain). These results correlate well with the inhibition experiments and further indicate that most, if not all, of the O₂ utilization activity can be attributed to operation of the citric acid cycle.

The oxaloacetate \rightarrow α -ketoglutarate branch of the citric acid cycle. The segment of the citric acid cycle (Fig. 2) concerned with conversion of oxaloacetate + acetyl-CoA to KG can be conveniently referred to as the tricarboxylic acid or "C₆" branch. In *R. capsulata*, operation of this branch is essential for the utilization of N₂ or NH₄⁺ as the sole nitrogen sources for growth. These are assimilated via the glutamine synthetase-glutamate synthase reaction sequence (16), and KG is a substrate of the latter enzyme. Accordingly, mutants with defects in the C₆ branch would be expected to require exogenous provision of KG or of L-glutamate for growth. This has been observed for mutant

WE29, which lacks both CS and ICD (7). The experimental results of Fig. 3 show the effect of increasing L-glutamate concentration on cell yields of WE29 in the minimal malate + NH₄⁺ medium (RCVB) under photosynthetic growth conditions; wild-type strains showed excellent growth in the absence of added glutamate.

In this investigation, mutants deficient in only CS activity were isolated as fluoroacetate-resist-

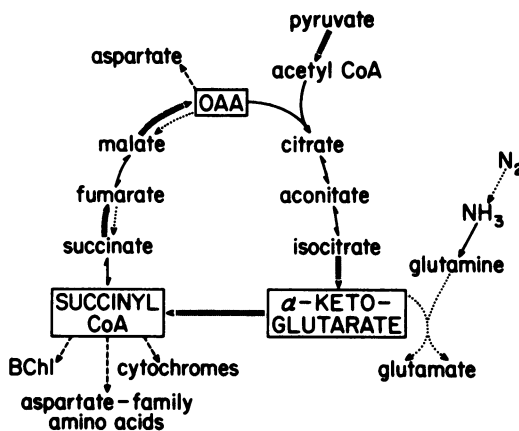


FIG. 2. The citric acid cycle and related metabolic sequences. Oxidative reactions are indicated by hatched arrows, and reductive reactions or sequences are shown by dotted arrows (OAA, oxaloacetate). Major biosynthetic products derived from the intermediates oxaloacetate, KG, and succinyl-CoA are indicated.

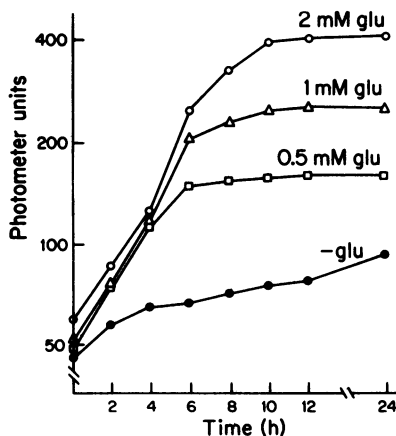


FIG. 3. Effect of L-glutamate (glu) on photosynthetic growth of mutant WE29. RCVB medium was supplemented with L-glutamate at the concentrations indicated. Inoculum: Cells grown photosynthetically in RCVB medium plus 10 mM L-glutamate were harvested by centrifugation and resuspended in RCVB medium.

ant derivatives of the wild type (see Materials and Methods). Such mutants, typified by strain T500, showed the same special growth requirement as WE29, namely, an exogenous source of KG such as L-glutamate. Levels of activities of C₆ branch enzymes in extracts of B10, WE29, and T500 cells (grown photosynthetically in RCVB medium supplemented with 10 mM L-glutamate) are shown in Table 2. The dependence of mutant WE29 and T500 on an exogenous source of L-glutamate for photosynthetic growth demonstrates the biosynthetic role of the C₆ branch of the citric acid cycle.

Mutants of *R. capsulata* defective in the C₆ branch would be expected to grow poorly, if at all, aerobically in darkness on malate even when supplied with L-glutamate since in such circumstances the energy supply depends on operation of the complete cycle. KGD⁻ mutants, in fact, cannot grow aerobically in darkness on carbon/energy sources that must be catabolized via the citric acid cycle (6a), and similar observations were made with T500. In RCVB medium supplemented with 10 mM L-glutamate, the CS-negative mutant grew aerobically to a very limited extent with a doubling time of about 24 h (the doubling time of the wild type in the same medium is ca. 3 h).

Biosynthesis of citric acid cycle enzymes in *R. capsulata*: CS and ICD. As indicators of C₆ branch enzymes, levels of CS and ICD were assayed in extracts of wild-type *R. capsulata* grown under a wide variety of nutritional conditions. These encompassed three modes of energy conversion, namely, photosynthetic, dark respiratory, and dark fermentative. The growth conditions used and the enzyme-specific activities observed are listed in Table 3, which also gives the ratios of ICD to CS activities. In general, specific activities of both ICD and CS were higher in extracts of aerobically grown cells than in those from cells grown photosynthetically. The activities in "aerobic extracts," however, varied more widely, probably reflecting differences among cultures in respect to medium pH and other conditions that change as cell density increases during growth. Despite the wide variation in nutritional circumstances, the ratio of ICD to CS activities was relatively constant. This is also seen in the differential plot shown in Fig. 4. An approximately linear relationship of the kind observed is interpreted as indicating coordinate expression of genes coding for the two enzymatic activities in question (3, 15). The scatter of values from the line of the order seen in Fig. 4 is commonly observed in other systems where enzymes are coordinately synthesized (3, 12, 38).

TABLE 2. Activities of C₆ branch citric acid cycle enzymes in extracts of *R. capsulata* wild-type and mutant strains

Strain	Enzyme activity ^a		
	CS	Aconitate hydratase	ICD
B10 (wild type)	142	67	627
WE29	<1	85	<1
T500	<1	37	541

^a Values expressed in terms of nanomoles of substrate transformed per minute per milligram of protein.

Factors affecting KGD synthesis in *R. capsulata*. In *R. capsulata*, succinyl-CoA appears to be produced from malate and fumarate only via KGD activity (6a) and is required as a biosynthetic precursor under both respiratory (dark) and photosynthetic growth conditions. It might be supposed that with light as the energy source for growth, synthesis of KGD would be appreciably depressed as compared to cells growing aerobically in darkness on typical organic substrates, that is, under conditions in which KGD activity is also necessary for citric acid cycle-supported energy conversion. This notion was investigated by determination of KGD activity levels in cells cultivated on various carbon sources in the alternative growth modes (see Table 4). Except for two special situations (with succinate or L-glutamate as carbon sources), extracts of "aerobic dark cells" showed KGD activities three- to fivefold higher than extracts from cells grown photosynthetically. Thus, the relative KGD levels in aerobic and photosynthetic cells roughly parallel the relative respiratory rates observed in cells grown in the two modes (Table 1).

Exceptions to the general pattern of results in Table 4 were observed with cultures grown aerobically on succinate and photosynthetically on L-glutamate as sole carbon sources. Succinate is, of course, derived from the succinyl-CoA generated by KGD activity, and when the C₄ acid served as the sole carbon source aerobically, the enzyme level was significantly decreased. Glutamate as a sole carbon source under photosynthetic conditions, on the other hand, appeared to induce KGD synthesis.

Keevil et al. (18) have recently reported that even trace concentrations of O₂ (ca. 20 ppm) in the gas phase can lead to detectably increased KGD levels in *Citrobacter freundii* cells growing in "anaerobic" cultures. This is also true for *R. capsulata* as evidenced from experiments which will be noted here in brief summary. *R. capsu-*

TABLE 3. CS and ICD activities in extracts of *R. capsulata* cells grown under various conditions

C/N sources for growth ^a	Growth conditions ^b	Symbol in Fig. 4	Culture density ^c	Activity ^d		ICD/CS
				ICD	CS	
Malate/NH ₄ ⁺	An/L	1	300	389	80	4.8
Malate/NH ₄ ⁺	An/L	2	256	463	90	5.2
Malate/NH ₄ ⁺	An/L	3	310	634	80	8.0
Malate/NH ₄ ⁺	An/L	4	138	640	142	4.5
Malate/NH ₄ ⁺	An/L	5	315	405	65	6.3
Lactate/NH ₄ ⁺	An/L	6	280	659	92	7.2
Malate/glutamate	An/L	7	294	683	95	7.2
Malate/glutamate	An/L	8	264	507	94	5.4
Malate + KG/NH ₄ ⁺	An/L	9	305	487	98	5.0
Glucose/NH ₄ ⁺	An/L	10	126	549	115	4.8
Pyruvate/NH ₄ ⁺	An/L	11	192	369	92	4.0
CO ₂ /NH ₄ ⁺ e	An/L	12	117	477	96	5.0
Malate/N ₂	An/L	13	115	580	107	5.4
Fructose/NH ₄ ⁺ f	An/D	14	71	493	129	3.8
Malate/NH ₄ ⁺	Aer/D	a	175	989	185	5.3
Malate/NH ₄ ⁺	Aer/D	b	275	997	257	3.9
Malate/NH ₄ ⁺	Aer/D	c	153	497	103	4.8
Malate/NH ₄ ⁺	Aer/D	d	240	457	192	2.4
Malate/NH ₄ ⁺	Aer/D	e	300	471	165	2.9
Malate/glutamate	Aer/D	f	320	1,080	284	3.8
Malate/glutamate	Aer/D	g	385	1,010	281	3.6
Lactate/NH ₄ ⁺	Aer/D	h	455	1,630	415	4.0
Lactate/NH ₄ ⁺	Aer/D	i	300	914	287	3.2
Pyruvate/NH ₄ ⁺	Aer/D	j	190	809	143	5.7

^a All media contained the mineral salts and vitamins specified for RCVB medium.

^b An/L, Anaerobic/light; An/D, anaerobic/dark; Aer/D, aerobic/dark.

^c Photometer units at time of cell harvest.

^d Activities are expressed in terms of nanomoles of substrate transformed per minute per milligram of protein.

^e Gas phase, 5% CO₂ + 95% H₂.

^f Trimethylamine-*N*-oxide was used as the accessory oxidant for fermentative growth (23).

lata B10 was grown photosynthetically in continuous culture essentially as described by Aiking and Sojka (1), using RCVB medium modified so as to contain only 6 mM DL-malate (the limiting nutrient). Special precautions were taken, however, to remove O₂ from the gas phase of ultrapure argon, and insofar as possible, all supply lines and connections were constructed of glass tubing. At a dilution rate of 0.05 h⁻¹ (doubling time of 14 h), the KGD specific activity in extracts of the cells from several experiments was 15 ± 4 nmol·min⁻¹·mg protein⁻¹. This activity corresponds to that observed in photosynthetic batch-culture cells (Table 4). When the glass tubing that served as the medium supply line was replaced with latex rubber tubing, the KGD level in extracts of cells harvested later increased to a specific activity of 25 ± 0.6. The quantity of O₂ that diffuses through the rubber tubing connection in such experiments is quite low and not easily measurable. The presence of a very low pO₂, however, is indicated by the fact that after replacement of glass with rubber tubing, the (continuous) culture color becomes more reddish owing to an O₂-

dependent production of red carotenoids (H. Aiking, J. Cox, and G. Sojka, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, I123, p. 107). Molecular oxygen is clearly an important regulatory signal for synthesis of KGD and, consequently, for expression of overall citric acid cycle activity.

Photosynthetic growth of KGD⁻ mutants. KGD11 and similar mutants cannot grow aerobically in darkness on succinate, malate, or pyruvate (6a). Experiments with KGD11 as the test organism showed, however, that despite the apparent absence of KGD, this strain can grow photoheterotrophically on malate, fumarate, or succinate as sole carbon sources. Photosynthetic growth on succinate can be readily rationalized since the succinyl-CoA required for biosynthesis can be generated directly from succinate through the action of succinyl-CoA synthetase. Since *R. capsulata* has no demonstrable fumarate reductase activity (6a), however, it is at first glance difficult to see how a KGD⁻ mutant can grow on fumarate or malate. How is succinyl-CoA generated from fumarate or malate in a mutant lacking both fumarate reductase and KGD activities? Isocitrate lyase activity was

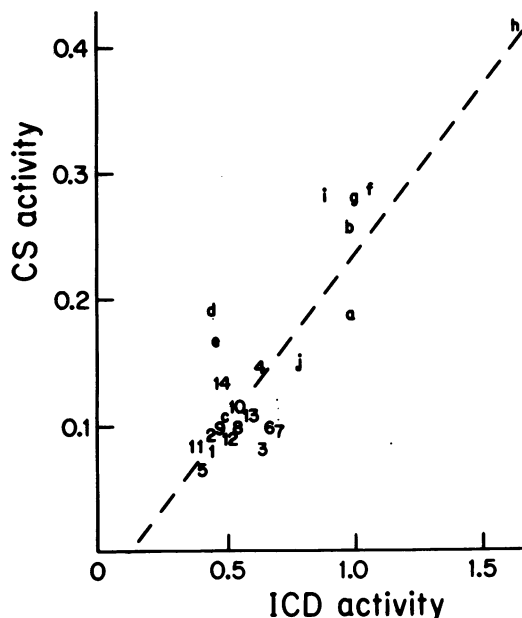


FIG. 4. Differential plot of ICD and CS activities measured in extracts of *R. capsulata* cells grown under various conditions. The letters and numbers specify the results obtained in the experiments of Table 3.

TABLE 4. KGD activities in extracts of *R. capsulata* cells grown under various conditions

Carbon source for growth ^a	KGD activities ^b in extracts of cells grown:	
	An/L	Aer/D
Fructose	20 (± 2)	54 (± 8)
Lactate	12 (± 2)	61 (± 12)
Pyruvate	17 (± 6)	60 (± 15)
Malate	14 (± 3)	54 (± 18)
Succinate	15 (± 6)	24 (± 1)
Glutamate	34 (± 6)	53 (± 14)

^a In all instances, the medium contained 7.5 mM $(\text{NH}_4)_2\text{SO}_4$.

^b Values expressed in terms of nanomoles of NADH per minute per milligram of protein. Each value is the mean of results from three to seven separate experiments; standard deviations are given in parentheses.

considered as a possible *in vivo* source of succinate despite the fact that the enzyme is not produced in wild-type *R. capsulata* cells grown photoheterotrophically on organic carbon sources other than acetate (28). The lyase was readily demonstrable in extracts of *R. capsulata* B10 cells that had been acetate adapted as described by Nielsen and Sojka (28), but could not be detected in extracts of KGD11 grown photosynthetically on malate.

The possibility that the commercially obtained DL-malate used for preparation of culture media contained sufficient succinate contamination to meet the biosynthetic succinyl-CoA requirement was entertained, and, indeed, small quantities of succinate were detectable by gas chromatographic analysis. This led to experiments designed to show that mutants such as KGD11 should be unable to grow photoautotrophically on CO_2 plus H_2 , that is, in media devoid of organic substrates.

Contrary to expectations, KGD11 showed the capacity to grow quite well as a photoautotroph under a gas phase of 5% CO_2 in H_2 . Moreover, from Fig. 5 it can be seen that although photosynthetic growth of KGD11 on $\text{CO}_2 + \text{H}_2$ was slower than that of the wild-type strain, development of the mutant could be sustained through numerous doublings in successive subcultures. As far as is known, BChl synthesis in *R. capsulata* and closely related organisms occurs primarily via the "succinyl-CoA + glycine" pathway and, consequently, BChl levels can be used as an index of *in vivo* KGD activity. In the experiment of Fig. 5, the B10 inoculum cells had a specific BChl content (micrograms of BChl per

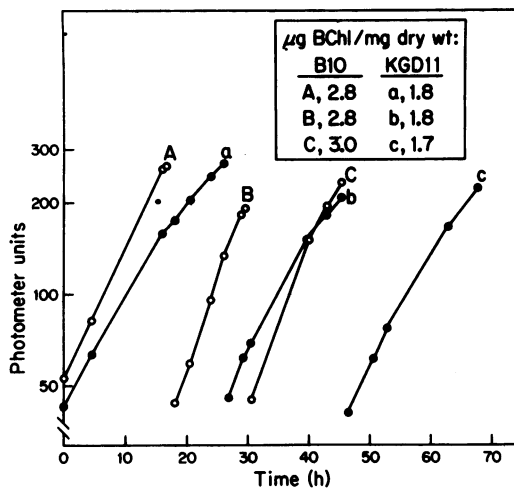


FIG. 5. Growth kinetics and BChl contents of *R. capsulata* wild-type strain B10 (○) and mutant KGD11 (●) under photoautotrophic conditions. Cells were grown under an atmosphere of 5% CO_2 in H_2 in RCVB minus malate medium, as described in the text. Samples were taken for BChl determination (see inset) and for serial subculture (A \rightarrow B \rightarrow C; a \rightarrow b \rightarrow c) at the times corresponding to the last point of each growth curve. Inocula: Cells grown photosynthetically in RCVB medium modified by substitution of 30 mM succinate for malate were sedimented by centrifugation and resuspended in RCVB minus malate medium with increased phosphate buffer content (20 mM).

milligram [dry weight] of cells) of 2.7, whereas the value for the KGD11 inoculum cells was 2.2. From the BChl values shown at the top of Fig. 5, it is evident that the BChl content of B10 was maintained at the normal level during photoautotrophic subculture. In KGD11, the specific BChl content diminished from the initial value of 2.2 in the succinate-grown inoculum to 1.7 in the third photoautotrophic subculture. The latter content was obviously still sufficient to support relatively good photoautotrophic growth, but since the BChl level was significantly lower than the wild-type value, it can be concluded that the rate of succinyl-CoA generation in growing cells of the mutant was appreciably diminished. In the experiment of Fig. 5, controls were included to determine the reversion frequencies of KGD11 under the conditions used, and these were of the order of 8×10^{-6} or less; such frequencies cannot account for a significant fraction of the BChl observed in photoautotrophically grown mutant populations.

DISCUSSION

Organic acids such as malate, lactate, and pyruvate are readily used by most representatives of the *Rhodospirillaceae* as carbon sources for photoheterotrophic growth, or as carbon and energy sources for aerobic dark growth. It seems to be generally assumed that catabolism of the organic acids occurs via the citric acid cycle, but this view is largely an extrapolation from studies on the metabolism of *Rhodospirillum rubrum* (4, 29). The present investigation provides evidence for operation of the cycle as a major mechanism in the dark aerobic (heterotrophic) metabolism of a typical and widely investigated species of the family *Rhodospirillaceae*. As in other kinds of cells, the citric acid cycle can fulfill several metabolic functions, primarily (i) generation of reduced pyridine nucleotide to support "electrophosphorylation" (13) and (ii) production of biosynthetic intermediates (mainly oxaloacetate, succinyl-CoA, and KG). The central importance of the cycle, or of component sequences, also is indicated by the fact that the activities of various enzymes are regulated by intermediates of the cycle (5, 25).

Since it is a precursor of the glutamate needed for protein synthesis, KG is required in substantial quantity for cell growth. Thus, photosynthetic growth is severely inhibited in mutants that have effective mutational blocks in the C₆ branch of the cycle, unless the cells are supplemented with exogenous glutamate (or KG). Mutants of this kind are also unable to grow aerobically in darkness on organic acids, even when

supplied with glutamate, due to failure of the energy conversion function of citric acid cycle activity.

In cells growing photoheterotrophically with light as the energy source, the KGD reaction is significant only from a biosynthetic standpoint, that is, for generation of succinyl-CoA. The latter is used primarily for synthesis of BChl and heme proteins, which in photosynthetically grown *Rhodospseudomonas* are found to the extent of ca. 10 nmol of BChl·mg protein⁻¹ (11, 34) and ca. 0.5 nmol of total heme·mg protein⁻¹ (30). From these values, the minimum rate of succinyl-CoA synthesis necessary for maintaining the 2-h doubling time characteristic of wild-type *R. capsulata* in RCVB medium (24) can be calculated to be ca. 0.7 nmol·min⁻¹·mg protein⁻¹ (at saturating light intensity; at low light intensity with a doubling time of 5 h, the calculated value is ca. 0.6). The quantity of succinyl-CoA required for biosynthesis is clearly of a different order of magnitude than that of KG. In *Rhodospirillaceae* growing photosynthetically, the maximum potential rate of KGD activity is much higher than the minimum needed to produce the required succinyl-CoA (6a). Thus, typical specific activities of KGD in extracts of *R. capsulata* are of the order of 15. Such values, of course, represent maximal activities observed in vitro with all substrates and cofactors at saturating concentrations. We assume that the in vivo rates must be considerably lower and subject to a variety of regulatory controls.

Calculations similar to those noted above can be made for mutant KGD11 growing photoautotrophically (on CO₂ + H₂) with a doubling time of 8 h and a somewhat diminished BChl content (Fig. 5). In this case, the required in vivo rate of succinyl-CoA production is ca. 0.03 nmol·min⁻¹·mg protein⁻¹. Such low rates cannot be detected in cell extracts with available procedures, and this explains the seeming paradox of the capacity of KGD11 to grow photoautotrophically despite the "absence" of KGD activity and of fumarate reductase activity. Accordingly, in mutants of this kind, very low rates of KGD activity could account for the succinyl-CoA needed for biosyntheses. Even when there is a severe genetic block, "mutant enzymes" can have low residual activity (leakiness), and it is also possible that succinyl-CoA could be slowly produced by the pyruvate dehydrogenase complex or in some other way. It is relevant that observations similar to those under discussion have been made with *E. coli*. Creaghan and Guest (9) constructed mutants of *E. coli* lacking KGD, fumarate reductase, succinate dehydrogenase, and isocitrate lyase activities, and these

still could grow slowly with glucose as the sole carbon and energy source. The cells showed a reduced heme content as compared with wild type, and it was concluded that leaks through the mutational blocks or an unknown metabolic pathway could supply succinate (or succinyl-CoA) at a low rate. The existence in green plants of a mechanism for conversion of KG to the porphyrin precursor δ -aminolevulinic acid not involving succinyl-CoA has been established (6, 17, 21), but the significance, if any, of this pathway in photosynthetic bacteria is still unclear (for recent studies in this connection, see reference 8). Investigations on purple photosynthetic bacteria indicate that in these organisms, the major (if not sole) source of δ -aminolevulinic acid is the succinyl-CoA + glycine condensation reaction (20).

Although KGD⁻ mutants of *R. capsulata* virtually devoid of KGD activity apparently produce sufficient succinyl-CoA for biosynthetic purposes when light is the energy source, the metabolic lesion in such strains is lethal for the aerobic dark growth mode because the bioenergetic function of the citric acid cycle cannot be maintained. The increase of KGD activity seen in extracts of cells grown aerobically as compared to extracts from "photosynthetic cells" is consistent with the need for greatly increased carbon flow via the cycle to maintain an adequate supply of reducing equivalents for the energy-converting respiratory apparatus.

The various controls that affect formation of citric acid cycle enzymes and their activities are still not well understood (for a recent discussion of regulation of the cycle in mammalian systems, see reference 37). Deeper insights into this aspect of cell metabolism should be facilitated by more detailed study of organisms like *R. capsulata* that have well-developed alternative mechanisms of energy conversion. In this respect, the effects of O₂ on *R. capsulata* are of special interest. The observed increase in activity of several citric acid cycle enzymes as well as of the NADH oxidase system in cells grown aerobically suggests that exposure to O₂ induces a multicomponent metabolic system of considerable complexity. Molecular oxygen appears to exert regulatory effects even when present at very low concentration, and this may well have meaning for further analysis of the biochemical evolution of aerobic systems from fermentative precursors.

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