L-Malate Oxidation by the Electron Transport Fraction of *Azotobacter vinelandii*

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The membrane-bound L-malate oxidoreductase of *Azotobacter vinelandii* strain O was found to be a flavoprotein-dependent enzyme associated with the electron transport system (R_3) of this organism. The particulate R_3 fraction, which possessed the L-malate oxidoreductase, carried out the cyanide-sensitive oxidation of L-malate, D-lactate, reduced nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate, succinate, cytochrome *c*, tetramethyl-*p*-phenylenediamine, and *p*-phenylenediamine, with molecular O_2 as the terminal electron acceptor. D-Malate was not oxidized, but L-malate was oxidized to oxalacetate. Phenazine methosulfate (PMS), vitamin K_3 , $K_3Fe(CN)_6$, nitro blue tetrazolium, and dichloro-indophenol all served as good terminal electron acceptors for the L-malate oxidoreductase. Cytochrome *c* was a poor electron acceptor. Extensive studies on the L-malate oxidase and PMS and K_3 reductases revealed that all were stimulated specifically by flavine adenine dinucleotide and nonspecifically by di- or trivalent cations, i.e., Ca^{++} , Ba^{++} , Mn^{++} , Mg^{++} , Fe^{+++} , Ni^{++} , and Al^{+++} . All these activities were markedly sensitive to ethylenediaminetetraacetate (EDTA). The V_{max} values for the L-malate oxidase, PMS, and vitamin K_3 reductases were, respectively, 3.4, 15.1, and 45.5 μ moles of substrate oxidized per min per mg of protein at 37 C. Spectral studies revealed that the *Azotobacter* R_3 flavoprotein and cytochromes (a_2 , a_1 , b_1 , c_4 , and c_5) were reduced by L-malate. L-Malate oxidase activity was sensitive to various inhibitors of the electron transport system, namely, *p*-chloromercuriphenylsulfonic acid, chlorpromazine, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide, antimycin A, and KCN. Minor inhibitory effects were noted with the inhibitors 4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione, rotenone, and Amytal.

In mammalian and most microbial systems, L-malate is oxidized to oxalacetate or pyruvate and CO_2 by the soluble nicotinamide adenine dinucleotide (NAD)-dependent malate dehydrogenase (EC 1.1.1.37), malate dehydrogenase (decarboxylating) (EC 1.1.1.38 and 1.1.1.39), or by the soluble nicotinamide adenine dinucleotide phosphate (NADP)-dependent malate dehydrogenase (decarboxylating) (EC 1.1.1.40). In bacteria, however, there is another enzyme, the L-malate oxidase or oxidoreductase (EC 1.1.3.3), a flavoprotein-dependent enzyme which catalyzes the oxidation of L-malate to oxalacetate. This enzyme has been studied in *Mycobacterium phlei* (3-5, 11, 33, 34, 49), *M. avium* (32, 47, 48, 50), *Acetobacter xylinum* (6-10, 31), *Micrococcus lysodieticus* (13, 14, 18-22, 36, 39), *Pseudomonas ovalis* (16, 17, 41), *Azotobacter agilis* (15, 40, 43), and *A. vinelandii* (1, 2, 12, 25, 26, 46; P. Jurtschuk, C. H. Denton, and A. J. Bednarz, *Bacteriol. Proc.*, p. 104, 1967). Preliminary indications are that this

oxidoreductase may be either soluble or particulate and is closely associated with or part of the electron transport system of microorganisms.

Further studies on the L-malate oxidoreductase in microbial systems revealed that oxidative phosphorylation can occur during this oxidation without the involvement of a pyridine nucleotide (9, 11). Cytochromes (7, 8, 11, 16, 18, 26), ubiquinone or coenzyme Q (8, 17, 25, 41), naphthoquinone or vitamin K (4, 17, 48, 49), nonheme iron (7, 31, 33, 34), and flavoproteins are involved in the overall reaction (7, 11, 16, 17, 22, 31, 41, 47). Furthermore, L-malate oxidation in microbial particulate fractions may be associated with or be part of the electron transport chain that carries out reduced nicotinamide adenine dinucleotide (NADH) oxidation (3, 20, 36). In some of these microbial systems, the flavoprotein moiety of the L-malate oxidoreductase is easily dissociable; restoration of activity is accomplished specifically by the addition of flavine adenine dinucleotide (FAD; 7, 32). Phospholipid

was also reported to be a component of this system. Numerous reports in the literature suggested that this enzyme may be reactivated by phospholipids (5, 41, 47).

Malate oxidation has long been known in *Azotobacter* species (1, 2, 12, 15, 25, 26, 40, 43, 46; P. Jurtshuk, C. H. Denton, and A. J. Bednarz, *Bacteriol. Proc.*, p. 104, 1967). The soluble NAD- and NADP-dependent (decarboxylating) L-malate dehydrogenases and the flavoprotein-containing L-malate oxidoreductase are present in high concentrations. The purpose of this investigation was to study the flavoprotein-dependent L-malate oxidoreductase of *A. vinelandii* strain O with respect to the kinetic properties of this enzyme, its ability to utilize various electron acceptors, its response to cofactors, its sensitivity to inhibitors and chelators, and its relationship to the *Azotobacter* electron transport system.

MATERIALS AND METHODS

Chemicals. Abbreviations used in the text are as follows: *p*-CMB, *p*-chloromercuriphenyl sulfonic acid; DCIP, 2,6-dichloroindophenol; EDTA, ethylenediaminetetraacetic acid (disodium salt); FAD; FMN, flavine mononucleotide; HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; MB, methylene blue; MTT, 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide; NBT, nitro blue tetrazolium; PMS, phenazine methosulfate; PPD, *p*-phenylenediamine; TFTB, 4,4',4-trifluoro-1-(2-thienyl)-1,3-butanedione; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride; and K_3 or vitamin K_3 , menadione, or 2-methyl-1,4-naphthoquinone. The sources of the chemicals were as follows: EDTA, FAD, FMN, Trizma base, *p*-CMB, and *o*-phenanthroline from Sigma Chemical Co., St. Louis, Mo.; riboflavine and 8-hydroxyquinoline from Eastman Organic Chemicals Co., Rochester, N.Y.; L-malate from Calbiochem, Los Angeles, Calif.; NAD and NADP from P-L Biochemicals, Inc., Milwaukee, Wis.; α,α -dipyridyl from Matheson Co., Inc., Joliet, Ill. Sources for all other chemicals are listed elsewhere (28).

Chemical methods. FAD, FMN, and riboflavine were prepared in deionized water and standardized spectrophotometrically at 450 nm by the use of the extinction coefficient of 10.3 per cm per mM (37). The chelators, 8-hydroxyquinoline and *o*-phenanthroline, as well as the electron transport inhibitors (Amytal, antimycin A, TFTB, HQNO, and rotenone), *p*-CMB, and vitamin K_3 were prepared in 95% ethyl alcohol.

The dinitrophenylhydrazine (DNP) derivative of the end product formed from L-malate oxidation was prepared in the conventional manner (45) and chromatographically characterized in the solvent systems previously described (44). Protein was determined by a modification of the biuret method of Gornall, Bardawill, and David (23).

Spectral characterization. The difference spectra (reduced minus oxidized) of the *Azotobacter* R_3 fraction were measured in a Cary model 14 recording

spectrophotometer. Details and conditions for these steady-state reductions are presented in Fig. 1.

Preparation of *A. vinelandii* cell-free extracts. The preparation of the *Azotobacter* R_3 fraction has been described previously (29, 30). This fraction is similar to the electron transport particles isolated and described by other workers (2, 12, 15, 25, 43, 46). The R_3 fraction appeared as a reddish-brown, translucent pellet that was obtained from sonically disrupted resting cells by differential centrifugation. This fraction did not sediment after 20 min at $37,000 \times g$ but was sedimented by centrifugation at $144,000 \times g$ for 2 hr. Of all the fractions examined, the R_3 exhibited the highest specific activities for NADH and succinate (27), D-lactate (28), L-malate (P. Jurtshuk, C. H. Denton, and A. J. Bednarz, *Bacteriol. Proc.*, p. 104, 1967), cytochrome *c* (30), and TMPD and PPD oxidases (27). A fractionation scheme showing the distribution of activity units for L-malate oxidation in cell-free extracts was drawn up (Table 4).

Enzyme assays. L-Malate oxidase activity was measured manometrically in 15-ml Warburg flasks with air as the gas phase. The main compartment contained 33.3 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 8.0), 0.04 mM FAD, and 2.0 mM metal ion (usually Ca^{++}) when desired, 0.18 or 0.35 mg of protein of the *Azotobacter* R_3 fraction

FIG. 1. Difference spectrum (reduced minus oxidized) exhibiting steady-state reduction in the *Azotobacter* R_3 electron transport fraction. The spectrum designated by "m" represents the reduction achieved by 0.09 M L-malate and the spectrum represented by "d" shows the chemical reduction achieved by the addition of a few crystals of sodium dithionite. The concentration of the *Azotobacter* R_3 fraction was 8.0 mg of protein in 1 ml of 0.02 M phosphate buffer (pH 7.5).

depending upon the presence or absence of cofactors, and deionized water to 3.0 ml. The side arm contained 50 μ moles of L-malate and the center well contained 0.2 ml of 2.0 M KOH. The reaction mixtures for the L-malate PMS and L-malate K_3 reductase assays were exactly as above, except that 2.0 mM KCN was added to the main compartment and either 2 ml of 1% (w/v) PMS was added to a side arm separate from the substrate or 0.03 ml of 2% (w/v) K_3 (in 95% ethyl alcohol) was added to the main compartment. The amount of *Azobacter* R_3 fraction added ranged from 0.04 to 0.15 mg of protein per 3 ml in the PMS reductase assay and from 0.02 to 0.07 mg of protein per 3 ml in the K_3 reductase assay. All reactions were initiated by the addition of substrate from the side arm after an initial equilibration period of 7 min. PMS was tipped into the main compartment at the same time as was the substrate in the L-malate PMS reductase assay. All specific activities are expressed as microatoms of oxygen per minute per milligram of protein at 30 or at 37 C. All inhibitor titration studies were carried out at 30 C, and the *Azobacter* R_3 fraction was incubated with the inhibitor and both cofactors in the main compartment during the equilibration period prior to the addition of the substrate.

Spectrophotometric assays were carried out at 25 C in quartz cuvettes. The final reaction volume was 1 ml. All reactions were initiated by the addition of enzyme, and all rates were corrected for any nonenzymatic rate. Routinely, the assays were performed in 50 mM Tris buffer (pH 8.0) with final concentrations of 60 to 70 μ M FAD, 1.0 mM $MgCl_2$, 1.0 mM KCN, and 10.0 mM L-malate. The reaction mixture for the PMS-mediated L-malate cytochrome *c* and DCIP reductase assays contained, in addition to the above, 0.165 mM PMS, and either 0.7 mg of cytochrome *c* per ml or 0.04 mM DCIP. The L-malate cytochrome *c* and L-malate DCIP reductase assays were carried out as described above, but with the elimination of PMS from the assay cuvette. The reduction of DCIP was monitored at 600 nm and, for all calculations, the extinction coefficient used was 20.5 per cm per mM (51). The reduction of cytochrome *c* was measured at 550 nm and the extinction coefficient used was 18.5 per cm per mM (38). The L-malate NBT reductase activity was measured by monitoring the increase in absorption at 530 nm with 84 μ M NBT, and calculations were based on the extinction coefficient of 18.0 per cm per mM (35). The concentration of L-malate used for the NBT reductase assay was 75 mM. The reaction mixture for the L-malate $K_3Fe(CN)_6$ reductase assay contained 40 mM Tris buffer (pH 8.0), 5.0 mM L-malate, 1.0 $MgCl_2$, 1.6 mM $K_3Fe(CN)_6$, and 60 μ M FAD. The reaction was monitored at 420 nm and the specific activity was calculated by the use of an extinction coefficient for ferricyanide of 1.0 per cm per mM (24, 51).

RESULTS

Stoichiometry of the reaction. L-Malate was oxidized by the L-malate oxidase in the *Azobacter* R_3 fraction according to the following reaction:

The enzymatic oxidation was found to be stoichiometric since 30 μ moles of L-malate was oxidized completely with the resultant uptake of 15 μ moles of O_2 . The keto end product formed could be trapped as the DNP derivative (45). Paper chromatographic analyses of R_F values, with three solvent systems (44), revealed that the keto-DNP derivative formed from L-malate oxidation was identical to a known authentic sample of 2,4-dinitrophenylhydrazone of oxalacetate.

Specificity of the enzyme for L-malate. The malate oxidoreductase activity in the *Azobacter* R_3 fraction was specific for L-malate. The unnatural isomer, D-malate, was not oxidized by this enzyme fraction (28).

Effect of metal ions and FAD in L-malate oxidation. The oxidation of L-malate by the *Azobacter* R_3 fraction was assayed readily by using molecular O_2 , PMS, and K_3 as electron acceptors. L-Malate oxidation was stimulated by the addition of FAD or metal ion, or both, and this stimulation occurred regardless of the electron acceptor used (Table 1). The enzymatic activity was found to be maximum only when both FAD and metal ion were present in the reaction vessel. The stimulation by metal ions was non-specific and a large number of cations could substitute for Ca^{++} ion. Only Ba^{++} , however, was as effective as Ca^{++} in stimulating L-malate oxidation. As would be expected from the above findings, L-malate oxidation by the *Azobacter* R_3 fraction was sensitive to chelating agents, namely, EDTA, *o*-phenanthroline, 8-hydroxyquinoline, and to a lesser extent to α, α -dipyridyl.

In contrast to the stimulation observed with metal ions, it was found that stimulation by FAD

TABLE 1. Effect of FAD and metal ions on L-malate oxidation by the *Azobacter* R_3 fraction

Assay system	Specific activity ^a		
	O_2	PMS	K_3
Control	0.43	2.88	1.11
Control + FAD (4×10^{-5} M)	0.90	6.04	17.14
Control + CaCl_2 (2×10^{-3} M)	1.29	4.72	8.43
Control + FAD + CaCl_2	1.98	9.41	23.81

^a Expressed in microatoms of oxygen per minute per milligram of protein at 30 C.

was highly specific (Table 2). Neither FMN nor riboflavin could substitute for FAD in stimulating L-malate oxidation, and neither NAD nor NADP influenced this oxidation. Stimulation of the L-malate oxidase activity by FAD, either in the presence or absence of metal ion, was sensitive to cyanide, suggesting that terminal oxidation via the electron transport system was still involved for the stimulated rates.

Development of assay and enzyme kinetics. Experiments were performed which were designed to define the kinetic characteristics of the *Azotobacter* R₃ L-malate oxidoreductase. Each artificial electron acceptor (PMS and vitamin K₃), as well as the substrate L-malate, was titrated by the use of the manometric assays. The $V_{\max}$ and K_m values were calculated (Table 3).

The $V_{\max}$ values calculated, with L-malate as the variable for the malate oxidase and the malate-K₃ and malate-PMS reductase assays, were slightly higher than the values obtained with the standard manometric assay used throughout this study. Substrate inhibition was not observed at concentration levels well in excess of 16.7 mM. This concentration of L-malate was found to be saturating and was used routinely for the standard assays.

The specific activity for the standard L-malate PMS reductase assay was 11.0 μ atoms of oxygen per min per mg of protein at 37 C, at a PMS concentration of 2.2 mM. The theoretical $V_{\max}$ for this assay, calculated at infinite PMS concentration, was 15.1 and the K_m value for PMS was 0.0028 M.

The kinetic parameters for the L-malate K₃ reductase assay, calculated at infinite vitamin K₃ concentration, were a $V_{\max}$ of 45.5 and K_m for K₃

TABLE 2. Effect of FAD, FMN, and riboflavin on L-malate oxidation by the *Azotobacter* R₃ fraction

Assay system	Specific activity ^a		
	O ₂	PMS	K ₃
Control	0.36	1.52	3.00
Control + CN (2×10^{-3} M)	0		
Control + riboflavin (10^{-4} M)	0.36	1.36	2.98
Control + FMN (10^{-4} M)	0.33	1.66	2.39
Control + FAD (10^{-4} M)	0.63	3.61	8.75
Control + FAD + CN (2×10^{-3} M)	0		
Control + FAD + Ca ⁺⁺ (2×10^{-3} M)	1.01	8.91	19.21
Control + FAD + Ca ⁺⁺ + CN (2×10^{-3} M)	0		

^a See Table 1.

TABLE 3. Kinetic parameters established for L-malate oxidation by the *Azotobacter* R₃ electron transport fraction

Variable assay component	Kinetic parameter	L-Malate oxidation assay ^a		
		Oxidase	PMS reductase	K ₃ reductase
L-Malate	$V_{\max}^b$	3.4	11.5 ^c	26.3 ^d
	K_m	0.0041	0.0032	0.0071
PMS	$V_{\max}$		15.1 ^e	
	K_m		0.0028	
K ₃	$V_{\max}$			45.5 ^f
	K_m			0.0018

^a Assays were performed with 4×10^{-5} M FAD and 2×10^{-3} M CaCl₂.

^b Expressed in micrograms of oxygen per minute per milligram of protein at 37 C.

^c The final concentration of PMS in the assay was 2.2 mM.

^d The final concentration of K₃ in the assay was 1.2 mM.

^e The final concentration of L-malate was 16.7 mM.

^f Concentrations greater than 1.2 mM of K₃ were inhibitory for the K₃ reductase assay.

of 0.0018 M. Concentrations of vitamin K₃ in excess of 1.2 mM were inhibitory, and, for this reason, activities close to the theoretical $V_{\max}$ value could never be obtained for the L-malate-K₃ reductase assay. Instead, specific activities of approximately 50% of the $V_{\max}$ value, or 26.3 μ atoms of oxygen per min per mg of protein, were obtained routinely with the standard assay in which a 1.2 mM concentration of vitamin K₃ and 16.7 mM L-malate were used.

Spectrophotometric assays. L-Malate oxidation in the *Azotobacter* R₃ fraction was examined also with various other artificial electron acceptors. The highest turnover rates were obtained with the PMS-mediated L-malate cytochrome *c* or DCIP reductases. For these two assays, specific activities of 2.05 and 1.65 μ moles, respectively, of L-malate (or $2 e^-$) oxidized per min per mg of protein at 25 C were obtained. The specific activity for the L-malate K₃Fe(CN)₆ reductase was 0.36 and the activities for L-malate oxidation with DCIP and NBT as the sole electron acceptors were 0.09 and 0.08, respectively. Cytochrome *c* served as the poorest acceptor and gave a specific activity value of 0.04 μ mole of L-malate oxidized per min per mg of protein at 25 C.

Distribution of activities for L-malate oxidation. A fractionation scheme, which shows the distribution of the various activities for L-malate oxidation in cell-free extracts of *A. vinelandii*, was devised. The fractionation, shown in Table 4, was carried out by differential centrifugation of son-

ically disrupted resting cells of *A. vinelandii*. The supernatant and residue fractions were designated "S" and "R," respectively. S_1 was the original sonic-treated extract obtained from the disrupted *Azotobacter* cells after centrifugation at $4,300 \times g$ for 10 min. The S_1 fraction was centrifuged further at $37,000 \times g$ for 20 min, and the residue R_2 fraction from this centrifugation was obtained. The resultant supernatant S_2 fraction was then centrifuged for 2 hr at $144,000 \times g$ and the R_3 and S_3 fractions were obtained. A more complete description of this differential centrifugation procedure was published elsewhere (27, 29).

In essence, all the activity associated with L-malate oxidation, regardless of the acceptor used, concentrated in the particulate R_3 fraction (Table 4). Specific activities for all the L-malate oxidation assays were always highest with this fraction, and the predominant portion of the activity units concentrated here.

Difference spectra of the *Azotobacter* R_3 fraction. An analysis of the difference spectra of the *Azotobacter* R_3 fraction revealed that the membrane-bound cytochrome and flavoprotein components were reduced readily by L-malate. Partial steady-state reduction was achieved by the addition of L-malate ("m") and the subsequent reduction that was attained by the addition of sodium dithionite ("d"; Fig. 1). The addition of L-malate completely reduced cytochrome a_2 (629 nm) and cytochrome a_1 (600 nm) but partially reduced cytochromes b_1 (561 and 531 nm) and $c_4 + c_5$ (553 and 523 nm). Only a small portion of the flavoprotein (and possibly nonheme iron) was reduced by L-malate, as shown by the trough in the 457-nm region. No appreciable steady-state reduction of the electron carriers was detected when cyanide

(10^{-3} M) was added to the test cuvette in the absence of L-malate or when the test cuvette was made anaerobic by evacuation. When, however, cyanide was added during steady-state reduction by L-malate, all of the cytochrome components were reduced to the dithionite level, although no effect was noted on the extent of bleaching in the flavoprotein region. It can be concluded by these steady-state reduction studies that the cytochrome and flavoprotein components of the *Azotobacter* R_3 electron transport fraction were reduced substantially, although not maximally, by L-malate.

Sensitivity to electron transport inhibitors. The L-malate oxidase activity of the *Azotobacter* R_3 fraction was examined for its sensitivity to electron transport inhibitors. These titrations (Fig. 2) were made in the presence of both FAD and CaCl_2 , and the values shown in brackets represent the molar concentration at which the 50% inhibition values were estimated by interpolation. As shown, chlorpromazine and *p*-CMB were the most effective inhibitors. The next most effective inhibitors of the L-malate oxidase activity were HQNO (19.1 μg per mg of protein), KCN, and antimycin A (34.3 μg per mg of protein). Other electron transport inhibitors were tested, but these were found to be less effective. Amytal at 2×10^{-3} M caused only a 9% inhibition, whereas TFTB at a concentration of 4.0×10^{-4} M (239 μg per mg of protein) inhibited the L-malate oxidase by 33%. Rotenone, at 2×10^{-4} and 1×10^{-3} M, inhibited the L-malate oxidase activity by 50 and 65%, respectively.

Sensitivity to chelating agents. The L-malate oxidase was most sensitive to EDTA; 5×10^{-5} M caused 40% inhibition. Other chelators, namely, *o*-phenanthroline and 8-hydroxyquinoline, at a concentration level of 10^{-4} M, inhibited the L-malate oxidase activity by 20 and 8%, respec-

TABLE 4. Distribution of activity units for L-malate oxidation in fractions obtained by differential centrifugation from sonically disrupted resting cells of *Azotobacter vinelandii*

Fraction	Protein recovery	Specific activity ^a and recovery of activity units					
		O_2	TU ^b	PMS	TU ^b	K_3	TU ^b
	%		%		%		%
S_1	100	0.14	100	2.08	100	14.99	100
S_2	62	0.12	54	2.32	67	10.03	42
R_2	26	0.13	24	1.01	13	3.67	24
S_2	100	0.12	100	2.32	100	10.03	100
S_3	57	0.001	3	0.89	23	1.92	11
R_3	48	0.24	96	4.53	98	17.30	83

^a See Table 1.

^b Total units (TU) were calculated by multiplying total protein concentration by specific activity.

FIG. 2. Effect of increasing concentrations of electron transport inhibitors on the L-malate oxidase activity of *Azotobacter* R_3 fraction. The inhibitor titrations were performed at 30 C in the presence of both Ca^{++} ion (2×10^{-3} M) and FAD (4×10^{-5} M). The values in brackets indicate the molar concentration of inhibitor at which a 50% inhibition was estimated by interpolation.

tively. At 3.3×10^{-4} M, α, α -dipyridyl caused only a 6% inhibition.

DISCUSSION

The L-malate oxidoreductase of *A. vinelandii* is a particulate enzyme apparently bound to the electron transport system. Studies from numerous laboratories revealed that the enzyme fraction possessing L-malate oxidoreductase activity also contained high concentrations of flavoprotein, coenzyme Q_8 , cytochromes b_1 , $c_4 + c_5$, o , a_1 , and a_2 , and nonheme iron. The L-malate oxidoreductase of *A. vinelandii* strain O utilized vitamin K_3 , PMS, molecular O_2 , $K_3Fe(CN)_6$, DCIP, NBT, and cytochrome c as electron acceptors. The L-malate vitamin K_3 reductase from the *A. vinelandii* R_3 fraction was unlike that found in *Acetobacter xylinum* as it was not sensitive to cyanide.

The *Azotobacter* L-malate oxidase was sensitive to such inhibitors as *p*-CMB, chlorpromazine, HQNO, KCN, and antimycin A (Fig. 1). This is in contrast to the L-malate oxidoreductase of *M. lysodeikticus*, which was insensitive to both antimycin A and cyanide (21).

As with the L-malate oxidoreductase of *Mycobacterium phlei*, *M. avium*, *Acetobacter xylinum*, and *P. ovalis*, the L-malate oxidoreductase in *Azotobacter vinelandii* strain O was specifically stimulated by FAD. This is in contrast to the findings reported for *A. vinelandii* strain 8660 NCIB in which the L-malate oxidoreductase in cell-free extracts was not stimulated by externally added FAD (25). Jones and Redfearn (25, 26) examined this particulate enzyme in strain 8660 and showed that it was neither NAD- nor NADP-dependent and that it did not require a supernatant fraction for the rapid oxidation of L-malate. They reported that FAD did not stimulate the particulate L-malate oxidoreductase of *A. vinelandii* strain 8660 when enzyme assays were performed by the use of an oxygen electrode. A similar lack of FAD stimulation was also observed when the L-malate oxidoreductase of the R_3 fraction of *A. vinelandii* strain O was assayed by the polarographic technique. However, FAD stimulation could be demonstrated if the enzyme from strain O was dialyzed extensively before assay with the oxygen electrode. This treatment resulted in a marked loss of enzymatic activity, a small part of which was restorable by adding FAD. Both *Azotobacter* strains contained the same cytochromes, flavine and ubiquinone Q_8 , but no menaquinone (K_2) could be detected in *A. vinelandii* strain 8660, although it was found in strain O (25).

The L-malate oxidoreductase of the *Azotobacter* R_3 fraction was stimulated nonspecifically

by externally added divalent cations. Plaut and Lardy (42) reported that certain divalent cations increased the rate of enzymatic decarboxylation of oxalacetate to pyruvate and CO_2 in cell-free extracts of *A. vinelandii*. Furthermore, higher concentrations of these same metal ions increased even the nonenzymatic decarboxylation rate of this highly unstable acid. The metal ion stimulation observed with the L-malate oxidoreductase of the *Azotobacter* R_3 might be attributed to the decarboxylation of oxalacetate, which is the primary end product of L-malate oxidation. The increased rate of removal of oxalacetate by decarboxylation could drive the initial reaction, the oxidation of L-malate to oxalacetate. This type of metal ion stimulation was not reported for the L-malate oxidoreductase in other microorganisms.

Unlike the flavine found in the enzyme preparation of *Mycobacterium avium* and *Acetobacter xylinum*, the flavine moiety of the *Azotobacter* L-malate oxidoreductase in the R_3 fraction could not be dissociated by dialysis or by ammonium sulfate-acid fractionation (37). The *Azotobacter* L-malate oxidoreductase in the R_3 fraction remained active during the ammonium sulfate-acid treatment, and the FAD stimulation of the treated enzyme was almost identical to that noted for the untreated R_3 preparation. In contrast to the enzyme from *Micrococcus lysodeikticus* (13) and *Mycobacterium phlei* (3-5, 33), the L-malate oxidoreductase from the *Azotobacter* R_3 fraction from strain O did not depend upon any factors in the supernatant liquid for activity.

The clarified supernatant liquid or S_3 fraction of *A. vinelandii* contained slight but significant amounts of L-malate, vitamin K_3 , and PMS reductase activities (Table 4). This suggests that the L-malate oxidoreductase of the R_3 fraction has been partially dissociated from the electron transport chain by the sonic-treatment procedure used in preparing the enzyme. Such soluble L-malate vitamin K reductases have also been described in *M. phlei* (3, 5, 33), in *A. xylinum* (7, 10, 31), in *M. avium* (47), and in *P. ovalis* (17, 41). As in these other microorganisms, the soluble (S_3) fraction of *Azotobacter vinelandii* contained no significant L-malate oxidase activity. Residual amounts of the *b*- and *c*-type cytochromes are present in the S_3 fraction, although cytochromes a_1 , a_2 , and *o* appear to be absent. These latter cytochromes are believed to be responsible for terminal oxidase function.

Specific activities for L-malate oxidation by the *Azotobacter* R_3 fraction were unusually high when compared to the activities reported for other microorganisms. This could be a reflection of the high metabolic activity that is commonly found in *Azotobacter* species.

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