THE PARTICIPATION OF VITAMIN K IN MALATE OXIDATION BY ACETOBACTER XYLINUM

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The cytochrome chain-linked dehydrogenation of malate to oxaloacetate (OAA) in A. xylinum has been shown to be irreversible. It is catalyzed by a FAD-flavoprotein, which is not affected by high concentrations of OAA (Benziman and Galanter, 1964; Benziman and Abeliovitz, 1964). These properties are in contrast to those of the NAD-linked malic dehydrogenase of animal tissues and other microorganisms. The present communication presents evidence for the participation of a vitamin K-like compound in the malate oxidase system in A. xylinum, which catalyzes the transfer of electrons from malate to molecular oxygen.

Materials and Methods. Extracts of Succinate-grown cells of A. xylinum were prepared as previously described (Benziman and Galanter, 1964). Malate oxidase activity was measured by conventional manometric techniques. Reaction velocity was calculated from oxygen uptake or from amount of OAA formed during the first 10 min after addition of substrate. Decarboxylation of OAA was prevented by the addition of ethylenediaminetetraacetic acid (EDTA) to reaction mixtures (Benziman and Heller, 1964). Malate-vitamin K_3 reductase and K_3H_2 (reduced vitamin K_3) oxidase activities were determined spectrophotometrically at 262 mμ by measurement of the reduction of K_3 or the oxidation of K_3H_2, respectively, according to Colpa-Boonstra and Slater (1958). OAA was determined as described previously (Benziman and Abeliovitz, 1964). Protein was determined according to Lowry et al. (1951). Irradiation of extracts at 360 mμ was performed according

to Brodie and Ballantine (1960). K_3H_2 and phthiocol were prepared according to Fieser (1940).

Results. Preliminary experiments carried out with sonic extracts revealed that as the sonication time was increased malate oxidase activity of the extracts decreased significantly. Thus extracts obtained by submitting cells to sonication for 40 min had one fourth the activity of extracts obtained from a 10 min sonication period. Activity could be restored to such extracts by the addition of low concentrations of vitamin K_3 (2-methyl-1, 4-naphthoquinone) or higher concentrations of vitamin K_1 (2-methyl-3-phythyl-1, 4-naphthoquinone).

Irradiation of extracts with light at 360 mμ resulted in a decrease of over 80% of malate oxidase activity (Table 1). Addition of vitamin K_3 markedly reactivated the irradiated extracts. Vitamin K_1 was less effective than K_3, both in the stimulation of the activity of nonirradiated extracts and in the activation of irradiated preparations. Vitamin K_1 was active only when suspended in phospholipid (asolectin or cardiolipin). K_1 suspended in detergents like Triton X-100 or Tween 80 showed no activity. Other quinones tested showed no activity. These included 1, 4-naphthoquinone, 1, 2-naphthoquinone, benzoquinone, K_3 metabisulfite, Synkavit, Coenzyme Q_{10}, and phthiocol.

Table 1

Effect of Vitamin K on Malate Oxidase Activity of Irradiated and Nonirradiated Extracts

Additions	Activity	
	Nonirradiated	Irradiated
None	0.030	0.005
$K_3(10^{-4}M)$	0.170	0.120
$K_1(5\times10^{-3}M)$	0.060	0.020

Incubation mixture (1 ml) contained: 0.1 M phosphate buffer, pH 7.0, 0.05 M EDTA, extract (4 mg protein) and 0.02 M L-malate. K_3 was added in 3 μl of methanol. K_1 was suspended in 2% asolectin. Irradiation time 90 min. Activity is expressed as μmoles malate oxidized per min per mg protein.

Direct evidence was obtained for the reduction of vitamin K_3 during malate oxidation and for the oxidation of reduced vitamin K_3 (K_3H_2) by the extracts (Table 2).

Table 2

Oxidative Activities of A. xylinum extracts

Enzyme System	Activity
Malate $(+K_3) \rightarrow O_2$	0.112
Malate $\rightarrow K_3$	0.123
$K_3H_2 \rightarrow O_2$	0.335

Malate oxidase activity was determined at pH 6.0. Conditions as in Table 1. Malate-K_3 reductase assay mixture contained in 1 ml: 0.1 M phosphate buffer, pH 6.0, 5×10^{-5}M K_3, 10^{-2}M KCN, 2×10^{-3}M EDTA and extract (0.05 mg protein). Activity is expressed as μmoles K_3 reduced per min per mg protein. K_3H_2 oxydase assay mixture contained in 1 ml: 0.1 M phosphate buffer, pH 6.0, 2×10^{-3}M EDTA, 5×10^{-5}M K_3H_2 (in 3μl methanol) and extract (0.05 mg protein). Activity corrected for autooxidation is expressed as μmoles K_3H_2 oxidized per min per mg protein.

The rates of these reactions are compatible with the overall rate of malate oxidation in the presence of K_3. The malate-K_3 reaction seems to be rate limiting for the overall malate-O_2 reaction. The stoichiometry of malate oxidation was found to be the same in the presence or absence of K_3, namely, one atom of oxygen consumed per mole of OAA formed.

The effects of various electron-transport inhibitors on the different oxidative activities is given in Table 3. Amytal, dicoumarol, 2-n-heptyl-4-hydrozyquinoline-N-oxide (HQNO), cyanide, azide and p-hydroxymercuribenzoate (HMB), were found to exert similar inhibitory effects on malate oxidase activity in the presence and in the absence of K_3. All the inhibitors which affected one of the partial reactions (malate $\rightarrow$ K_3 or $K_3H_2 \rightarrow O_2$) similarly inhibited the overall malate $\rightarrow$ O_2 reaction. The type of inhibition caused by dicoumarol was shown to be competitive with vitamin K_3 by a Lineweaver-Burk plot.

Table 3

Effect of Various Inhibitors on Oxidative Activities of A. xylinum extracts

Enzyme system	Amytal (2×10^{-3}M)	Dicoumarol (10^{-5}M)	HQNO (4×10^{-5}M)	HMB (10^{-4}M)	CN^- (10^{-3}M)
	Percent inhibition				
Malate → O_2	90	82	84	75	100
Malate (+K_3) → O_2	90	80	85	73	100
Malate → K_3	92	80	5	72	0
K_3H_2 → O_2	0	0	88	10	100

The incubation conditions were the same as for Table 2. Extract was preincubated with the inhibitors for 5 min.

Discussion. Treatment of bacterial extracts with light at 360 mμ was reported (Brodie and Ballantine, 1960; Downey, 1960) to inactivate bound vitamin K-like compounds without affecting other components of the respiratory chain, like flavins and cytochromes. The results of Table 1 thus suggest that a vitamin K-like compound is a functional intermediate in the electron transport system catalyzing electron transfer from malate to oxygen in A. xylinum. Inactivation of this quinone by irradiation resulted in a loss of malate oxidase activity, which could be restored by replacing the inactivated quinone with exogenous vitamin K_3 or vitamin K_1. That a vitamin K-like compound is involved in our system is further suggested by the observation that dicoumarol, a competitive inhibitor of vitamin K, inhibited malate oxidation, even in a system not supplemented with K_3. This observation is at variance with the report of Conover and Ernster (1962) that dicoumarol inhibits DPNH oxidase activity of rat liver mitochondria only in a system supplemented with vitamin K_3. The stimulation by vitamin K_3 of malate oxidation in nonirradiated extracts can be explained as being due to an increase in the concentration of quinones available for electron transport. In addition, the rates of the enzy-

matic reduction of K_3 and the subsequent oxidation of K_3H_2 may be faster than the rates of the respective reactions which occur with the natural quinone in the absence of K_3.

The malate oxidase reaction in the presence of K_3 seems to occur with K_3 acting directly as an intermediary electron carrier. This is strongly suggested by the compatibility of the rates of the malate-K_3 reductase and the K_3H_2 oxidase reactions with the overall rate of malate oxidation. The inhibition by cyanide and HQNO of malate oxidation in the presence of K_3 and of K_3H_2 oxidation indicate that K_3 mediates electron transport to oxygen through the cytochrome chain.

Amytal was previously shown (Benziman and Galanter, 1964) not to inhibit the malate-ferricyanide interaction in A. xylinum, although it inhibits the malate oxidase reaction. Thus its inhibition of the malate oxidase activity should be at a site on the respiratory chain, which is on the oxygen side of the flavoprotein, presumably before cytochrome b (Hatefi et al., 1962 ; Packer et al., 1960). The lack of inhibition by Amytal of K_3H_2 oxidation would place K_3 at a site on the respiratory chain after the site of Amytal inhibition. On the other hand, the inhibition of K_3H_2 oxidase by HQNO which is generally believed to block electron transport between cytochrome b and cytochrome c (Lightbown and Jackson, 1956) indicates that K_3 acts before cytochrome c and possibly before cytochrome b (Conover and Ernster 1962, Colpa-Boonstra and Slater, 1953). Thus the observed pattern of inhibition places K_3 and probably the natural quinone in A. xylinum between the primary flavoprotein dehydrogenase and cytochrome c.

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