OXIDATIVE PHOSPHORYLATION IN FRACTIONATED BACTERIAL SYSTEMS XII. THE PROPERTIES OF MALATE-VITAMIN K REDUCTASE*

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Studies of the soluble components required for restoration of oxidative phosphorylation revealed the presence of a new enzyme tentatively referred to as malate-vitamin K reductase, which was associated with the components necessary for restoration of activity with malate. At least three different bacterial enzymes have been described which are capable of oxidizing malate. Korkes et al. (1950) described an DPN-dependent malic enzyme from Lactobacillus arabinosus. A soluble DPN-linked malic dehydrogenase was found in Micrococcus leisodeikticus by Cohn (1956). In addition, Cohn described a particle bound malic dehydrogenase which did not require DPN. The latter enzyme appears to be similar to the FAD-dependent, particle bound malic dehydrogenase from M. avium (Kimura and Tobari, 1963).

The malate-vitamin K reductase from Mycobacterium phlei is unique with respect to the other malic dehydrogenases since it requires vitamin K as the electron acceptor. The participation of naphthoquinones with this enzyme is of particular interest since vitamin K is also required for restoration of oxidative phosphorylation in M. phlei (Brodie and Ballantine, 1960).

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Materials and Methods

Malate-vitamin K reductase was purified from crude M. phlei supernatant fluid following fractionation with ammonium sulfate and chromatography on DEAE-cellulose (Asano and Brodie, 1963). The enzyme was assayed spectrophotometrically by following the changes in OD at 565 mμ. The reaction mixture contained purified enzymes, MTT tetrazolium salt,* FAD, Tris buffer pH 7.2, and vitamin K_1 suspended in phospholipid.

Results

The reduction of MTT-tetrazolium by malate with either crude or purified enzyme requires the addition of vitamin K_1 and FAD, Table I. In the absence of vitamin K_1 reduction of the dye does not occur. Optimal rates of reduction of dye were achieved when vitamin K_1 was suspended in phospholipid (Asolectin,** Cardiolipin and Lecithin). In the absence of vitamin K_1 but with phospholipid reduction did not occur. Vitamin K_1 suspended in dialyzed supernatant showed little activity. In addition, FAD was required for the enzyme activity. Substitution of FMN for FAD resulted in an appreciable lower rate of dye reduction.

TABLE I

Requirements of FAD and vitamin K_1 for malate-vitamin K reductase activity

Vitamin K_1	FAD	Phospholipid	ΔE_{565}
--	10^{-5}M	Asolectin 2.5 mg	0.000
2.0 mg (2.9×10^{-3}M)	"	"	0.750
--	"	Lecithin 1.8 mg	0.004
2.0 mg	"	"	0.189
--	"	Cardiolipin 1.6 mg	0.000
2.0 mg	"	"	0.651
2.0 mg	"	- -	0.011
2.0 mg	--	Asolectin 2.5 mg	0.019
2.0 mg	FMN 10^{-5}M	"	0.032

Vitamin K_1 was suspended in the enzyme solution. The reaction mixture contains: dialyzed supernatant, 0.10 ml; Tris buffer (pH 7.2), 6×10^{-2}M; $MgCl_2$, 3×10^{-3}M; KCl, 3×10^{-2}M; malate, 2×10^{-2}M; MTT, 2.8×10^{-4} M; and total volume 1.5 ml in 1 cm light-pass cuvettes. Reaction was followed by Cary model 11 recording spectrophotometer at 23° C.

* 3 (4,5 Dimethyl thiazolyl 1-2) 2,5-diphenyl tetrazolium bromide.
** Asolectin (Associated Concentrates, Woodside, N.Y.).

The malate-vitamin K_1 reductase was found to be less specific for quinones than that observed for restoration of coupled phosphorylation (Table II). However, the affinity constant for vitamin K_1 with the enzyme (Km) was 4.9×10^{-4}M, and is similar to that observed for oxidation (8.6×10^{-4}M) and phosphorylation (6.9×10^{-4}M) of overall reaction (Brodie and Ballantine, 1960). Menadione in solution was also found to serve as an electron carrier. The Km value for menadione was, however, lower than that observed with vitamin K_1. Nevertheless, the Vmax value for vitamin K_1 (746 mμ moles/min/mg protein) was higher than that found with menadione (385 mμ moles/min/mg protein). Coenzyme Q_6 and Q_{10} although active exhibit a lag period of one to three minutes (Table II) before reduction occurs. The reduction of MTT by reduced coenzyme Q appears to be non-enzymatic, thus the lag period may be due to the period required for accumulation of reduced Q. α-Tocopherol was without effect.

TABLE II

Quinone specificity

Quinone	ΔE_{565}
---	0.000
Vitamin K_1 (6×10^{-4}M)	0.489
Menadione (10^{-4}M)	0.440
Coenzyme Q_6 (5.7×10^{-4}M)	0 → 0.201*
Coenzyme Q_6 (1.14×10^{-3}M)	0 → 0.462*
Coenzyme Q_{10} (3.5×10^{-4}M)	0 → 0.230*
α-Tocopherol (6.2×10^{-4}M)	0.000

* Measured 3 min. after addition of malate. The reaction mixture was as shown in legend of Table I with FAD (10^{-5}M) and Asolectin (2.5 mg).

Effects of inhibitors on the enzyme activity (Table III) were similar to that observed with coupled phosphorylation with malate as an electron donor (Asano and Brodie, 1963). Amytal, atebrin, Dicumarol, quinoline-N-oxide (NOQNO), Tween 80, BRIJ-35, and pCMB were found to be effective inhibitors of both activities. NOQNO has two sites of action with the M. phlei system. At low concentration (1 μg/mg protein) this reagent inhibits at the cytochrome b region, whereas at high concentrations (5-10 μg) NOQNO inhibition occurs before the

quinone level (unpublished observations). It is of interest to note that the non-ionic detergents (Tween 80 and BRIJ-35) are effective inhibitors of the main pathway of oxidation at low concentrations. These compounds have been used as suspending medium for vitamin K_1 (Martius, 1954; Wosilait, 1960; Weber and Rosso, 1963).

TABLE III
Effects of inhibitors

Inhibitors	Concentration (M)	Per cent of inhibition
Amytal	10^{-2}	55
Atebrin	2×10^{-3}	83
Dicumarol	5×10^{-4}	68
NOQNO	7 μg/mg protein	82
TTB	5×10^{-4}	2
Tween 80	0.13%	100
BRIJ-35	0.33%	100
pCMB	5×10^{-4}	90

Other electron carriers were tested for the ability to serve as electron acceptors. Dichlorophenolindophenol was found to be as effective as MTT-tetrazolium, whereas cytochrome c was found to be a poor electron acceptor. The activity was found to be specific for L-malate. D-malate, DPNH, TPNH, oxalacetate, pyruvate, fumarate, succinate, α-ketoglutarate, acetaldehyde, β-hydroxybutyrate and formate were inactive as the electron donors. The end products of the malate-vitamin K reductase were found to be oxalacetate and either reduced dye or quinone.

Discussion

The malate-coupling factor (Asano and Brodie, 1963) and the malate-vitamin K reductase from M. phlei have similar physical and biochemical properties. Both enzymes require vitamin K for activity and exhibit the same pattern of inhibition with respiratory inhibitors. Nevertheless, the specificity of the coupling factor for naphthoquinones was found to be more specific than the malate-K reductase. This enzyme, unlike the typical malic dehydrogenase, does not involve DPN but requires FAD. The malate-K reductase in association with the particles would require an

additional enzyme for oxidation of reduced vitamin K. Further details of the malate-vitamin K reductase will be published elsewhere.

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References

Asano, A., and Brodie, A. F., Biochem. Biophys. Res. Commun., in press (1963).

Brodie, A. F., and Ballantine, J., J. Biol. Chem. 235, 232 (1960).

Cohn, D. V., J. Biol. Chem. 221, 413 (1956).

Kimura, T., and Tobari, J., Biochim. Biophys. Acta, 73, 399 (1963).

Korkes, S., del Campillo, A., and Ochoa, S., J. Biol. Chem. 187, 891 (1950).

Martius, C., and Strufe, R., Biochem. Zeit. 326, 24 (1954).

Weber, M. M., Rosso, G., and Noll, H., Biochim. Biophys. Acta 71, 355 (1963)

Wosilait, W. D., J. Biol. Chem. 235, 1196 (1960).