THE PROPERTIES OF THE NON-PHOSPHORYLATIVE ELECTRON TRANSPORT BYPASS ENZYMES OF MYCOBACTERIUM PHLEI *

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The system from Mycobacterium phlei which couples phosphorylation to oxidation has been resolved into two components, a particulate and supernatant fraction (Brodie, 1959). Both fractions were required for restoration of oxidative phosphorylation. The particulate fraction was shown to contain the bulk of the respiratory carriers (Asano and Brodie, 1964) whereas the soluble fraction was shown to contain both oxidative enzymes and coupling factor proteins (Brodie, 1959; Asano and Brodie, 1963, 1964). Although NADH oxidation occurred with either fraction alone the nature of the electron transport sequence from NADH differed in the two fractions. The NADH oxidative pathway of the particles was found to be similar to that of mammalian mitochondria and was coupled to the synthesis of ATP. The soluble fraction oxidized NADH by a non-phosphorylative pathway.

The soluble enzymes which mediate the oxidation of NADH and NADPH are referred to as "bypass" enzymes since they transfer electrons directly to oxygen and thus bypass the particulate chain entirely, or re-enter the particulate chain at the cytochrome c region and thus bypass the electron carriers between NADH and cytochrome c. The loss of sites of phosphorylation and presence of these electrons transport bypass reactions in bacteria may both account in part for the low P:O ratios observed with bacterial systems. This is particularly evident with systems like

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M. phlei which have respiratory chains similar to those of mammalian mitochondria but nevertheless exhibit lower P:O ratios. Increased P:O ratios have been obtained with the system from Escherichia coli following the removal of the soluble bypass enzymes (Kashket and Brodie, 1963). The bypass enzymes found in M. phlei and E. coli appear to be similar to the pyridine nucleotide reductase described by Wosilait and Nason, 1954, and to DT-diaphorase of mammalian tissues (Ernster, et. al., 1960). The properties of two different bypass enzymes of the soluble fraction from M. phlei are presented in the present communication.

MATERIALS AND METHODS

Mycobacterium phlei, ATCC 354, was grown and cell-free homogenates prepared as previously described (Brodie and Gray, 1956 a). The crude supernatant fluid, obtained by centrifugation of the homogenate at 144,000 x g for 90 minutes was dialyzed at 2°C for 48 hours. The dialyzed fluid was treated with solid ammonium sulfate to 30 per cent saturation and centrifuged at 20,000 x g. The precipitate was discarded and the supernatant solution brought to 60 per cent saturation. The resulting precipitate was collected, dissolved in distilled water, and dialyzed against cold distilled water for 48 hours. The pH of the fluid was adjusted to 8.2 with M Tris-HCl buffer and the material fractionated on a DEAE-cellulose column (1.6 x 13 cm) which was previously buffered with Tris-HCl (pH 8.2). The column was washed with 10 ml of 0.01 M Tris (pH 8.2) and developed with increasing chloride concentration. The non-linear elution system consisted of a mixing chamber containing 250 ml of 0.01 M Tris buffer (pH 8.2) and a reservoir containing 250 ml of 0.35 N KCl in the same buffer. Fractions were collected in 15 ml aliquots.

NADH oxidation was measured spectrophotometrically by following the change in absorption at 340 mμ. The system consisted of 0.1 ml of DEAE fractionated supernatant fluid (0.25 to 0.4 mg of protein), 0.6 μmole of NADH, 100 μmoles of Tris-HCl buffer (pH 8.2) and water to a final volume of 1.5 ml. The cofactors and menadione were added at the concentrations indicated in the tables. The solution of menadione was prepared by the method of Wosilait (1960). The rate of reduction of endogenous cytochrome c of the particulate fraction by NADH was measured with a dual wavelength spectrophotometer at 551-540 mμ. Electron transport via the particulate

NAD^+-linked chain was blocked with amytal or 2-n-nonylhydroxyquinoline N-oxide (NHQNO).

RESULTS AND DISCUSSION

Ammonium sulfate fractionation of the supernatant fluid from M. phlei resulted in a purification of the factors necessary for restoration of oxidative phosphorylation with particles. Menadione reductase activity was found to be associated with the fractionated factors (Brodie and Gray, 1956 b ; Weber and Brodie, 1957). Further fractionation of the supernatant fraction on DEAE-cellulose has provided a means of separating this bypass activity from the components necessary for restoration of coupled phosphorylation with malate as an electron donor (Asano and Brodie; 1963). In addition, the chromatographic procedure resulted in the resolution of two distinct protein fractions which exhibited menadione reductase activity, figure 1. The two reductases were found to differ in their response to added cofactors, to menadione and in their sensitivity to p-CMB.

The first fraction, referred to as peak I (figure 1), was found to oxidize NADH

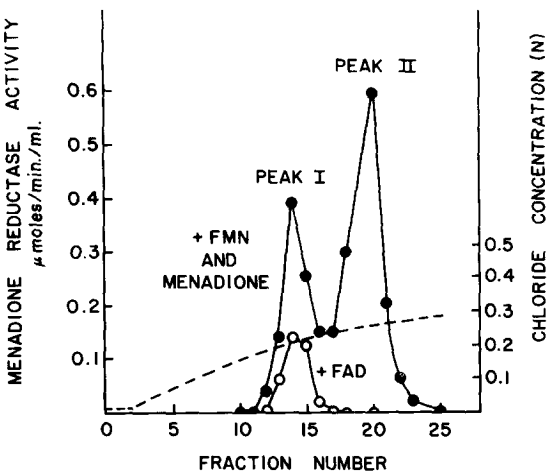

Figure 1. Separation of the Soluble Menadione Reductase from M. phlei by DEAE-Cellulose Chromatography. The conditions are described in Materials and Methods.

in the absence of menadione provided that either FMN or FAD was added to the assay system, Table I. The rate of oxidation of NADH with this enzyme and FMN

was 3 times greater than that observed with FAD. Further addition of menadione resulted in a 50 per cent increase in the rate of oxidation of NADH. In contrast, the reductase activity of the peak II required menadione for oxidation of NADH. This activity was increased 3.5 times by the addition of FMN and was not affected by FAD. A soluble component with absorption at 420 mμ was found in the supernatant fraction which appears to be non-heme iron. This material was reduced by NADH and oxidized by menadione or phenazine methosulfate. FMN was required for reduction of dye but not for reduction of this non-heme component. This component may be similar to the compound described by Sutton (1963) in extracts of M. phlei which was necessary for glucose-6-phosphate oxidation.

TABLE I

The Properties of Two Bypass Enzymes from M. phlei

Additions	Activity ($\Delta E_{340\ m\mu}$/min/mg protein)	
	peak I	peak II
None	0.0	0.0
FMN	3.52	0.20
FAD	1.10	0.0
Menadione	0.82	1.76
FMN + Menadione	6.35	6.40
FAD + Menadione	3.07	1.68

The system contained 0.02 μmole of either FMN or FAD and 0.1 μmole of menadione as indicated.

The two menadione reductases were further differentiated by their sensitivity to p-CMB, Table II. Greater sensitivity to the sulfhydryl agent was observed with the reductase from the peak I fraction. Inhibition with this enzyme by p-CMB was observed in the absence of menadione or with menadione and either FAD or FMN. The sensitivity of the non-phosphorylative oxidative pathway to p-CMB was previously noted (Brodie, 1959).

TABLE II

p-CMB Inhibition of the Reductases from M. phlei

Additions	p-CMB (M)	Per Cent Inhibition peak I	peak II
FMN + Menadione	3×10^{-4}	75	30
FAD + Menadione	3×10^{-4}	75	--
Menadione	3×10^{-4}	39	+ 18*

* Stimulation

Condition similar to those reported in Table I.

The significance of the "bypass" enzymes in cellular metabolism is not understood at present. A role for DT-diaphorase in extramitochondrial oxidation of NADH has been suggested by Conover, 1961 and Ernster, 1961; however, no natural mediator has been found. The enzyme from peak I fraction is of particular interest since it was found to transfer electrons in the absence of an artificial electron acceptor. Reduction of the endogenous cytochrome c of the parti-

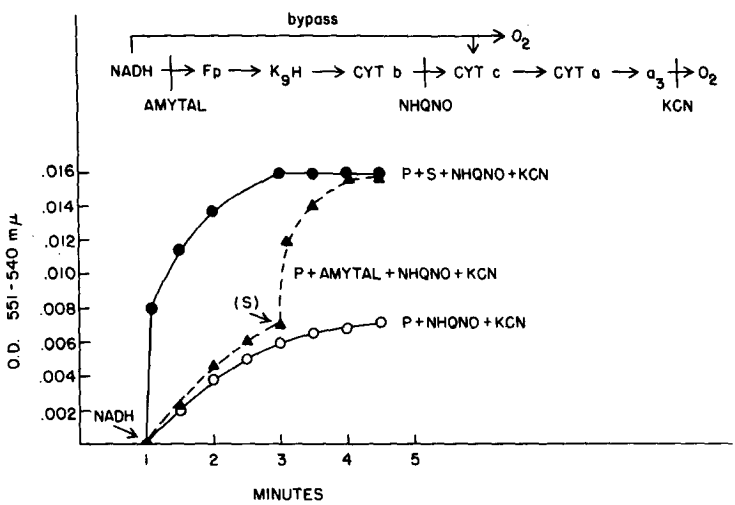

Figure 2. Reduction of endogenous cytochrome c by NADH with an Amytal and Quinoline N-oxide (NHQNO) blocked system.

The system consisted of 100 μmoles of Tris-HCl buffer (pH 7.2), 30 μmoles of $MgCl_2$, 100 μmoles of KCl, 0.3 ml of particles (P) (7.6 mg of protein), 5 μmoles of KCN, 20 μg of 2-n-nonylhydroxyquinoline N-oxide or 1 μmole of amytal as indicated and water to a final volume of 2 ml. In addition, the system contained ammonium sulfate fractionated supernatant fluid (S) as indicated (1.5 mg of protein) and the reaction started by the addition of NADH (0.6 μmole).

culate chain was previously shown to occur with NADH (Asano and Brodie, 1964) or NADPH (Murthy and Brodie, 1964) by a KCN insensitive non-phosphorylative pathway. In the presence of NHQNO or Amytal,agents which block the major respiratory chain of the particles before cytochrome c, reduction of endogenous cytochrome c occurred at a slow rate, figure 2. However, stimulation of this rate of reduction of the particulate cytochrome c occurred on addition of the fractionated supernatant material. Thus, the soluble enzyme can mediate the transfer of electrons from NADH to oxygen or in the presence of particles may enter the chain at the cytochrome c level as indicated in figure 2.

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