Pathways involved in cytochrome *c* reduction in *Mycobacterium phlei**

It has recently been shown[1] that coupled oxidative phosphorylation in sonically-disrupted *Mycobacterium phlei* cells required soluble supernatant factors in addition to the bacterial particles. It was also demonstrated that during fractionation procedures of the supernatant, the fractions which gave optimal P/O ratios when added to the bacterial particles always contained menadione reductase activity. On the basis of the report by WOSILAIT AND NASON[2] on the dinitrophenol-sensitivity of menadione reductase from *E. coli*, and the suggested involvement of vitamin K in oxidative phosphorylation by MARTIUS[3], it was of interest to study this and other electron transport enzyme systems which might be involved in oxidative and phosphorylative activities in *M. phlei*.

As can be seen in Fig. 1A, comparing curves I and II, when menadione was added to the crude lipid-free and particle-free supernatant, there was a marked stimulation in the rate of cytochrome *c* reduction. Dialysis of the crude supernate or fractionation with ammonium sulfate with subsequent dialysis resulted in a preparation which did not catalyze the reduction of exogenous cytochrome *c*. As seen in curve III, addition of menadione restored the ability of the enzyme to catalyze this reduction. The essentiality of reduced diphosphopyridine nucleotide (DPNH) in the reaction is shown in curve IV. Reduced triphosphopyridine nucleotide would not serve as electron donor in the reaction.

Attempts to reactivate the cytochrome *c* reductase, in a fractionated enzyme preparation, by the addition of a crude boiled extract revealed the presence of a relatively heat-stable enzyme.

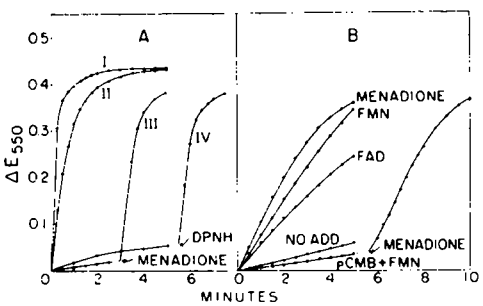

Fig. 1. The effect of menadione and flavins on cytochrome *c* reduction. A. The reaction mixtures indicated by the curves contained 500 μmoles K phosphate, pH 9.1, 0.2 ml 1.0% cytochrome *c* (Sigma), and the following in a total volume of 3.0 ml. Curve I: 0.4 μmole DPNH, 1.0 μmole menadione sodium bisulfite, and 0.05 ml crude enzyme. Curve II: same concentration of DPNH and enzyme, but no menadione. Curve III: 0.4 μmole DPNH, and 0.05 ml of crude dialyzed enzyme; 1.0 μmole menadione added at the arrow. Curve IV: 1.0 μmole menadione, and the same enzyme as in III; 0.4 μmole DPNH added at the arrow. All reactions were started with enzyme. B. All reaction mixtures, in a total volume of 3.0 ml, contained the same concentrations of phosphate and cytochrome *c* as in Fig. 1A, 0.4 μmole DPNH, 0.06 ml crude dialyzed enzyme, and as noted on the curves No Addition, and 0.03 μmole of either menadione, FMN, or FAD. 0.3 μmole pCMB was used as indicated. All reactions were started with DPNH after a 5 min incubation period of enzyme with either menadione, FAD, FMN, or pCMB + FMN.

After heating at 100° C for 5 and 12 min, 95 and 50% of the activity remained. It was subsequently found that the heat-stable property resided in the "menadione-dependent cytochrome *c* reductase".

Catalysis of cytochrome *c* reduction by the crude extract, which does not require but is stimulated by menadione, was inhibited with 10^{-4} *M* *p*-chloromercuribenzoate (pCMB). The addition of menadione reversed this inhibition. It was therefore suggestive that two enzymes were involved in catalyzing the reduction of cytochrome *c*. As shown in Fig. 1B, a dialyzed lipid-free crude supernatant preparation possessed negligible cytochrome *c* reductase activity in the absence of menadione as indicated by the curve "No Add.". Although flavin mononucleotide (FMN) was more effective than flavin adenine dinucleotide (FAD) in replacing menadione for restoration of cytochrome reductase activity, it was inhibited by pCMB. The reactivation by menadione, on the contrary, was not inhibited. Although not indicated in the figure, riboflavin could not replace FMN or FAD in the reaction. These results confirm the presence of two enzymes capable of catalyzing the reduction of cytochrome *c*: an FMN cytochrome *c* reductase, and a "menadione-dependent cytochrome *c* reductase". The reaction involving menadione for the reduction of cytochrome *c* can be expressed as follows:

(1) DPNH + H^+ + menadione → DPN^+ + menadione H_2

(2) menadione H_2 + 2 cyt. *c* Fe^{+++} → menadione + 2 cyt. *c* Fe^{++} + $2H^+$

Net: DPNH + 2 cyt. *c* Fe^{+++} → DPN^+ + 2 cyt. *c* Fe^{++} + H^+

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Reaction (1) is presumably catalyzed by a flavoprotein, since diaphorase activity, and the transfer reaction[4] (which involves a transfer of electrons from DPNH to the 3-acetyl pyridine analog of DPN) can be demonstrated in the ammonium sulfate fractionated and dialyzed extracts. At present it is not known whether reaction (2) is enzymic or non-enzymic in nature. Although, in soluble preparations, it is probably non-enzymic, the recent demonstration by COLPA-BOONSTRA AND SLATER[5] of a reduced menadione oxidase offers an alternative possibility. If reaction (2) proves to be non-enzymic, then the menadione reductase (reaction (1)) like Straub's diaphorase, catalyzes the reduction of menadione, which in turn reduces cytochrome *c*[6].

The possible role of these soluble enzymes in coupled oxidative phosphorylation in this organism will be discussed in a more detailed paper to follow.

MORTON M. WEBER
ARNOLD F. BRODIE

Department of Bacteriology and Immunology and Leonard Wood Memorial Laboratory, Harvard Medical School, Boston, Mass. (U.S.A.)

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The role of vitamin K_1 in coupled oxidative phosphorylation*

Roles for vitamin K analogues in electron transport[1,2,3] and in oxidative phosphorylation[4,5,6] have been suggested. Evidence will be presented in this communication that vitamin K_1 is an essential coenzyme necessary for coupled oxidative phosphorylation in extracts of *Mycobacterium phlei*. The bacterial system requires active subcellular particles[7,8] and the soluble enzymes of the supernatant which include menadione reductase[5].

Evidence that vitamin K_1 plays an essential role as a physiological electron carrier, and is a key compound in oxidative phosphorylation, now has been obtained in several ways: (1) restoration of oxidative phosphorylation in light-inactivated preparations by the addition of vitamin K_1; (2) the reconstitution of coupled phosphorylation by adding K_1 after extensive fractionation of the supernatant; and (3) the specific requirement for certain configurations which occur in vitamin K_1, but not in related compounds.

Exposure of isolated particles and active supernatant to light emitted at 3600 A (Gates Raymaster Lamp, tube B) results in a complete loss of oxidation and phosphorylation. Addition of vitamin K_1 suspended in the inactive lipids obtained from extracts after centrifugation[5] completely restores both oxidation and coupled phosphorylation (Table I).

Exposure of the lipid-K_1 suspension to light at 3600 A results in complete inactivation. Lipid-extractable material obtained from untreated (active) supernatants has an absorption spectrum similar to that of the K-like compounds. Furthermore, this exposure to light alters in a similar manner the characteristic absorption spectrum of the lipid-extractable material from supernatant and of vitamin K_1.

This requirement for vitamin K_1 can also be demonstrated in the presence of enzymes obtained by ammonium sulfate fractionation and in which restoration of activity previously required the addition of heated crude supernatant. Both oxidation and phosphate esterification are restored by the addition of vitamin K_1.

After exposure of both particle and supernatant to light, the bacterial system exhibits a specific dependence on vitamin K_1 for coupled oxidative phosphorylation. Lapachol methyl ester and vitamin K_1 diacetate, compounds which closely resemble vitamin K_1 in the R_2 and R_3 position of the naphthoquinone, show some activity. Vitamin K_2, menadione, menadione diphosphate, dimethyl naphthoquinone, phthicol, lapachol, quinone and α-tocopherol are inactive. The system reactivated with vitamin K_1 is uncoupled by dinitrophenol and dicumarol; it is not affected by antimycin A. Addition of riboflavin monophosphate (FMN) to the light-treated particles and supernatant restores oxidation but not the ability to esterify inorganic phosphate.

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