RESPIRATORY CHAIN LINKED FERRICYANIDE REDUCTION DRIVES ACTIVE TRANSPORT IN MEMBRANE VESICLES FROM *BACILLUS SUBTILIS*

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1. Introduction

Active transport of amino acids [1–4], mono-, di- and tricarboxylic acids [3,5,6] in membrane vesicles from *Bacillus subtilis* can be energized by electron transfer in the respiratory chain with oxygen as terminal electron acceptor. The best electron donors for the energization of transport are reduced β-nicotinamide-adenine-dinucleotide (NADH) and the artificial electron donor ascorbate-phenazine methosulphate (Asc-PMS) [1,2,4].

For the interpretation of experiments on integrated functions in membrane vesicles it is essential to have information about the orientation of the vesicle membrane with respect to that of intact cells. In our studies with membrane vesicles from *B. subtilis*, we have presented evidence by freeze-etch electron microscopy [7] and by studies on the localization of membrane-bound proteins [8] that essentially all membrane vesicles are oriented as the cytoplasmic membrane of whole cells. A similar conclusion was reached for membrane vesicles from *E. coli* [9–13]. However, other investigators concluded that a significant fraction of the vesicles are inverted or that enzymes which are localized at the inner side of the cytoplasmic membrane in intact cells move to the outside during the isolation of the vesicles [14–18]. An important line of evidence for this conclusion is based on measurements of the activity of membrane-bound dehydrogenases with the membrane-impermeable electron acceptor ferricyanide [14,15]. With this electron acceptor no dehydrogenase activity could be detected in either whole cells or spheroplasts from *E. coli*, unless permeability barriers were destroyed by toluene. However, in membrane vesicles, isolated with the lysozyme-EDTA procedure according to Kaback [19], approximately 50% of the total dehydrogenase activity was accessible to ferricyanide even before toluenization.

In this paper we will demonstrate in membrane vesicles from *B. subtilis*, that ferricyanide accepts electrons from cytochromes at the terminal oxidase-side of the respiratory chain and not at all from NADH dehydrogenases directly. Electron transfer from NADH to ferricyanide under anaerobic conditions can even generate the energy for active transport in membrane vesicles from this strict aerobic organism. Dehydrogenase activity measurements with ferricyanide, therefore, do not supply information about the localization of these enzymes in membrane vesicles or about the orientation of the vesicles.

2. Materials and methods

Bacillus subtilis W23 was grown at 37°C in a nutrient sporulation medium [20]. *B. subtilis aro D* (RB163) was kindly supplied by Dr H. W. Taber (Dept. of Microbiology, University of Rochester, Rochester, New York). This mutant was grown at 37°C in a medium containing 1.0% Bacto-Tryptose (Difco), 0.3% Beefextract (Oxoid Ltd., London SE 1, England) and 0.5% NaCl. Cells were harvested at the end of logarithmic growth and membrane vesicles were isolated as described previously [6,7]. The membrane vesicle preparations were stored in liquid nitrogen. In all experiments membrane vesicles were thawed quickly in a waterbath at 46°C; only samples which were frozen and thawed once were used.

Uptake experiments were performed as described

previously under aerobic [2,5,6] and anaerobic [21, 22] conditions. In all experiments L-[U-^{14}C] glutamate (The Radiochemical Centre, Amersham, Buckinghamshire, England) with a specific activity of 290 mCi/mmol was used at a concentration of 8.6 μM. In uptake experiments in the presence of ferricyanide, this electron acceptor was added 2 min before the addition of NADH (Boehringer, Mannheim, West Germany). The effects of respiratory chain inhibitors on the initial rates of transport were determined as described previously [2,5] with the exception that the effect of K-cyanide was studied in an incubation mixture of pH 7.0.

Oxygen consumption experiments were performed as described previously [5,8] in an incubation mixture which contained 0.1–0.2 mg membrane protein/ml.

The NADH-dependent reduction of ferricyanide was measured spectrophotometrically in a double beam spectrophotometer as described previously [8]. The reaction mixture contained in 50 mM K-phosphate (pH 7.2): 0.05–0.1 mg membrane protein/ml, 0.32 mM K-ferricyanide and 0.4 mM NADH.

Anaerobic reduction of respiratory chain components in membrane vesicles were measured with an Aminco-Chance split beam spectrophotometer at room temperature. The oxidized/oxidized base-line was adjusted for aerobic membrane suspensions (1.0 ml) containing 50 mM K-phosphate (pH 6.6) and 2–3 mg membrane protein/ml in both sample and reference beams. NADH (10 mM) was added to the sample cell and the spectrum was scanned until no further absorbance changes were observed, indicating that an anaerobic steady state had been reached. Subsequently NADH (10 mM) was added to the reference cell. When the reduced/reduced difference spectrum was equal to the baseline, ferricyanide (10 mM) was added to the sample cell. The oxidized/reduced difference spectrum was recorded when no changes in the spectrum could be observed (usually after 5 min.).

In all inhibition studies, the respiratory chain inhibitors (with the exception of K-cyanide) were dissolved in ethanol. Control experiments were performed in the presence of ethanol at the same concentration (< 1% v/v) and inhibition percentages were calculated with respect to these control values. Freshly prepared solutions of NADH, K-ferricyanide, and K-cyanide were used in all experiments.

3. Results

Membrane vesicles from *B. subtilis* W23 contain a high NADH-dehydrogenase activity and oxidation of NADH occurs via the respiratory chain at a high rate (table 1) [2,4,8]. In the presence of the electron acceptor ferricyanide, this oxidation rate is decreased by 35%. Under anaerobic conditions a high rate of NADH-dependent ferricyanide reduction is observed (table 1), indicating that ferricyanide functions as a very effective electron acceptor from NADH in *B. subtilis* membrane vesicles.

In the presence of NADH, membrane vesicles of *B. subtilis* W23 catalyze the active transport of L-glutamate very effectively (fig.1A). Supplementation of the reaction mixture with 10 mM ferricyanide has hardly any effect on the uptake of L-glu with NADH as electron donor. These results indicate that under aerobic conditions ferricyanide does not interfere with electron flow through the energy generating part of the respiratory chain, when NADH is the electron donor. Fig.1B shows the results of uptake experiments performed under anaerobic conditions. In the absence of an electron acceptor no stimulation of transport above endogenous levels is observed with NADH. In the presence of ferricyanide, however, a marked stimulation of uptake is observed with NADH. The initial rate of L-glu transport with NADH-ferricyanide under anaerobic conditions varies in different vesicle preparations between 30–100% of the rate obtained

Table 1
Rates of NADH-dependent oxygen consumption and NADH-dependent ferricyanide reduction by membrane vesicles from *Bacillus subtilis* W23 and *Bacillus subtilis aro D* (RB 163)

	Rate of NADH oxidation with the electron acceptor (nmoles NADH/mg membrane proteins × min.)	
Membrane vesicles isolated from Strain	Oxygen	Ferricyanide
W23	520	400
aro D (RB 163)	48	9
aro D (RB 163) + 200 μM menadione	826	845

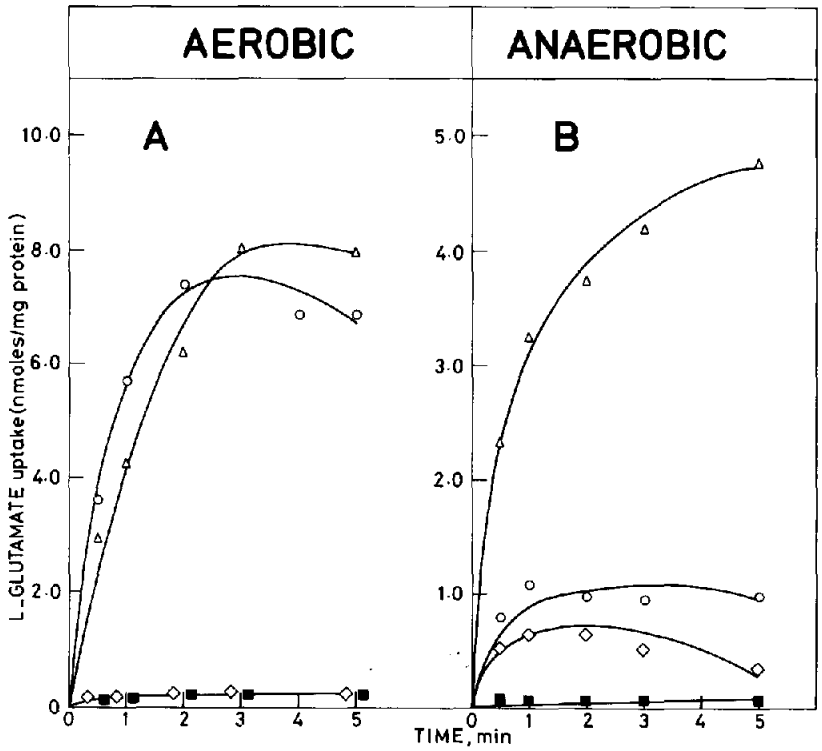

Fig. 1. Effect of K-ferricyanide on NADH-driven L-glutamate uptake under aerobic and anaerobic conditions by membrane vesicles from *B. subtilis W23*. (○) NADH (10 mM). (△) NADH (10 mM) with ferricyanide (10 mM). (■) No energy source added. (◇) Ferricyanide (10 mM).

with NADH under aerobic conditions. These observations strongly indicate that electrons from NADH are transferred to ferricyanide, partially at least, through the energy generating part of the respiratory chain.

In order to obtain more information about the site(s) of the respiratory chain from which ferricyanide can accept electrons, studies were performed with membrane vesicles from the menaquinone deficient mutant *B. subtilis aro D* (RB 163). As a consequence of the menaquinone deficiency these membrane vesicles oxidize NADH at a low rate. When membrane vesicles from this mutant are supplied with the menaquinone analogue, menadione (200 μM) a mared increase of the rate of NADH oxidation is observed and the rate of oxygen consumption is increased to a rate which is even higher than the rates obtained with membrane vesicles of *B. subtilis* W23 [6] (table 1). These results indicate that membrane vesicles of *B. subtilis aro D* (RB 163) contain a high activity of NADH-dehydrogenase. The results of transport experiments in membrane vesicles of this mutant are in agreement with these observations. In the presence of NADH a low stimulation of L-glu is observed and supplementation of the reaction mixture with menadione results in initial rates of transport which are higher than the rates obtained in membrane vesicles of *B. subtilis* W23 [6]. Similar results were obtained for the NADH-dependent ferricyanide reduction (table 1). Membrane vesicles of this mutant reduce ferricyanide in the presence of NADH under anaerobic conditions at a low rate and a marked increase of the rate of ferricyanide reduction is observed upon reconstitution with menadione. These observations demonstrate that ferricyanide does not accept electrons directly from NADH-dehydrogenase itself in the vesicle membrane and indicate that electrons are transferred to ferricyanide *via* other electron carriers of the respiratory chain. It is noteworthy in this

Table 2
Inhibition of L-glutamate transport, NADH oxidation and NADH-dependent ferricyanide reduction in membrane vesicles from *Bacillus subtilis* W23

Inhibitor	Concentration (mM)	L-Glu-uptake in the presence of		NADH oxidation with the electron acceptors	
		NADH	NADH-ferricyanide	Oxygen	Ferricyanide
		Percentage inhibition			
Rotenon	0.3	72	69	75	50
HOQNO	0.02	73	95	63	40
Antimycin A	0.2	n.d.	n.d.	74	80
	0.4	99	97	n.d.	n.d.
K-cyanide	20	51	65	65	15

n.d. = not determined

respect that ferricyanide is reduced at a high rate by the solubilized enzyme in the presence of NADH (A. Bisschop, H. Muilerman, W. N. Konings, unpublished observations).

Further information about the site at which ferricyanide accepts electrons is obtained from studies with respiratory chain inhibitors. The effects of known inhibitors of the respiratory chain of *B. subtilis* [22] on the rate of anaerobic NADH-ferricyanide driven transport and NADH-dependent ferricyanide reduction were compared with the effects on the rates of aerobic NADH-driven transport and the oxidation rate of NADH (table 2). Rotenone, *N*-heptyl-hydroxy-quinoline-*N*-oxide (HOQNO) and Antimycin A all inhibit the NADH-oxygen and NADH-ferricyanide dependent reactions. K-cyanide also inhibits strongly these reactions with the exception for the NADH-dependent ferricyanide reduction. At this moment no explanation can be offered for this observation. From these results it can be concluded that the site of coupling between the respiratory chain and ferricyanide lies after the inhibition site of Antimycin A i.e. cytochrome *b* and possibly before the inhibition site of cyanide i.e. cytochrome *a*, the terminal oxidase.

This conclusion is supported by direct measurements of the reduction of cytochromes in membrane vesicles from *B. subtilis* in the presence of NADH and NADH-ferricyanide. In the presence of NADH almost complete reduction of all cytochromes present in the membrane vesicles is observed (fig.2) [2]. Upon the addition of ferricyanide complete reoxidation occurs of the bands for cytochrome *b*, *c* and c_1 at 528 and 560 nm while the band for cytochrome *a* at 603 nm is reoxidized for 70–85%.

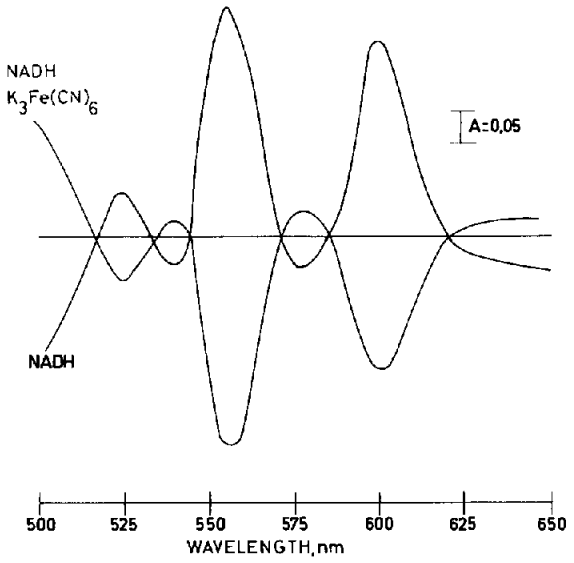

Fig.2. Effect of K-ferricyanide on the NADH-reduced cytochromes in membrane vesicles from *B. subtilis* W23. (NADH) Spectrum reduced by NADH (10 mM) versus oxidized spectrum (NADH-$K_3Fe(CN)_6$) Spectrum reduced by NADH (10 mM) versus spectrum after addition of NADH (10 mM) and ferricyanide (10 mM).

4. Discussion

Ferricyanide has been widely used as a membrane-impermeable electron acceptor. Since this compound is assumed to accept electrons directly from dehydrogenases it seems to be an ideal tool for measurements of the activity of dehydrogenases which are localized at the outside of membrane vesicles. Consequently, for dehydrogenases which are normally localized at the inner surface of the cytoplasmic membrane, ferricyanide can be used for the determination of the fraction of the vesicles which is inverted.

Ferricyanide is a redox dye with a midpoint potential (E'_0 (pH 7) of + 360 mV [24]) and might therefore function as an electron acceptor from respiratory chain intermediates as close to oxygen as cytochrome *a* [23]. A localization of such an intermediate at the outside of the membrane would lead to the reduction of ferricyanide in the presence of an electron donor. Such a localization of certain electron transfer intermediates is one of the essential features of the chemiosmotic coupling theory as postulated by Mitchell [25]. In a situation where electrons are transferred from a respiratory chain intermediate to ferricyanide, ferricyanide reduction experiments do not supply information about the localization of membrane-bound dehydrogenases.

The experiments described in this paper demonstrate that such a situation exists in membrane vesicles from *B. subtilis*. In the presence of ferricyanide NADH catalyzes under anaerobic conditions active transport of amino acids in membrane vesicles from *B. subtilis*. Only right-side-out membrane vesicles can perform active transport processes and therefore it can be concluded that ferricyanide accepts electrons from respiratory chain intermediate(s) located beyond the energy coupling site(s) in right-side-out oriented vesicles. The stimulation of transport under anaerobic conditions with NADH-ferricyanide varies in different vesicle preparations. At this moment no explanation can be offered for this observation.

The results of the experiments with the menaquinone mutant *B. subtilis aro D* (RB163) demonstrate that electrons are hardly accepted from NADH-dehydrogenase in the vesicle membrane directly. However, ferricyanide functions as an effective electron acceptor from the solubilized enzyme. This strongly supports previously obtained evidence for a localization of NADH-dehydrogenase at the inner side of the membrane in these right-side-out oriented vesicles.

After restoration of the NADH-oxidase activity in the vesicles of the menaquinone mutant electron transfer from NADH to ferricyanide occurs, which again is an indication for electron transfer to ferricyanide via a terminal part of the respiratory chain. The studies with respiratory chain inhibitors on NADH-ferricyanide dependent functions and the spectral studies on the effects of ferricyanide on the reduction of the cytochromes in the presence of NADH indicate that the site of coupling with ferricyanide lies, most likely, just before the terminal oxidase, cytochrome *a*. According to the scheme of the respiratory chain, proposed by Miki [23], this means that cytochrome c_1 is exposed to the outside of the membrane.

In a previous publication [8] it was demonstrated that an intermediate of the respiratory chain before the energy coupling site(s) is exposed to the outside of the vesicles of *B. subtilis*. This observation, together with the data presented in this paper, indicates that respiratory chain intermediates are arranged alternately at the inner side and the outer side of the membrane, as was proposed by Mitchell [25].

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