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Relation Between Reduced Nicotinamide Adenine Dinucleotide Oxidation and Amino Acid Transport in Membrane Vesicles from *Bacillus subtilis*

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The rate of reduced nicotinamide adenine dinucleotide (NADH) oxidation by membrane vesicles from *Bacillus subtilis* W23 increases three- to fourfold during logarithmic growth, reaching maximal levels in early stationary phase. Initial rates of L-proline, L-alanine, and L-glutamate transport energized by NADH closely parallel the increase in NADH oxidation. In vesicles prepared at different stages of growth, a constant number of NADH molecules varying from 150 to 260 have to be oxidized to transport one molecule of amino acid. Membrane vesicles from *B. subtilis aroD* (strain RB163), a mutant defective in menaquinone synthesis, do not transport amino acids in the presence of NADH. Ascorbate plus phenazine methosulfate, however, energizes amino acid transport equally well as in vesicles of *B. subtilis* W23. NADH oxidation and NADH-driven amino acid transport can be restored instantaneously by the addition of menadione (vitamin K_3).

For the study of the mechanism of active transport through the bacterial cytoplasmic membrane, a model system was developed by Kaback, consisting of isolated membrane vesicles (8). Membrane vesicles from a wide variety of microorganisms (11, 19, 25) retain membrane functions such as active transport of amino acids, sugars, and other metabolites (1, 10, 18), oxidation of several substrates, and phospholipid biosynthesis (25, 26).

In vesicles of the gram-positive organism *B. subtilis*, transport of amino (11, 14, 15, 18) and mono-, di-, and tricarboxylic acids (A. Bisschop and W. N. Konings, manuscript in preparation) is coupled to the oxidation of electron donors via respiratory chain-linked electron transport. In addition to the physiological electron donors NADH and L-α-glycerophosphate, artificial electron donors (e.g., reduced 5-*N*-methyl phenazonium methyl-sulfate [PMS] and 5-*N*-methyl phenazonium-3-sulfonate [W. N. Konings, manuscript in preparation]) are also effective in energizing transport of these metabolites (11, 14, 15). In the course of our studies, we have observed that membrane vesicles prepared from *B. subtilis* W23 grown on the same medium, considerably differed in transport activities for amino acids and dicarboxylic acids and also in oxidation rates of several substrates. It also was observed that during vegetative growth the concentration of several cytochromes increased markedly in membranes of *B. subtilis* (2, 27) and consequently the rate of oxygen consumption did also. In two strains of *B. subtilis*, menaquinone-7, a membrane-associated component of the cytochrome-linked electron transport chain, also reaches maximal cellular concentrations during early stationary phase, and these changes closely parallel the changes in the cytochrome content of the cytoplasmic membrane (5).

As presented in this paper, we investigated the correlation between the rate of NADH oxidation and the rate of amino acid transport.

MATERIALS AND METHODS

Cell growth and membrane isolation. *B. subtilis aroD* (RB163) cells were grown on a tryptone medium (12). This mutant strain, defective in menaquinone synthesis (4, 5), was kindly donated by H. W. Taber. Membrane vesicles were prepared from these cells at the end of logarithmic growth. *B. subtilis* W23 cells were grown on nutrient sporulation medium (6) in a Microferm laboratory fermenter (New Brunswick Scientific Co., Inc., New Brunswick, N. J.) under vigorous aeration at 37 C. The *B. subtilis* W23 culture was inoculated at an absorbancy at 663 nm (A_{663}) of 0.0003 with overnight nutrient sporulation medium-grown cells, and growth was followed turbidometrically. At different stages of growth (see Fig. 1), 0.4 to 1.5 liters was sampled, cells were harvested by centrifugation, and membrane vesicles were prepared by the isolation procedure described previously by Kon-

ings et al. (13). Protein concentrations were measured according to Lowry's method (16). The membrane vesicles have a low endogenous respiration and amino acid transport activity and are free of intact cells and spores as checked by phase contrast microscopy. No significant differences in behavior with respect to lysis and lysozyme treatment could be observed between the individual samples. Under the culture conditions employed, few refractile spores were formed (less than 1% of the total viable count) as judged by phase contrast microscopy and counts of heat-resistant particles. The final membrane preparations were free of spores and whole cells.

Counting of heat-resistant spores. The heat-resistant particle fraction of the total viable count was determined by plating on nutrient sporulation medium agar plates a sample of cells that were exposed for 15 min at 75 C.

Transport studies. Amino acid transport experiments were performed as described previously (11, 15, 18) with 0.03 to 0.09 mg of membrane protein in a final incubation volume of 50 μliters at 25 C. Amino acid transport assays in intact *B. subtilis* W23 cells were performed at 37 C in the same way as described for membrane vesicles. Initial rates of transport were calculated from the amino acid uptake after 15 s in cells that were washed twice with 0.1 M K-phosphate (pH 6.6) at room temperature. The filters were dried at 105 C immediately after the transport assay.

The effect of inhibitors on the initial rates (1 min) of transport in membrane vesicles was studied by the addition of these inhibitors just before 2-min preincubation of the membrane vesicles. KCN, however, was added just before the addition of ^{14}C-labeled amino acid to prevent the escape of HCN from the mixture. Control values were determined in the absence of inhibitors but in the presence of the same amount of solvent in which the corresponding inhibitor was dissolved. Rotenone and 2-heptyl-4-hydroxyquinoline-*N*-oxide were dissolved in dimethylsulfoxide; antimycin A was dissolved in ethanol; NaN_3 and KCN were dissolved in water; and inhibitors were added to the reaction mixture in small volumes (5 μliters).

O_2 consumption measurements. O_2 utilization of membrane vesicles preparations was measured polarographically with a Clark-type oxygen electrode (YSI, model 53 Yellow Springs Instruments Co., Yellow Springs, Ohio) connected to a Servogor recorder (model RE 511) as described previously (18).

NADH dehydrogenase (reduced nicotinamide adenine dinucleotide:ferricyanide oxidoreductase, EC 1.6.99.2) was measured by following the anaerobic reduction of ferricyanide at 420 nm with a Perkin-Elmer 124 double-beam spectrophotometer connected with a Hitachi QPD 33 recorder. The reaction mixture of 3 ml contained 0.16 mM $K_3Fe(CN)_6$, 50 mM potassium phosphate (pH 6.6) and 10 mM $MgSO_4$. NADH (0.2 mM final concentration) was added and the endogenous rate was followed for several minutes. The reaction was started by the addition of 0.23 mg of membrane protein. The rate was extrapolated from initial velocity, and for final calculations an extinction coefficient of 1.0 $mM^{-1}\ cm^{-1}$ was used (3).

Restoration of NADH oxidation and NADH-stimulated amino acid transport. NADH oxidation and NADH-driven amino acid transport in membrane vesicles of *B. subtilis aroD* (RB163) (4) was measured in the absence and in the presence of 0.1 mM menadione (vitamin K_3).

Materials. All ^{14}C-labeled amino acids, uniformly labeled, were purchased from the Radiochemical Centre (Amersham, Buckinghamshire, England). Specific activities of L-proline, L-alanine, and L-glutamic acid were 290, 173, and 265 mCi/mmol respectively. Nutrient broth was a product of Difco (Difco Laboratories, Detroit, Mich.); β-nicotiamide adenine dinucleotide (NADH) was from Boehringer (Mannheim, Germany); PMS, and $K_3Fe(CN)_6$ were from BDH Chemicals Ltd. (Poole, England); and 2-heptyl-4-hydroxyquinoline-*N*-oxide, antimycin A and 2-methyl-1,4-naphtoquinone (menadione) were from Sigma Chemical Company (St. Louis, Mo.). Ribonuclease (RNase) and deoxyribonuclease (DNase) were products of Miles Laboratories Ltd. (Berkshire, England). Rotenone was obtained from Aldrich-Europe (Beerse, Belgium). Other chemicals were purchased from E. Merck (Darmstadt, Germany).

RESULTS

During the growth cycle of *B. subtilis* W23, the rate of amino acid uptake per milligram of membrane protein changes considerably. The initial rate of L-glutamate uptake per milligram of protein increases almost threefold during logarithmic growth and decreases again when the culture enters the stationary phase (Fig. 1). To investigate whether these changes are due to changes in the energy-supplying membrane-bound respiratory chain or in the number of transport carrier proteins or both, the oxidation rate of NADH and the initial rates of amino acid transport were studied in membrane vesicles isolated during different stages of growth (Fig. 1).

NADH oxidation. Membrane vesicles of *B. subtilis* oxidize NADH via a membrane-bound respiratory chain. The rate of NADH-oxidation is used as a standard for the number of functional respiratory chains in the membrane. During growth the rate of NADH oxidation changes considerably and a three- to fourfold increase is observed during logarithmic growth. Maximal rate of NADH oxidation is reached in early stationary phase, after which the activity remains constant (Fig. 2). The rate of NADH oxidation is linear with the protein concentration for each membrane preparation up to 0.7 mg of membrane protein per ml, indicating that the observed differences are not due to differences in the protein content between the individual preparations.

Qualitatively similar results are obtained with the non-physiological electron donor sys-

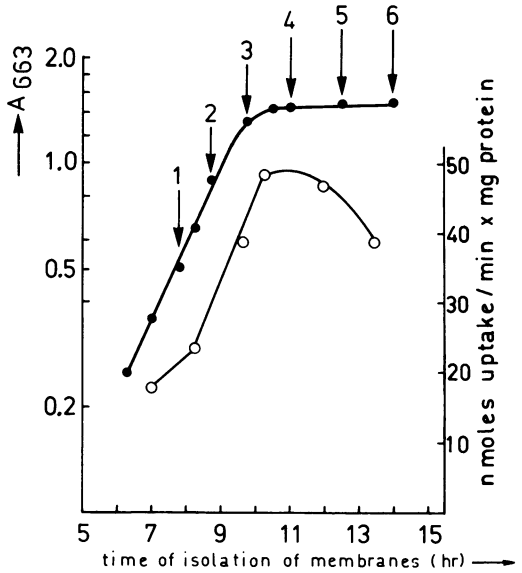

FIG. 1. *Growth curve of B. subtilis and initial rate of* L*-glutamate transport. Symbols:* (●) *growth curve of B. subtilis W23 on nutrient sporulation medium. Arrows indicate the culture densities at which membrane vesicles were prepared;* (○) *initial uptake rates of* L*-glutamate (1.89 × 10⁻⁵ M) in intact cells of B. subtilis at different stages of growth. Uptake experiments were performed as described in text. Protein concentration: 0.1 to 0.12 mg/ml.*

tem, ascorbate plus phenazine methosulfate (Asc-PMS). Reduced PMS is auto-oxidizable, but it also donates electrons via the respiratory chain to oxygen (14). The fraction of reduced PMS oxidized via the respiratory chain increases during logarithmic growth in a similar way as the NADH oxidation rate. The high rate of auto-oxidation of reduced PMS, however, does not enable accurate determinations of the Asc-PMS oxidation via the respiratory chain.

NADH-driven amino acid transport. Membrane vesicles of *B. subtilis* catalyze active transport of amino acids in the presence of NADH or Asc-PMS (11, 12, 14, 15, 18). The initial rates of transport of three amino acids (e.g., L-proline, L-alanine, and L-glutamic acid) which are transported by three independent systems (15) were investigated in the different vesicle preparations (Fig. 3). During logarithmic growth, a three- to fourfold increase in NADH-dependent transport activity of each amino acid occurs after which the transport rates remain constant. A similar increase in amino acid transport activity during exponential growth is observed with Asc-PMS as energy source (data not shown).

Efficiency of NADH-driven amino acid transport. The observations made in Fig. 2 and 3 suggest that a positive correlation exists between NADH oxidation and NADH-driven amino acid transport. For each membrane preparation the number of NADH molecules that are oxidized in order to transport one molecule of amino acid is determined (Fig. 4). The efficiency of the NADH oxidation in energizing transport varies for the different amino acids, but for each amino acid the efficiency is constant during all stages of growth. To transport one molecule of proline, the oxidation of 260

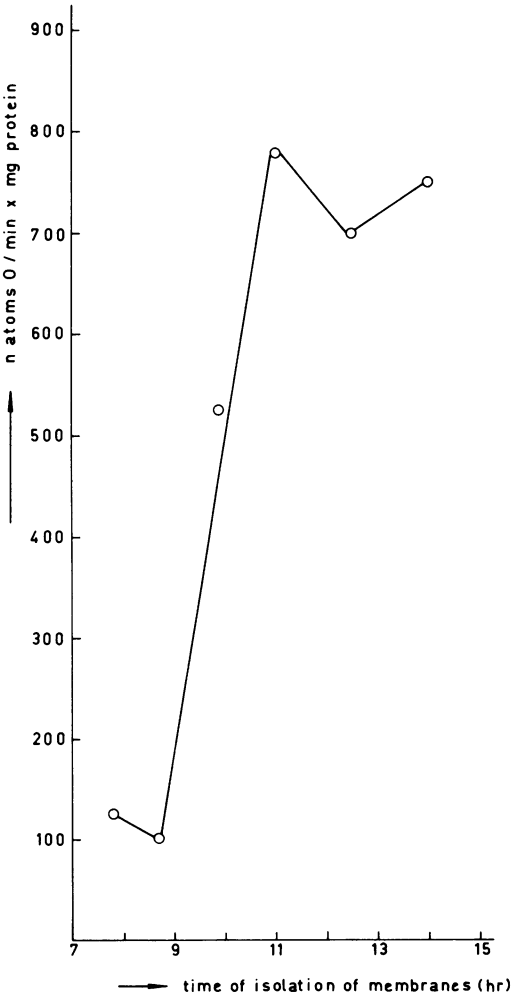

FIG. 2. *Oxidation rate of NADH at 25 C by membrane vesicles of B. subtilis, isolated at different stages of growth. NADH concentration was 10 mM and membrane protein concentrations were 0.1 to 0.3 mg/ml. The experimental points are determined in the vesicles preparations indicated in Fig. 1.*

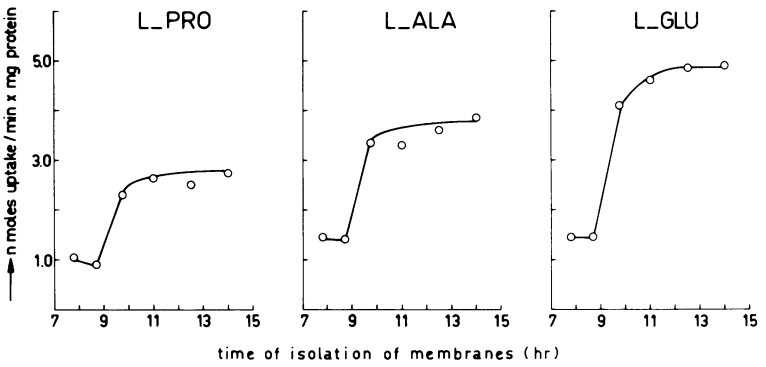

FIG. 3. *Initial uptake rates of* L-*proline,* L-*alanine, and* L-*glutamate by membrane vesicles isolated at different stages of growth. The experimental points are determined in the vesicles preparations indicated in Fig. 1. Concentrations: NADH, 20 mM;* L-*proline, 1.72* $\times$ 10^{-5} *M;* L-*alanine, 2.89* $\times$ 10^{-5} *M;* L-*glutamate, 1.89* $\times$ 10^{-5} *M.*

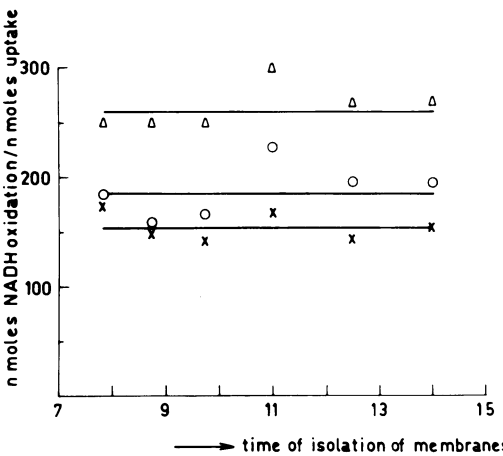

FIG. 4. *Number of molecules of NADH oxidized per transported molecule of* L-*proline* (Δ), L-*alanine* (O), *and* L-*glutamate* (×) *by membrane vesicles isolated from cells at different growth stages (see Fig. 1).*

molecules of NADH is required; for L-alanine 180 molecules must be oxidized and for L-glutamate 150 molecules are required.

Inhibition of oxidation and transport. To obtain more insight in the pathway of NADH oxidation and NADH-driven transport, the effects of respiratory chain inhibitors on these activities were studied. NADH oxidation can occur in *B. subtilis* via two routes: either from the flavoprotein N via cytochrome *b*-560, cytochrome *c*-554, and cytochrome *c*-550, to the terminal oxidase, or via flavoprotein N directly to cytochrome *c*-554 or to cytochrome *c*-550 and then to the terminal oxidase, the so-called "bypass" (20). Antimycin A specifically blocks electron flow between cytochrome *b*-560 and cytochrome *c*-554, and consequently does not effect NADH oxidation via the bypass (21). Other respiratory chain inhibitors block electron flow in both routes. The data presented in Table 1 demonstrate that antimycin A inhibits NADH oxidation and NADH-energized transport to different extents at different growth stages, indicating that less NADH is oxidized via the bypass in stationary phase than in logarithmic phase. Other electron transfer chain inhibitors inhibit in all samples to the same extent. Sodium azide inhibits L-glutamate transport rate significantly (58 to 65%), while the oxidation of NADH is only slightly inhibited (14 to 16%).

Restoration of NADH oxidation and NADH-driven amino acid transport in membrane vesicles of a menaquinone-less mutant. Farrand et al. (4) isolated *B. subtilis aroD* (RB163), a mutant defective in menaquinone synthesis, and showed that this mutant oxidizes NADH at a low rate when grown in the absence of skihimic acid, tryptophan, and histidine (5). Consequently, membrane vesicles of this mutant, isolated from cells at the end of logarithmic growth, oxidized NADH at a low rate, although a high NADH-dehydrogenase activity is present in these vesicles (Table 2). In agreement with this observation, the rate of NADH-driven amino acid transport is low (Fig. 5A). However, a high rate of amino acid transport occurs in the presence of Asc-PMS, indicating that menaquinone is required for NADH oxidation, but not for Asc-PMS oxidation via the respiratory chain. Analogous results were obtained by H. R. Kaback (unpublished experiments).

NADH oxidation in these membrane vesicles can be restored instantaneously by the addition of 0.1 mM menadione (vitamine K_3) and a 10-fold-higher NADH oxidation rate is obtained. Upon restoration of NADH oxidation,

TABLE 1. *Effect of metabolic inhibitors on NADH oxidation and initial rate of NADH-driven L-glutamate transport*

Inhibitor	Concn (mM)	Inhibition (%)					
		Sample[a] 1		Sample 4		Sample 6	
		Transport	Oxidation	Transport	Oxidation	Transport	Oxidation
Antimycin A	0.4	60	59	94	78	94	91
Rotenone	0.3	70	79	82	79	75	71
HOQNO[b]	0.03	75	88	84	91	88	81
NaN_3	10	64	14	65	14	58	16
KCN	10	56	67	55	53	70	68

[a] Sample numbers correspond with numbers indicated in Fig. 1.
[b] HOQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide.

TABLE 2. *NADH oxidation rates[a] and NADH dehydrogenase[b] activity in membrane vesicles of B. subtilis strains aroD (RB163) and W23*

Membrane vesicles of:	Enzyme activity	nmol of NADH/min × mg of membrane protein
B. subtilis aroD (RB 163)[c]	NADH dehydrogenase	194
	NADH oxidation	48
	NADH oxidation in the presence of menadione	490
B. subtilis W23[d]	NADH oxidation	195
	NADH oxidation in the presence of menadione	252

[a] Oxidation rates were determined with 10 mM NADH in the presence or absence of 0.1 mM menadione.
[b] Determined with ferricyanide assay as described in text.
[c] Cells were harvested at end of logarithmic growth (A_{660} = 1.25).
[d] Cells were harvested in mid-log phase (A_{660} = 1.0).

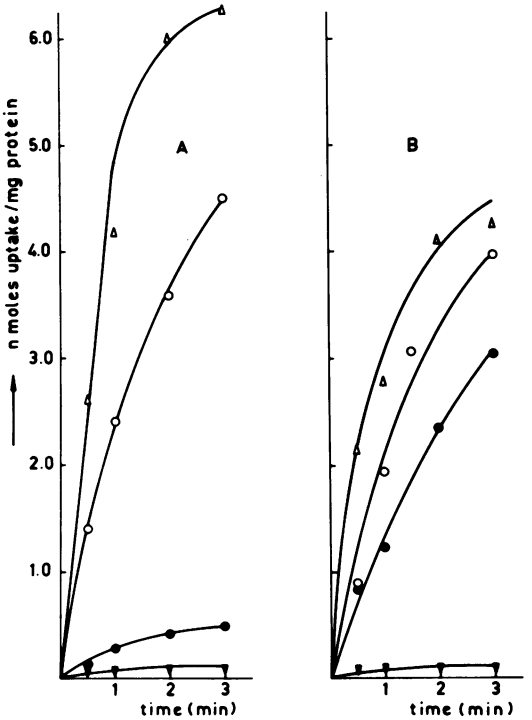

FIG. 5. *L-Glutamate uptake (1.89 × 10^{-5} M) by membrane vesicles from (A) B. subtilis aroD (RB163) and from (B) B. subtilis W23, in the presence of (Δ) ascorbate (10 mM) plus PMS (100 μM); (●) NADH (20 mM); (○) NADH (20 mM) + menadione (0.1 mM); (▼) no energy source added.*

NADH-driven L-glutamate transport is also restored (Fig. 5A) with a 10-fold-higher initial rate; hardly any stimulation of Asc-PMS-driven L-glutamate transport is observed. As expected, addition of 0.1 mM menadione to membrane vesicles of *B. subtilis* W23 grown under the same conditions as the mutant cells results in only a slight stimulation of NADH-driven L-glutamate transport (Fig. 5B).

DISCUSSION

The data presented clearly indicate that in a growing *B. subtilis* culture a considerable increase in NADH oxidation occurs during the transition from logarithmic phase to stationary phase, suggesting an increase of functional electron transport chains per milligram of membrane protein. This observation is in agreement with studies of Tochikubo (27) and Chaix and Petit (2) concerning the cytochrome content of *B. subtilis* at various stages of growth. During logarithmic growth also, considerable changes in the rate of amino acid transport occur.

Qualitatively similar changes are observed in membrane vesicles. Furthermore, the rate of amino acid transport, via three distinct transport systems energized by NADH, closely parallels the changes in oxidation rates by the membrane vesicles. It is very unlikely that the differences are due to variations in the isolation procedure, because the isolation method employed is simple, gentle, and standardized. These changes in NADH oxidation rate and NADH-driven transport are most likely not due to early sporulation events, because the growth conditions employed resulted in a low rate of sporulation and heat-resistant spores were first observed at T_3. Furthermore, during stationary phase no changes in NADH oxidation and NADH-stimulated transport were observed.

Transport activity can be varied in principle by variations of: (i) the energy supply (e.g., respiratory chain); (ii) the carrier protein content; and (iii) both energy supply and carrier protein content simultaneously. The constant efficiency of NADH in energizing amino acid transport in *B. subtilis* membrane vesicles most simply can be explained by a regulation at the level of the energy-generating system: the electron transport chain. This implies the presence in the cytoplasmic membrane of more amino acid carrier protein than can be energized by electron flow. The observation that *E. coli* membrane vesicles contain β-galactoside carriers, the M proteins, more than seven- to eightfold relative to D-lactate dehydrogenase (24) suggests that such a regulation is very likely. However, our data do not allow one to distinguish between the alternative possibilities; such a distinction can only be made when a method for the determination of the number of specific carrier proteins per milligram of membrane protein is available.

Most respiratory chain inhibitors inhibit the rate of oxidation to an extent similar to the rate of amino acid transport (Table 1). Antimycin A inhibits, according to Miki et al. (20, 21), only electron transfer from NADH via cytochrome *b* to the terminal oxidase and not via the bypass, while other inhibitors like cyanide, an inhibitor of the terminal oxidase, inhibit electron transfer in both pathways. This suggests that NADH oxidation via both pathways is equally effective in energizing transport. Sodium azide inhibits electron flow only slightly but inhibits L-glutamate transport to about the same level as cyanide. This suggests that azide does not directly effect electron flow but behaves as an uncoupler (9). It was already shown before (11) that uncouplers are very effective inhibitors of amino acid transport in membrane vesicles.

The oxidation of NADH requires the presence of menaquinone (21). In membrane vesicles lacking menaquinone, NADH oxidation is strongly limited, and addition of vitamin K_3 to these vesicles strongly stimulates NADH oxidation and also to the same extent the NADH-driven L-glutamate transport (Fig. 5A). Some stimulation of NADH-driven amino acid transport upon the addition of menadione is also observed in vesicles of *B. subtilis* W23 (Fig. 5B). This stimulation is possibly due to a restoration of menaquinone which is lost during the isolation procedure. MacLeod et al. found that in vesicles of *Bacillus licheniformis* NADH stimulation of L-glutamate transport was destroyed by exposure to light of 360 nm and addition of vitamin $K_{2(5)}$ or $K_{2(10)}$ restored this activity (17).

To transport one molecule of amino acid, 150 to 260 molecules of NADH have to be oxidized; this seems to be a very inefficient process. According to the redox model of Kaback (9), this means that only one out of 150 to 260 respiratory chains is coupled to a specific amino acid carrier. According to the chemiosmotic coupling theory of Mitchell (23), this means that only a small fraction of the protons ejected upon the respiration actually are used to drive transport of the amino acid present and that the majority of the protons flow back into the interior of the vesicles via other carriers in the absence of substrate (or via Ca^{2+}-Mg^{2+} adenosine triphosphatase although the vesicles do not perform oxidative phosphorylation) or that the experimental conditions allow a rapid dissipation of the proton motive force. Hampton and Freese (7) explained the inefficiency of NADH-driven amino acid transport by the presence of open or inverted membrane vesicles which oxidzie NADH but do not participate in amino acid uptake. However, it has been shown by freeze-etch electron microscopy that the gentle isolation procedure of the vesicles employed in this investigation (13) results in right-side out vesicles. Moreover, it was shown that no differences could be observed in the localization of several enzymes between membrane vesicles and the cytoplasmic membrane of intact cells (W. N. Konings, manuscript in preparation).

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