The increase of KCN-sensitive electron flow in the branched respiratory chain of *Micrococcus luteus* grown slowly in carbon-limited culture

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The strictly aerobic bacterium *Micrococcus luteus* was grown in the presence of lactate as sole carbon source under conditions of excess substrates (in batch culture) or under strict lactate (C) or ammonium (N) limitation (in a chemostat, $D = 0.02 \text{ h}^{-1}$). KCN (2 mM) stimulated the respiration of batch-grown and N-limited cells, and inhibited the oxidase activity of C-limited cells by 40–60%. The content and composition of cytochromes and the KCN-dependences of the oxidase activities were found to be the same for the membranes of C-limited and batch-grown cells. The KCN sensitivity of the oxidase activities of isolated membranes decreased in the order $\text{NADH} > \text{malate} > \text{lactate}$. NAD-dependent lactate and malate dehydrogenase activities were observed in the cytoplasm of C-limited cells but not in that of batch-grown cells. The stimulation of cell respiration by lactate, malate, pyruvate or ethanol caused a marked increase of the steady-state level of NAD^+ reduction only in C-limited cells. It is assumed that the slow growth of C-limited cells is accompanied by an increase in the formation of NADH, which is oxidized by the KCN-sensitive, tightly-coupled, main branch of the *M. luteus* respiratory chain. Under non-limited conditions of growth the respiration is due mainly to the direct oxidation of lactate via the weakly coupled alternative branch. The role of this switching of the electron flow from one pathway to the other during the adaptation of the bacteria to the slow C- and energy-limited growth is discussed.

Introduction

The transition of bacterial cultures from non-nutrient-limited, fast growth to slow, oligotrophic growth under conditions of substrate deficiency is a very common occurrence in natural conditions. In these cases the cells can adapt to the new conditions by changing their morphology (Morita, 1990), enzyme systems and metabolic pathways (Harder & Dijkhuizen, 1983). In particular, differences in the initial steps of the transformation of the substrate for energy generation under conditions of substrate excess versus those of substrate limitation have been observed in *Aerobacter aerogenes* (*Klebsiella pneumoniae*), *Pseudomonas aeruginosa* (Matin, 1981) and *Lactobacillus casei* (Harder & Dijkhuizen, 1983). However, little attention has been paid to the terminal stages of substrate transformation (respiratory substrate oxidation and oxidative phosphorylation) during this transition. Many bacteria have branched respiratory chains, with several terminal oxidases (Anraku, 1988). This branching could serve for such metabolic adaptation,

permitting a redistribution of electron flux and regulating energy supply to the cell (Tempest & Neijssel, 1984).

In the work described in this paper, we compared the respiratory chain composition and function of different respiratory branches in the strictly aerobic bacterium *Micrococcus luteus* cultured under conditions in which the energetic substrate is in excess, and under substrate limitation with very slow growth, more closely representing cells that one would find in the environment. The data obtained show the different contributions of the main and the alternative branches to the total respiration under C-limited and C-sufficient conditions.

Methods

Growth conditions. *Micrococcus luteus* (strain Flemming 2665) was grown in a medium composed of 0.4% NH_4Cl , 0.4% KH_2PO_4 , 0.001% biotin, 0.002% methionine, 0.004% thiamin, 1 mg $\text{FeSO}_4 \text{ l}^{-1}$, 70 mg $\text{MgSO}_4 \text{ l}^{-1}$, 0.02 mg $\text{CuSO}_4 \text{ l}^{-1}$, 0.5 mg $\text{MnCl}_2 \text{ l}^{-1}$ and 1% DL-lactate, pH 7.5 (adjusted with NaOH) in a 180 ml fermenter at 30 °C. The stirring speed was 300 r.p.m. The concentration of dissolved oxygen was not less than 80% of maximal saturation. Cells were harvested at the middle of the exponential growth phase (batch cells). For chemostat culture the same medium was used but with 0.2% lactate (C limitation)

Abbreviation: NQNO, 2-n-nonyl-4-hydroxyquinoline-N-oxide.

or with 0.01% NH_4Cl (N limitation). The medium was supplied to the same fermentation vessel (continuously, not by drops) with a Pharmacia P-3 pump at a constant rate of 4 ml h^{-1} (dilution rate, $D = 0.02 \text{ h}^{-1}$). The medium outflow from the chemostat was controlled by the same pump. Cells from chemostat were harvested after 5–6 volume changes ('limited cells').

Preparation of membrane and cytoplasmic fractions. Harvested cells were washed with 50 mM-potassium phosphate buffer, pH 7.4, by centrifugation. The pellet was suspended in buffer I, consisting of 40 mM-HEPES, 5 mM- MgSO_4 , pH 7.5, and incubated with lysozyme (1 mg per g wet cells) for 30 min at 25°C with a few crystals of DNAase. Membranes were sedimented by centrifugation at $22000 g$ for 30 min and the membrane pellet was suspended in buffer I (approx. $10 \text{ mg protein ml}^{-1}$). The cytoplasmic fraction (supernatant) was immediately used for testing.

Polarographic measurements. Oxygen consumption rates of cells and membranes were measured by a Clark oxygen electrode at 30°C . Reactions were carried out in 1 ml buffer I (for membranes). Cell respiration and its KCN sensitivity were analysed in 1 ml of material from the fermenter. The time period between cell withdrawal and analysis did not exceed 1 min.

Determination of cytochrome concentration. Difference spectra were recorded at 77 K with a Hitachi-557 spectrophotometer using 0.3 cm pathlength cuvettes. Each sample contained a membrane suspension in buffer I with 0.25 M-sucrose. Cytochromes were oxidized with potassium ferricyanide (5 mM) and reduced with sodium dithionite (a few crystals). CO-difference spectra were obtained as described by Tikhonova *et al.* (1981). The cytochrome content was calculated using the molar absorption coefficients ($\text{cm}^{-1} \text{ mM}^{-1}$) at 77 K: for cyt. *c*, 135; for cyt. *b*, 107 for cyt. *a*, 78, as calculated from the coefficients at 20°C of 21, 20, and $13 \text{ cm}^{-1} \text{ mM}^{-1}$ respectively.

Fluorimetric measurements of NAD(P) reductase. Malate-, lactate- and ethanol-dependent NADH production and isocitrate-dependent NADPH production catalysed by the cytoplasmic fraction were measured fluorimetrically at room temperature with a Hitachi MPF-4 fluorescence spectrophotometer (excitation wavelength 350 nm, slit 8 nm; emission wavelength 450 nm, slit 10 nm) with 0.01–0.1 mg cytoplasmic protein in 0.4 ml 50 mM-potassium phosphate buffer, pH 7.5, containing 1 mM-NAD(P). Substrate concentrations were: malate, 10 mM; lactate, 20 mM; ethanol, 50 mM; isocitrate, 2 mM.

Other enzyme activities. Fumarase (fumarate hydratase; (EC 4.2.1.2) and succinate thiokinase (EC 6.2.1.5) activities were measured spectrophotometrically at room temperature with a Beckman M 35 spectrophotometer by the increase of absorption at 250 nm (fumarase) and at 230 nm (thiokinase). The reaction mixture contained 0.2–0.8 mg cytoplasmic protein in 1 ml 50 mM-potassium phosphate buffer, pH 7.5. Reactions were initiated by addition of 10 mM-malate (fumarase) or 10 mM-succinate (thiokinase). The incubation medium for thiokinase activity measurement contained 0.4 mM-ATP and 10 mM-CoA. The molar absorption coefficient for succinyl-CoA was $4.5 \times 10^3 \text{ cm}^{-1} \text{ M}^{-1}$ (Bridger *et al.*, 1969).

Redox state of intracellular nicotinamide nucleotides. The harvested cells were washed with 50 mM-potassium phosphate buffer and tested fluorimetrically for the redox state of nicotinamide nucleotides according to Kučera *et al.* (1987) with a Hitachi MPF-4 fluorescent spectrophotometer (excitation wavelength 350 nm, emission wavelength 420 nm). The cylindrical cuvette (diameter 4 mm) contained 0.35 ml of the incubation medium (50 mM-potassium phosphate buffer, pH 7.5). Zero fluorescence corresponded to the medium without bacterial cells, and 100% fluorescence corresponded to the anaerobic cell suspension in the presence of lactate.

Fig. 1. Effect of KCN on the on O_2 -uptake rate of intact *M. luteus* cells. 1, C-limited cells; 2, N-limited cells; 3, batch cells. For experimental details see Methods.

Protein determination. The Lowry method was used, with human serum albumin as a standard.

Results

KCN sensitivity of respiration of batch-grown and limited cells

Fig. 1 (curve 1) shows a significant KCN inhibition of respiration by cells grown under C- (lactate-) limited conditions at KCN concentrations of 2–3 mM. Further increase of KCN concentration resulted in a slight additional effect. The inhibitory effect and the slope of the inhibition curve varied in different experiments depending on the time between the withdrawal of samples from the fermenter and their analysis. Reproducible results could be obtained only when the samples taken from the fermenter were tested within 1–1.5 min. Fig. 1, Curve 1, shows the results of one typical experiment from 10 cultivations. In contrast to lactate-limited cells, the respiration of the cells grown under C-sufficient conditions was resistant to these concentrations of KCN; in fact a 25–30% stimulation of respiration by low KCN concentrations was observed (Fig. 1, curve 3). The same pattern of inhibition was observed with N-limited cells growing under conditions of lactate excess (Fig. 1, curve 2). We have found (data not shown) that the addition of 1 mM-KCN induces approximately 80% reduction of oxidase aa_3 in both C- (or N-) limited and batch-grown cells when *b*-cytochromes, including oxidase *o* remain oxidized by O_2 .

The addition of the respiratory inhibitor 2-n-nonyl-4-hydroxyquinoline-*N*-oxide (NQNO) in the presence of KCN resulted in a dramatic fall in the rate of oxygen

Table 1. Oxidase activity and cytochrome content in cells and membranes of *M. luteus*

Data are presented as means $\pm$ SEM, with the numbers of determinations in parentheses. The assay method and absorption coefficients are given in Methods. Cytochrome maxima are given at 77K.

Cell type	Respiration					Cytochrome content of membranes (5) [nmol (g dry wt) ⁻¹]		
	Cells in growth medium (11)		Membranes (7) [natom O min ⁻¹ (nmol cyt. c) ⁻¹]					
	[natom O min ⁻¹ (mg dry wt) ⁻¹]	[natom O min ⁻¹ (nmol cyt. c) ⁻¹]	Lactate	Malate	NADH	Cyt. c ₅₅₀	Cyt. a ₆₀₀	Cyt. b ₅₅₆ (cyt. o)
Batch	220 $\pm$ 50	5800	230 $\pm$ 60	330 $\pm$ 60	730 $\pm$ 80	48 $\pm$ 12	38 $\pm$ 9	53 $\pm$ 7
C-limited	130 $\pm$ 30	7700	650 $\pm$ 100	760 $\pm$ 80	700 $\pm$ 100	17 $\pm$ 3	11.5 $\pm$ 3.9	17.5 $\pm$ 3

Fig. 2. Effect of KCN on the oxidase activities of C-limited *M. luteus* cells and of membranes isolated from these cells. A Dixon plot (V_0/V_{KCN} versus [KCN]) was used to calculate the K_i values reported in the text. V_0 is the initial oxidase activity and V_{KCN} is the oxidase activity in the presence of KCN. 1, Membrane NADH oxidase; 2, C-limited cells; 3, membrane malate oxidase; 4, membrane lactate oxidase. Data for curve 2 were taken from Fig. 1, curve 1. All membrane activities were measured in 40 mM-HEPES, 5 mM-MgSO₄, pH 7.5.

consumption by each of these types of *M. luteus* cells (5–10% of the initial rate at 1 mM-KCN and 4 mM-NQNO), which proves that the oxygen consumption occurred mainly via the respiratory chain (data not shown).

Fig. 2 shows the KCN inhibition of the O₂-uptake rate by C-limited cells (curve 2) and by membranes isolated from these cells oxidizing various exogenous substrates (curves 1, 3 and 4). The cyanide inhibition curve of lactate oxidation by membranes was almost monophasic (Fig. 2, curve 4) with a high apparent K_i value of approximately 24 mM. The inhibition curve of NADH oxidation was clearly biphasic (Fig. 2, curve 1), with a lower apparent K_i value of approximately 1–2 mM and a

higher K_i value close to K_i that obtained with lactate. The same inhibition pattern was observed with the C-limited cells (Fig. 2, curve 2). The first phase of KCN inhibition was only slightly expressed during the oxidation of malate by membranes (Fig. 2, curve 3). In accordance with the data obtained for intact cells, the addition of 4 mM-NQNO in the presence of 1 mM-KCN led to 95% inhibition of all substrate oxidase activities in isolated membranes (data not shown). The KCN inhibition patterns obtained for the membranes isolated from both batch-grown and C-limited cells were similar.

Characterization of respiration by cells and membranes, and their cytochrome composition

The oxygen uptake rate of cells grown in each of the three conditions, as measured in the growth medium, increased by up to 30–40% upon the addition of exogenous malate, succinate or pyruvate. The stimulation of the respiration of the cell culture by lactate was less pronounced in batch-grown and N-limited cells than in C-limited cells (40–50% and 150%, respectively); ethanol stimulated respiration only in C-limited cells (by up to 60%). The specific rate (per mol cyt. c) of oxygen reduction by C-limited cells was similar to that of batch-grown cells (Table 1).

The total cytochrome content (nmol per g dry wt) in the membranes of batch-grown cells was 2.5 times higher than that in limited cells; however (Table 1) we did not find any substantial differences in the relative stoichiometry or composition of the cytochromes (from low-temperature reduced-minus-oxidized and reduced-plus-CO-minus-reduced difference spectra; data not shown).

Redox state of pyridine nucleotides in batch-grown and C-limited cells

It was interesting to study the effect of exogenous substrates on NADH concentration in intact cells because of the similarity in KCN inhibition curves

Table 2. Specific activities [$\text{nmol substrate min}^{-1} (\text{mg protein})^{-1}$] of cytoplasmic enzymes of *M. luteus*

Cytoplasmic fraction from*:	Lactate dehydrogenase (NAD ⁺ -dependent)	Malate dehydrogenase (NAD ⁺ -dependent)	Isocitrate dehydrogenase (NAD ⁺ -dependent)	Fumarate hydratase†	Succinate thiokinase	Alcohol dehydrogenase
Batch cells (7)	0	0–5	50–82	31–48	13–20	148–355
C-limited cells (8)	3–8	18–40	61–100	120–400	20–36	280–450

* The number of experiments is given in parentheses.

† Activity presented as $1000 \times \Delta A_{250} \text{ min}^{-1} (\text{mg protein})^{-1}$. Experimental details are given in Methods.

Fig. 3. Time course of the steady-state reduction level of the intracellular pool of nicotinamide nucleotides in washed cells of *M. luteus*. 1, C-limited cells; 2, batch cells. Fluorimetric measurements were carried out as described in Methods. Excitation was at 340 nm, emission at 420 nm. The test cuvette contained 0.4 ml suspension (approximately 0.8 mg dry wt cells ml^{-1} in 50 mM-potassium phosphate buffer, pH 7.5). Additions (final concentrations): 10 mM-malate, 20 mM-lactate, 10 mM-pyruvate, 50 mM-ethanol, 0.5 mM-KCN. For curve 2 all additions were the same as for curve 1.

obtained for C-limited cell respiration and NADH oxidation in membranes. The reduced pyridine nucleotide concentrations in intact cells, determined fluorimetrically, are shown in Fig. 3. The steady-state pyridine nucleotide reduction level in washed, C-limited cells rose after the addition of pyruvate, lactate or ethanol (curve 1). The addition of 0.5 mM-KCN induced a marked increase in the level of pyridine nucleotide reduction in the cells. This effect of KCN was observed both in the absence of exogenous substrates and in the presence of any exogenous substrate tested. Most (80–90%) of the pyridine nucleotide pool was reduced in the presence of 0.5 mM-KCN. In contrast to the data obtained with C-limited cells, the steady-state level of pyridine nucleotide reduction in batch-grown cells could be slightly increased

by the addition of malate or ethanol, but not of lactate (Fig. 3, curve 2). The addition of 0.5 mM-KCN to batch-grown cells also led to a marked rise of the extent in pyridine nucleotide reduction.

Differences in the activities of some cytoplasmic enzymes between batch-grown and C-limited cells

Table 2 shows the greater activities of some TCA-cycle enzymes in the cytoplasm of C-limited cells in comparison with that in batch cells. The activity of NAD⁺-dependent malate dehydrogenase in the cytoplasm of C-limited cells was an order of magnitude higher than that in cytoplasm of batch-grown cells, and NAD⁺-dependent lactate dehydrogenase was measurable in C-limited cells only. NAD⁺-dependent lactate dehydrogenase and malate dehydrogenase activities were also immeasurably low in the cytoplasm of N-limited cells (not shown).

Discussion

Two terminal oxidases were previously found to function in the *M. luteus* respiratory chain: cytochrome *aa*₃ was sensitive to low KCN concentrations and cytochrome *o* was more resistant to KCN (Tikhonova *et al.*, 1981, Artzatbanov & Ostrovsky, 1990). In the present study we found that the *in situ* respiratory activities of C-limited, N-limited and batch-grown cells differ in their sensitivity to KCN (Fig. 1). Given similar turnover numbers for the oxidases in batch-grown and C-limited cells (Table 1), the observed difference could be explained by at least two mechanisms: (a) the composition of the respiratory chain (cyt. *o*/cyt. *a* ratio and the content of different cytochromes) depends on the growth rate, as was found for *Paracoccus denitrificans* (Cox *et al.*, 1978) and *Xanthomonas campestris* (Rye *et al.*, 1988); or (b) the contribution of different respiratory branches to the total respiration is different in batch-grown and C-limited cells. The lack of any marked differences in the low-

temperature spectra of C-limited and nonlimited cells (i.e. the same ratio of the amplitudes at 600 nm, cytochrome oxidase *aa*₃, to the amplitude at 556 nm, cytochrome oxidase *o*) are inconsistent with mechanism (a). [In accordance with earlier data (Tikhonova *et al.*, 1981) membranes of the cells grown on lactate as C-source had only two CO-binding components, corresponding to oxidases *o* and *aa*₃]. Cornish *et al.* (1987) also found no difference in the cytochrome composition of *Agrobacterium radiobacter* grown under C-limited and C-excess conditions. Thus, the results obtained indicate an increased contribution of the main cyanide-sensitive branch with cytochrome *aa*₃ to the total respiration under C-limited growth conditions.

It is well known that the contribution of different branches to the total respiration depends on the type of substrate utilized (Porte & Vignais, 1980; Wong & Maier, 1987; Artzbatanov & Petrov, 1990). We found (Fig. 2) that the KCN sensitivity of substrate oxidation by membranes decreased in the order NADH > malate > lactate. The similarity between the KCN inhibition patterns for C-limited cell respiration and for NADH oxidation by membranes allows us to assume that NADH is the main endogenous substrate oxidized by C-limited cells while lactate is the preferential substrate in C-sufficient cells. Apparently the first phase of the inhibition is associated with the blocking of cytochrome *aa*₃ with KCN. The remaining activity is provided by the functioning of cytochrome *o*. By contrast, the respiration of C-sufficient cells is provided mainly by the KCN resistant oxidation of lactate directly via the membrane-bound respiratory lactate dehydrogenase. Fluorimetric experiments indeed indicate that reduced pyridine nucleotides serve as an important intermediate substrate for the respiratory chain of C-limited cells. Thus, the provision of exogenous malate and lactate in the incubation medium stimulated the oxidase activities in both cell types (and finally led to anaerobic conditions) but caused an increase of steady-state pyridine nucleotide reduction level only in C-limited cells.

It should be noted that a significant reduction of pyridine nucleotides in the presence of 0.5 mM-KCN indicated the preferential oxidation of NADH by the main respiratory branch in the cell (NADPH was not oxidized by the membranes). The increase of activity of some cytoplasmic enzymes (especially NAD⁺-dependent enzymes: Table 2) may be responsible for the increased rate of NADH production in the cells grown under energy substrate limitation.

The changes in function of the electron-transport chain found under lactate-limited growth could in principle be due to a decrease in the cell doubling rate *per se*, as well as to substrate limitation. However, we found no marked difference in KCN sensitivity between

the respiration of exponential batch-grown and slowly-grown N-limited cells (Fig. 1, curves 3 and 2, respectively); similarly, NAD-dependent lactate dehydrogenase and malate dehydrogenase activities were absent from the cytoplasm of both N-limited and batch-grown cells. Moreover, the relatively high KCN sensitivity of lactate-limited cell respiration has been observed only at low growth rate ($D = 0.02\text{--}0.05\text{ h}^{-1}$). So, the changes in energy metabolism under discussion take place only at low growth rate of cultures limited by energy substrate. Under these conditions, the cells' metabolism is switched towards the 'NADH-type' respiration which is more energetically effective (more coupled) (Lawford *et al.*, 1976; Artzbatanov *et al.*, 1983). This could provide energy for the increased costs of protein synthesis (Bulthuis *et al.*, 1989) and for the energy-linked transport of substrates whose exogenous concentrations are low. The adaptation of the facultative methylotroph *Pseudomonas* AM1 to carbon-limited growth induced the synthesis of the membrane-bound cytochrome *c*, leading to the appearance of an additional coupling site (Keevil & Anthony, 1979). We found that the adaptation of *M. luteus* to slow, carbon- and energy-limited growth was accompanied by changes in the proportion of different endogenous substrates utilized by the branched respiratory chain without any marked modification of the cytochrome composition. It is interesting to note that a *Mycobacterium* strain with a slow growth rate has preferentially an NAD⁺-dependent malate dehydrogenase, in contrast to the membrane-bound FAD-linked malate dehydrogenase observed in a *Mycobacterium* strain with a high growth rate (Wheeler & Bharadwaj, 1983).

One can assume that under non-limited growth of the obligate aerobe *M. luteus* the existence of the alternative, weakly coupled respiratory branch allows the cell to accelerate the rate of substrate oxidation coupled with ATP synthesis and/or substrate level phosphorylation (fast rate but low P/O ratio) (see Kell, 1987).

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