THE ROLE OF CYTOCHROME b_1 IN NITRATE ASSIMILATION AND NITRATE RESPIRATION IN *KLEBSIELLA AEROGENES*

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1. Introduction

Klebsiella (=Aerobacter) aerogenes can utilize nitrate as the sole source of nitrogen under both aerobic and anaerobic conditions (nitrate assimilation). Under anaerobic conditions, nitrate also functions as a terminal electron acceptor (nitrate respiration). In both processes, nitrate is reduced to nitrite by a nitrate reductase, but in the assimilation process nitrite is reduced eventually to NH_4^+ by nitrite reductase [1, 2]. Previous studies [3–5] made it very likely that, although in both processes the same nitrate reductase is involved, the electron transport to nitrate proceeds differently in both cases, giving rise to different enzyme complexes.

This idea was further substantiated by the results of the experiments described in this paper. Evidence was obtained indicating that cytochrome b_1, although participating in the electron transfer to the nitrate reductase in the respiratory complex (respiratory nitrate reductase), is not involved in the nitrate assimilation process.

2. Experimental

K. aerogenes, strain S 45, was used in this study. Bacteria, executing nitrate assimilation, were grown aerobically in a minimal nitrate medium at 30° as described previously [3]. Bacteria, executing nitrate respiration, were grown in a minimal nitrate plus NH_4^+ medium under anaerobic conditions at 30° [3].

Dry weight determinations were performed as described by Hadjipetrou et al. [1].

Nitrate reductase activity with reduced benzyl viologen as an electron donor, was assayed as described before [3].

3. Results and discussion

One of the complications in the study of nitrate assimilation in *K. aerogenes*, is the high lability of the assimilatory nitrate reductase. Cell disruption, and even harvesting and washing of nitrate assimilating bacteria, lead to a considerable loss of the activity of the assimilatory nitrate reductase [3, 4]. Therefore, the composition of the electron transport chain in the assimilatory nitrate reduction process can only be studied in growing cultures. This limits the experimental approach to a large extent. First, in a growing culture (with a relatively low cell density), only absorbance changes of cytochrome b_1, and that in the Soret region can be measured. Secondly, the effect of nitrate, present in the growth medium, should be eliminated. This latter can be accomplished by the addition of azide to the medium. Using reduced benzyl viologen as an electron donor (transfers the electrons directly to the terminal nitrate reductase), we have found a complete inhibition of nitrate reductase activity by 5×10^{-4} M azide, both for the assimilatory and the respiratory enzyme. The inhibitory effect of azide on the assimilatory nitrate reduction is also illustrated in fig. 1. Azide almost completely inhibits aerobic growth in a minimal medium with nitrate as the sole source of nitrogen, while growth on nitrate or NH_4^+ is not influenced significantly. This suggests that neither the nitrate reductase, nor the assimilation of

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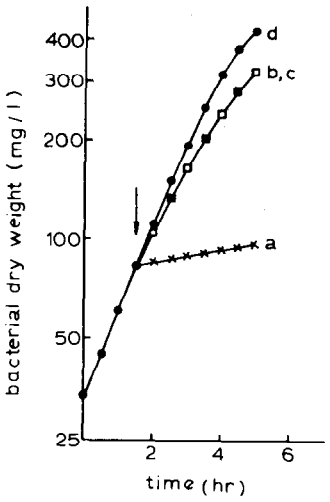

Fig. 1. The effect of azide on aerobic growth of *K. aerogenes* under various conditions. An exponentially growing aerobic culture in a minimal medium with nitrate as the sole source of nitrogen at 30° [3], was divided into 4 equal parts (indicated in the figure by an arrow). At the same time the four cultures were supplemented with, respectively: a) azide (5×10^{-4} M); b) NH_4^+ (4×10^{-3} M) plus aizde (5×10^{-4} M); c) nitrite (non-toxic concentration of 5×10^{-4} M) plus azide (5×10^{-4} M); d) Control (no additions). Incubation at 30° was continued, and growth was monitored as described previously [3].

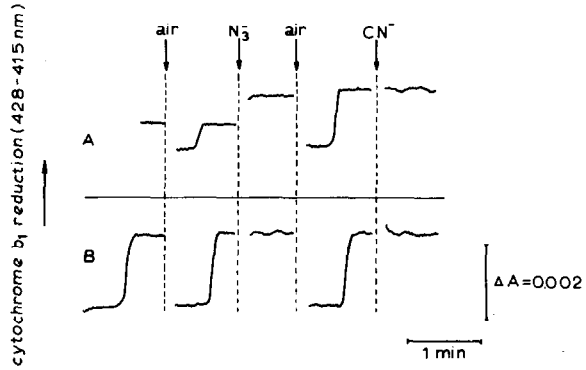

Fig. 2. The effect of azide on the reduction of cytochrome b_1 in growing cultures of *K. aerogenes*. An aliquot (3 ml) of a culture (cell density corresponding to 220 mg dry wt per l), executing nitrate respiration (fig. 2A), and a 3 ml-sample of a culture (400 mg dry wt per l), executing nitrate assimilation (fig. 2B), were placed in absorption cells (lightpath 10 mm). Changes in the absorbance at 428 nm (Soret-peak of cytochrome b_1) relative to 415 nm (isobestic point) were measured at 30° in an Aminco–Chance dual wavelength spectrophotometer. Additions were made as indicated in the figure. The final concentrations of azide and of cyanide were 5×10^{-4} M, and 10^{-2} M, respectively. The short duration (5 min) of the experiment in fig. 2B excludes a possible induction of the respiratory nitrate reductase, since this enzyme is synthesized only after a lagtime of 12 min [4].

NH_4^+, is inhibited by azide. Also, the oxygen consumption by whole cells (washed suspensions and in cultures), as measured with the Clark oxygen electrode at 30° was not inhibited by the same low concentration of azide. Thus, the addition of azide to cultures, growing under conditions of nitrate respiration or nitrate assimilation, will completely block the electron flow to nitrate, but not to oxygen.

The effect of azide on the reduction of cytochrome b_1, in cultures growing under conditions of nitrate reduction is presented in fig. 2. For the sake of comparison, the results obtained with a culture performing nitrate respiration are also presented in this figure; it was already established by other approaches that cytochrome b_1 participates in the respiratory nitrate reduction process [4, 6]. When an anaerobic, nitrate respiring culture is placed in the absorption cell (fig. 2A) the nitrate reducing steady state [7] of cytochrome b_1 is recorded. Aeration results in a transition to the aerobic steady state. The nitrate reducing steady state is restored after the oxygen has been used up. The addition of azide during the latter state, results in a further reduction of cytochrome b_1, because azide blocks the electron flow to nitrate. Subsequent aeration restores the aerobic steady state, indicating that cytochrome reoxidation is not prevented by azide. The high reduction level in the presence of azide represents the anaerobic state reduction of cytochrome b_1, since the addition of cyanide, a strong inhibitor of cytochrome oxidases, does not result in a further reduction of cytochrome b_1.

In an aerobic assimilating culture, cytochrome b_1 is present in the aerobic steady state level of reduction (beginning of trace in fig. 2B). When the oxygen tension approaches zero, cytochrome b_1 will be reduced, and then it may be present in the nitrate reducing steady state (the assimilatory nitrate reductase is not blocked by anaerobiosis) or in the anaerobic state. In contrast to the situation in fig. 2A, the addition of azide in this case does not result in a further reduction of cytochrome b_1, though azide was found to block the assimilatory nitrate reductase.

Therefore, the attained reduction level may represent the anaerobic state which is confirmed by the finding that the addition of cyanide or sodium dithionite did not have any influence on this reduction level of cytochrome b_1. Aeration of the sample, after the addition of azide, reoxidizes cytochrome b_1 to the original aerobic steady state level (fig. 2B). In summary, the results of fig. 2B show, that in nitrate assimilating cultures, the presence of nitrate does not have any influence on the reduction level of cytochrome b_1, or, nitrate, even in the presence of an active assimilatory nitrate reductase, cannot oxidize cytochrome b_1.

These findings permit the conclusion that cytochrome b_1, in contrast to its involvement in nitrate respiration, does not participate in the assimilatory nitrate reduction process in *K. aerogenes*. This supports our earlier conclusions, that the assimilatory and the respiratory nitrate reductase complexes have a different composition [3, 4]. The participation of cytochrome b_1 in nitrate respiration is in agreement with the energy-producing character of this process [8, 9] and offers an explanation for the observed competition for electrons between oxygen and nitrate, both *in vivo* [3] and *in vitro* [10]. *In vivo*, respiratory nitrate reduction is even completely blocked by oxygen, because of the preferential flow of electrons to oxygen [3]. In order to prevent such a drastic oxygen effect in the aerobic nitrate assimilation process, the assimilatory nitrate reductase should not accept electrons from carriers, participating in the electron transport chain to oxygen or, at least, not in the same way as the respiratory enzyme does. The presented results fit this idea and also indicate that nitrate assimilation is an energy consuming process [4, 8].

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