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Cupredoxines of obligate methylotroph

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Cells of type I obligate methylotroph *Methylobacillus flagellatum* KT, which can use only methanol or methylamine as carbon and energy sources, possess two types of blue copper proteins: amicyanin and azurin. During exponential phase of growth amicyanin was the only blue copper-containing protein in the cells from methylamine medium. Azurin-type proteins appeared in these cells when they reached the stationary growth phase. In contrast, amicyamin could not be detected in methylamine-grown cells when they are from stationary growth phase. Cells from methanol-containing medium possess only azurin, amicyanin could not be detected in all growth phases. Kinetic studies with methylamine dehydrogenase and amicyanin purified from *M. flagellatum* KT cells show that amicyanin may serve as a primary electron acceptor from methylamine and methylamine dehydrogenase in this type I of obligate methylotroph.

Cupredoxine; Obligate methylotroph; Amicyanin; Azurin

1. INTRODUCTION

The cupredoxins present in bacterial respiratory systems (type I or blue cuproproteins) contain one atom of copper which bind to a small protein, carry a single electron and exhibit a high redox potential [1]. These cupredoxins absorb in the near-red region of the spectrum (~600 nm) and thus appear blue, but the color completely disappears on reduction [2]. All these enzymes exhibit some of the structural and redox properties of the simple electron transfer proteins, like cytochromes, but they have in general been much less extensively investigated [3].

The type I blue copper, which was named amicyanin, was found to be a primary electron acceptor for *Methylobacterium extorquens* AM1 methylamine dehydrogenase and to have the ability to serve as an electron carrier between methylamine dehydrogenase and cytochrome *c* [4]. Growth in methylamine-containing medium leads to induction of synthesis of methylamine dehydrogenase together with amicyanin [5].

Gram-negative non-pigmented obligate methylotroph, organism 4025, which is unable to use methane, but able to grow on methylamine or methanol, produce very large amounts of blue copper proteins, amicyanin and azurin-type, when cells grow in high-copper centration medium [6-9]. The concentrations of blue copper proteins were highest at the copper concentrations which supported maximum growth of organism 4025, but during the growth on methanol or in the absence of copper amicyanin was not detectable. It was also demonstrated that iron may be involved in the synthesis of amicyanin and azurin in organism 4025 [10].

However, in obligate methylotroph bacterium W3A1 it has not been observed that amicyanin serves as electron acceptor for methylamine dehydrogenase, it was suggested that a cytochrome *c* might replace it [11]. This has also been suggested for *M. extorquens* AM1 during growth on low copper concentrations [5,12]. Even in the well-studied organism 4025 the function of amicyanin remains obscure after finding that amicyanin might be replaced by some unknown alternative electron acceptor.

The aim of this work was to demonstrate the existence of blue copper proteins during growth of type I obligate methylotrophs *M. flagellatum* KT on methanol and methylamine medium, and to show the possible involvement of these cupredoxines in methylamine oxidation.

2. MATERIALS AND METHODS

2.1. *Growth of bacteria, preparation of bacterial extracts and membranes*

The cultures of *M. flagellatum* KT were grown as batch cultures in mineral medium with methanol or methylamine as carbon and energy source, this strain was isolated from activated sludge of the wastewater treatment station in Moscow [13].

The cultivation medium contained (g/l): KH_2PO_4, 10.0; NaCl, 1.0; $MgSO_4.7H_2O$, 0.23; $(NH_4)_2SO_4$, 4.0; pH was 7.4 before autoclaving. Trace elements solution was added to the medium in final concentrations (mg/l): $FeSO_4.7H_2O$, 4.0; $CaCl_2.2H_2O$, 5.3; $Na_2MoO_4.2H_2O$, 0.04; $MnSO_4.5H_2O$, 0.2; $ZnSO_4.7H_2O$, 0.2; $CoCl_2.6H_2O$, 0.04; $CuSO_4.5H_2O$, 0.04; H_3BO_3, 0.03. Methanol (1%, v/v) or methylamine hydrochloride (1%, v/v) was added to the sterile medium after filter-sterilizing.

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Copper-deficient media were prepared by using Analar-grade reagents made up with deionized and distilled water in glassware washed in chromic acid. The concentration of copper in growth media to which no copper had been added was equivalent to less than 0.1 mg $CuSO_4.5H_2O$ per liter. In experiments with copper-deficient media copper was excluded from the trace elements solution.

Cells from different growth phases collected by centrifugation, washed two times with cold KH_2PO_4 buffer (50 ml, pH 7.2), suspended in the same buffer and disrupted by passing two times through X-press (LKB-Pharmacia, Sweden). The resulted homogenate was centrifuged at 20 000 × *g* (20 min, 4°C) to obtain the crude extract. Crude extract was centrifuged again at 200 000 × *g* (120 min, 4°C) to obtain a membrane fraction which was suspended in the KH_2PO_4 buffer and supernatant fraction where soluble components of respiratory chain were determined.

2.2. *Determination of cytochromes and blue copper proteins*

The concentrations of these proteins in the soluble fractions of bacteria were determined after their separation by chromatography as described [10]. The concentrations of cytochromes were measured using reduced minus oxidized difference spectra taken at room temperature. The absorption coefficients were taken as in [8] and [9].

3. RESULTS

3.1. *Optical properties of amicyanin and azurin-type blue protein from* M. flagellatum *KT*

The visible absorption spectra of amincyanin and azurin-type blue copper proteins from *M. flagellatum* KT cells grown on methanol or methylamine show two distinct absorption maxima: at 625 nm for amicyanin and 620 nm for azurin (figs 1, 2).

It was estimated that amicyanin and azurin are in soluble fractions of methanol or methylamine-grown cells of *M. flagellatum* KT and do not bind the membranes which were obtained by centrifugation of crude cell extracts at 200 000 × *g* for 2 h. It seems to be that amicyanin and azurin are both in periplasmic space of Gram-negative *M. flagellatum* KT.

3.2. *Cupredoxines composition of methylamine- and methanol-grown* M. flagellatum *KT cells*

It was interesting to know how the amicyanin and azurin content in the methylamine- and methanol-grown cells of *M. flagellatum* KT might be affected by aeration in the medium or growth phase of cultures.

The high aeration of the growth medium was obtained in the 10% filled baffled shake flasks where oxygen concentration in the medium was not less than 20% from saturation during all growth stages. The low aeration was obtained in the ordinary shake flasks which was 50% filled and where oxygen concentration was always below the limit of detection of a platinum/silver-chlorine electrode (Rank Brother, Bottisham, England).

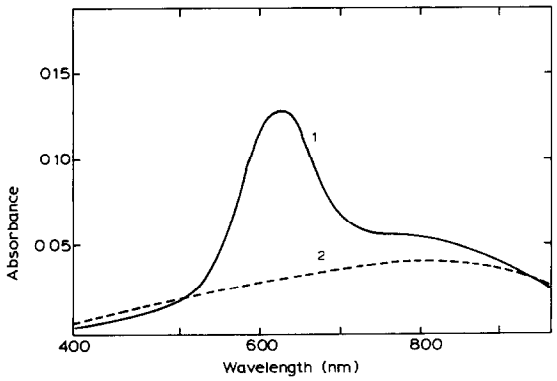

Fig.1. The absorption spectrum of partially purified amicyanin type blue copper protein from methylamine-grown cells of *M. flagellatum* KT. 1, Fully oxidized state; 2, fully reduced state.

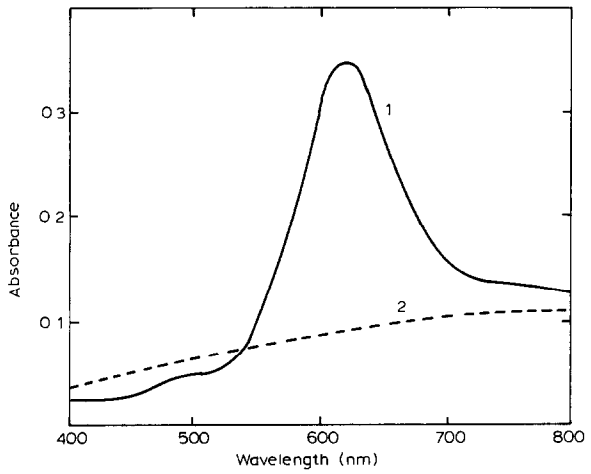

Fig.2. The absorption spectrum of partially purified azurin type blue copper protein from methanol-grown cells of *M. flagellatum* KT. 1, Fully oxidized state; 2, fully reduced state.

Table 1

Cupredoxines content in soluble fraction (200 000 × *g* for 2 h) of *M. flagellatum* KT cells after growth in various media in the presence of 1 μM of Cu^{2+} (nmol/mg protein)

		Methylamine-grown cells		Methanol-grown cells	
Aeration	Growth phase	Amicyanin	Azurin	Amicyanin	Azurin
High	Exponential	5.5	–[a]	–	0.39
	Stationary	2.2	–	–	<0.01
Low	Exponential	2.2	–	–	<0.01
	Stationary	–	0.2	–	<0.01

[a] The cupredoxines could not be detected

Table 2

Amicyanin content in soluble fractions (200 000 × *g* for 2 h) of methylamine-grown cells of *M. flagellatum* KT from exponential phase of growth depending on copper concentration in the media (mmol/mg protein)

Cu^{2+} concentration (μM)	Amicyanin content
0	<0.01
1	4.9
5	7.1
10	8.0

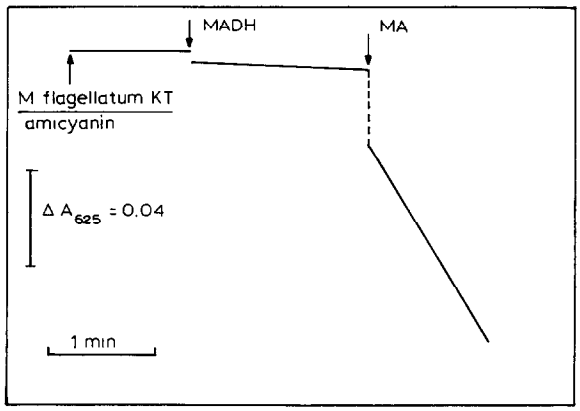

Fig.3. The kinetic studies of amicyanin reduction from methylamine and methylamine dehydrogenase of *M. flagellatum* KT. The reaction mixture contained 1 ml 10 mM KH_2PO_4 buffer (pH 7.0) and the reaction rate was measured at 25°C and 625 nm. Final concentrations of the reactants were: 5 μM of *M. flagellatum* KT amicyanin, 20 μM of *M. flagellatum* KT methylamine dehydrogenase (MADH) and 10 mM methylamine (MA).

In methylamine-grown cells of *M. flagellatum* KT the content of amicyanin was almost two times higher in the high-aerated cultures than in low-aerated ones. In stationary cultures amicyanin could not be detected (table 1). Azurin-like protein appeared in the cells of methylamine-grown cultures in the stationary phase of growth and its content was 10 times higher in high-aeration cultures.

Amicyanin could not be detected in methanol-grown cells at all growth phases and its content was low in low-aeration methylamine-grown cultures (table 1).

3.3. *Effect of copper in the medium on content of amicyanin and azurin-type blue proteins of* M. flagellatum *KT*

The effect of copper ($CuSO_4.5H_2O$) at concentrations of 0, 1, 5 and 10 μM on the content of amicyanin in the soluble fraction of *M. flagellatum* KT cells was examined. Amicyanin content was less than 0.01 nmol per mg of protein when no copper was added to the growth medium with methylamine (table 2). Amicyanin content in cells rose with increasing copper concentration in the medium, but the maximum growth was obtained when the copper concentration in medium was 5 μM. Azurin content in the cells varies and it seems that its concentration in the cells more dependend on the growth conditions, e.g. aeration, growth phase, etc. than on copper concentration in the medium.

It seems that azurin in *M. flagellatum* KT cells connected with methanol dehydrogenase (MDH) synthesis, as in the cells grown on methylamine MDH and azurin could be detected, but in the cells from methanol medium neither methylamine dehydrogenase, nor amicyanin could be detected in all growth conditions.

3.4. *Kinetic studies of amicyanin reduction by methylamine dehydrogenase from* M. flagellatum *KT*

The determine the role of amicyanin and its possible position in the electron transport chain of *M. flagellatum* KT growing on methylamine, kinetic studies of amicyanin reduction by methylamine dehydrogenase were carried out in vitro.

The results are shown in fig.3. Amicyanin was directly reduced in the reduced in the presence of methylamine and methylamine dehydrogenase, but not azurin-type blue protein.

4. DISCUSSION

In this study we can distinctly demonstrate that the two functionally different types of the blue copper proteins, amicyanin-type and azurin-type present in methylamine-grown *M. flagellatum* KT and their biosynthesis are controlled by carbon source in the growth medium and the growth phase. Amicyanin was synthesized in *M. flagellatum* KT during optimal growth phase and it was directly reduced by methylamine dehydrogenase in the presence of methylamine.

The azurin-type blue copper protein was only found in the stationary phase cells grown on methylamine. Thus, we can conclude that the azurin-type blue copper protein does not play an important role during methylotrophic growth of *M. flagellatum* KT on methylamine. The role of azurin-type blue copper proteins in the electron transport chain of obligate methylotrophs remains obscure and should be solved [5,10].

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