Jour. Microbiol. June 1996 p. 172-178 Vol. 34, No. 2

Effect of Copper on the Growth and Methanol Dehydrogenase Activity of *Methylobacillus* sp. Strain SK1 DSM 8269

Si W. Kim[1] and Young M. Kim*

Department of Biology and Bioproducts Research Center, Yonsei University, Seoul 120-749, and Department of Environmental Science, Chosun University, Kwangju 501-759, Korea[1]

(Received December 8, 1995/Accepted April 15, 1996)

***Methylobacillus* sp. strain SK1, which grows only on methanol, was found to grow in the absence of added copper. The doubling time (t_d=1.3 h) of the bacterium growing at the exponential growth phase at 30°C in the absence of copper was the same as that of the cell growing in the presence of copper. The bacterium growing after the exponential phase in the absence of copper, however, grew faster than the cell growing in the presence of copper. Cells harvested after the early stationary phase in the presence of copper were found to exhibit no methanol dehydrogenase (MDH) activity, but the amount and subunit structure of the enzyme in the cells were almost the same as that in cells harboring active MDH. Pellets of the cells harvested after the early stationary phase in the presence of copper were pale green. Cell-free extracts prepared from cells harvested at the early stationary phase in the presence of copper were pink and exhibited MDH activity, but it turned dark-green rapidly from the surface under air. The green-colored portions of the extracts showed no MDH activity and contained *c*-type cytochromes that were oxidized completely. The inactive MDH proteins in the green portions were found to have antigenic sites identical to those of the active one as the inactive MDHs in cells grown in the presence of copper. The bacterium was found to accumulate copper actively during the exponential growth phase. MDH prepared from cells grown in the presence or absence of copper was found to be more stable under nitrogen gas than under air. Methanol at 10 mM was found to enhance the stability of the MDH under air.**

Key words: Methanol, methylotroph, methanol dehydrogenase, copper, cytochrome *c*, *Methylobacillus* sp. SK1

Methylotrophic bacteria are a group of bacteria which are able to grow at the expense of compounds containing one or more carbon atoms but no carbon to carbon bonds as energy and carbon sources (1, 2). There have been many studies on the oxidation of methylotrophic substrates, especially of methane, methanol, and methylamine, in this group of bacteria at the biochemical, physiological, and molecular level (1, 2, 7, 16).

Among those studies, there are several interesting reports on the effect of copper on the growth of methanol-oxidizing bacteria. Copper added into the medium was found to increase the rate and extent of growth and stimulate synthesis of membrane-bound and soluble *c*-type cytochromes during growth of organism 4025 on methanol (5, 20). The aerobic suspension of the bacterium grown in the copper-containing medium was pale pink and contained a blue copper protein (5, 6, 15).

Methylobacillus sp. strain SK1 DSM 8269 is an obligate methanol-oxidizing bacterium isolated from soil samples collected in Kuala Lumpur, Malaysia (10). The bacterium is Gram-negative and grows only on methanol as the sole source of carbon and energy. We found during preliminary studies with the bacterium growing in the presence of copper that there were changes in color of crude cell extracts from pink to dark-green as the cells grew. The pink extracts revealed methanol dehydrogenase (MDH) activity but the green extracts did not. We, however, did not observe these changes in cells grown in the absence of added copper, implying that copper may be involved in these phenomena.

We, therefore, have determined to carry out this study to investigate the effect of copper on the growth and MDH stability in *Methylobacillus* sp. SK1 growing on methanol in the presence of a higher concentration of

* To whom correspondence should be addressed

copper than the one used in the earlier experiment. The present study has shown that copper has negative effects on the growth on methanol of *Methylobacillus* sp. strain SK1.

Materials and Methods

Bacterial strain and cultivation

Methylobacillus sp. strain SK1 DSM 8269 was batch cultured in a fermentor (7 liters; New Brunswick Scientific Co. MF-114) equipped with an oxygen electrode (Ingold 02-Sensor-12/320) or flasks at 30℃ in a modified mineral medium of Kim and Hegeman (12) supplemented with 1.0% methanol; the pH and phosphate concentration of the medium were 7.5 and 50 mM, respectively, and $NaNO_3$ (1.0 g per liter) was added as an additional mineral source. Copper-deficient medium was prepared by using ACS grade reagents (Sigma) and deionized water distilled in glassware washed in chromic acid. Copper-supplemented medium contained 1.0 mg $CuSO_4 \cdot 5H_2O$ per liter, except that the medium for copper accumulation test contained 2.0 mg $CuSO_4 \cdot 5H_2O$ per liter. The medium was agitated at 400 rpm. Growth was measured by turbidity determined at 500 nm using a spectrophotometer. Methanol in the media was analyzed with a Varian 3300 gas chromatograph equipped with a Porapak Q column (0.3×100 cm); the temperatures for column, injector and detector were 180℃, 210℃ and 210℃, respectively.

Preparation of crude cell-free extracts

Cells harvested at the appropriate growth phases were washed once with 100 mM Tris-hydrochloride buffer (pH 7.0). The washed cells were resuspended in the same buffer and disrupted by sonic treatment (10 s per ml). The suspension was centrifuged at 15,000×g for 30 min and the resulting supernatant was used as crude cell extracts. For copper determination, cells harvested were first washed twice by incubation for 1 h in 100 mM Tris-hydrochloride buffer (pH 7.0) containing 1.0 mM EDTA and then washed twice with the same buffer before sonic treatment.

MDH assay

MDH activity was assayed photometrically at 30℃ by monitoring the methanol-dependent reduction of 2, 6-dichlorophenol indophenol (ε_{600}=21.8 $mM^{-1} \cdot cm^{-1}$) as previously described by Anthony and Zatman (4). The reaction was started by addition of methanol to the reaction mixture containing crude cell extracts. One unit of enzyme activity was defined as the amount of enzyme required to reduce 1 nmole of dichlorophenol indophenol per min at 600 nm.

Electrophoresis

Nondenaturing polyacrylamide gel electrophoresis (PAGE) of crude cell extracts in 7.5% acrylamide gel was performed by the procedure developed for acidic proteins by Thomas and Hodes (19). Denaturing PAGE in 12.5% acrylamide gel was carried out according to Laemmli (14) in the presence of 0.1% sodium dodecyl sulfate (SDS). Proteins were stained with Coomassie brilliant blue R-250 (CBB) by the modified method (12) of Weber and Osborn (21). MDH was stained by activity after nondenaturing PAGE using methanol as a substrate by the method of Kim *et al.* (9).

Protein determination

Proteins in crude cell extracts were determined by the method of Lowry *et al.* (17) after boiling for 10 min in 20% NaOH (12). Bovine serum albumin was used as a standard.

Copper determination

Copper contents in the medium and cell-free extracts were determined at 324.7 nm using Inductively Coupled Plasma Spectrophotometer (Labtest Model Plasmascan 710, 27.12 MHz). Copper solution (1,000 ppm) purchased from Junsei, Japan was used as a standard. Argon gas was used as carrier (1.1 liter per min) and coolant (15 liter per min) gases. Samples treated with a mixture of HNO_3 (60%, v/v) and $HClO_4$ (60%, v/v) (HNO_3 : $HClO_4$=8 : 2) were boiled on hot plate and passed through 0.45 μm membrane filter prior to copper determination (8).

Immunodiffusion test

Antiserum against the purified MDH of *Methylobacillus* sp. SK1 (11) was prepared in a rabbit as described previously (12). Double immunodiffusion assays were performed in 1.2% agarose gel by a modified method (14) of Ouchterlony and Nilsson (18).

Results

Effect of copper on the rate and extent of growth

It was found that *Methylobacillus* sp. SK1 growing in the presence and absence of added copper grows at almost the same rate (t_d = 1.3 h) during the exponential growth phase. The growth rate of cells growing in the presence of copper, however, decreased more rapidly than that of the cells growing in the absence of copper after the exponential phase, resulting in lower cell density (A_{500} = 1.6) than that of the cells growing in the copper-deficient medium (A_{500} = 2.1) after 24 h cultivation

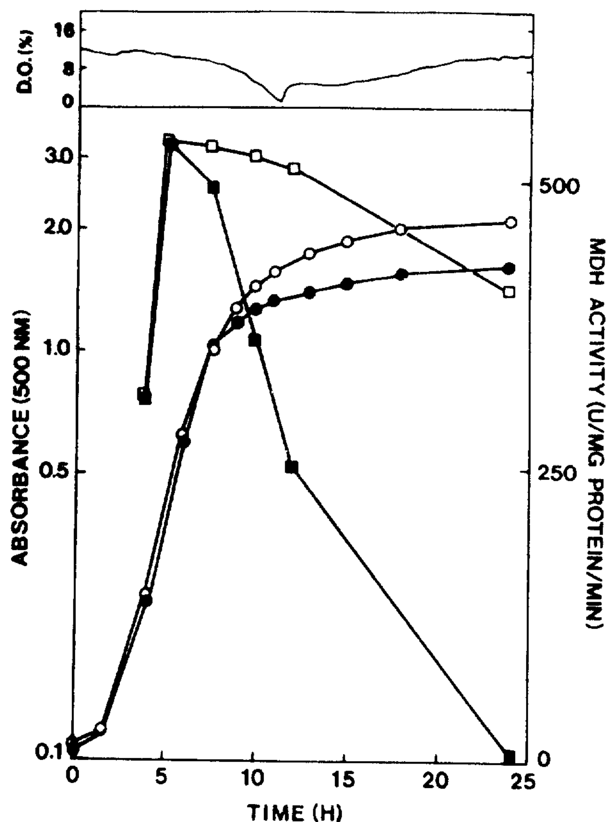

Fig. 1. Changes in the dissolved oxygen and MDH activity during growth of *Methylobacillus* sp. SK1. Cells were grown on methanol (1.0%) in the presence (-●-) or absence (-○-) of added copper. MDH activity in cell extracts prepared from cells grown in copper-supplemented (-■-) or -deficient (-□-) medium was assayed by the method as described in Materials and Methods. Dissolved oxygen was analyzed with an oxygen electrode equipped in a fermentor. The data represent the mean values of three tests.

(Fig. 1).

Dissolved oxygen and methanol concentration

It was found that the dissolved oxygen in the copper-supplemented and -deficient media was started to decrease from the mid-exponential phase and reached near zero between the exponential and stationary phases (Fig. 1). The dissolved oxygen then increased very rapidly to almost half the concentration of the fresh medium in 30 min as the cells grew slowly. The methanol concentration in the media was also found to decrease sharply during the exponential phase and became very low between the exponential and stationary phases (data not shown).

Variation in the MDH activity

The MDH activity in *Methylobacillus* sp. SK1 growing in the absence and presence of added copper was almost

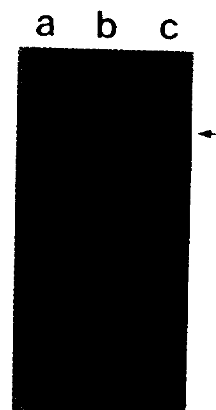

Fig. 2. Nondenaturing PAGE of MDH in cell extracts prepared from cells grown in the presence of copper. Gels containing 7.5% acrylamide were run according to Thomas and Hodes (19) and stained with CBB. 2 μg of purified MDH (a) and 80 μg protein of each sample prepared from cells harvested after 6 h (b) and 30 h (c) were applied. Arrow indicates MDH proteins.

the same during the exponential growth phase (Fig. 1). The enzyme activity in cells grown in the presence of copper began to decrease rapidly from the late exponential phase, whereas that in cells grown in the absence of copper decreased very slowly. Cells grown for over 24 h in the copper-supplemented medium were found to exhibit no MDH activity. Staining for MDH activity after nondenaturing PAGE also revealed that cell extracts prepared from cells grown for over 24 h in the presence of copper do not show MDH activity (data not shown). Analysis of acidic proteins by nondenaturing PAGE of crude cell extracts prepared from cells grown in the presence of copper, however, showed that there is no difference in the amount of MDH proteins in cells grown for over 24 h compared with that in cells harboring active MDH (Fig. 2). Two-dimensional nondenaturing-denaturing PAGE of MDH using the MDH band after nondenaturing PAGE revealed that the inactive MDH in cell extracts prepared from cells grown in the copper-supplemented medium for over 24 h also has the two nonidentical subunits as the active MDH (data not shown).

Cells grown in the copper-deficient medium for over 24 h, on the other hand, exhibited MDH activity even though the activity was much lower than that of the cells growing actively at the exponential growth phase (Fig. 1). The cells at all growth phases also contained almost equal amounts of MDH protein and showed the enzyme activity when it were subjected to stain by activity (data not shown).

Changes in color of cell-free extracts

The color of pellets of *Methylobacillus* sp. SK1 growing

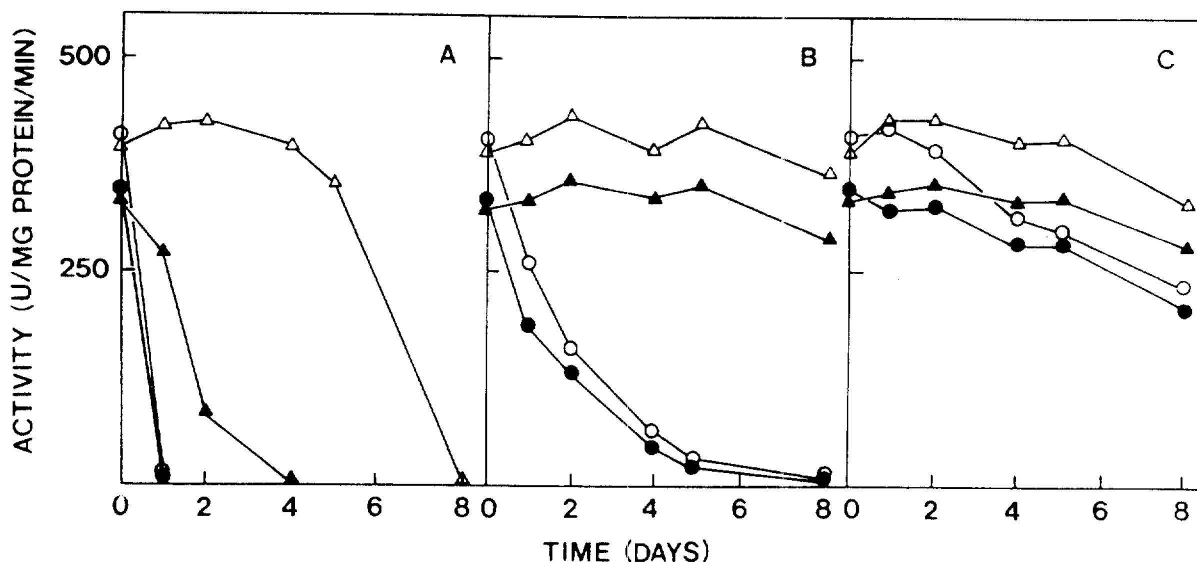

Fig. 3. Stability of MDH under different conditions. Stability of MDH in crude cell extracts prepared from cells growing at different growth phases in the presence or absence of copper was determined by comparing the MDH activity in the cell extracts after incubation of the extracts for appropriate time under air (A), nitrogen gas (B), or air in the presence of methanol (C). MDH activities in cell extracts prepared from cells growing at the mid-exponential (-△-) or early stationary phase (-○-) in the absence of copper and at the mid-exponential (-▲-) or early stationary (-●-) phase in the presence of copper were assayed by the procedure described in Materials and Methods.

in the presence of copper changed from pink to pale green after early stationary phase. Pellets of the cells growing at all growth phases in the absence of copper, however, were found to be pink; the color became reddish-pink at the stationary phase.

It was found that crude cell extracts prepared from cells harvested at the mid-exponential and early stationary phases in the presence of copper were pink at the early stage of preparation. When 3-ml portions of the cell extracts, especially those from cells harvested at the early stationary phase, were kept at 4℃ under air, the color changed rapidly to dark-green from the top of the extracts in an hour; the extracts from cells growing at the mid-exponential phase turned green very slowly. The extracts from cells harvested at the late stationary phase were not pink but completely dark-green. The cell extracts prepared from cells grown in the absence of copper, however, were found to remain pink for long time under air.

MDH activity and cytochrome *c* oxidation level after color changes

As the color of the extracts from cells harvested at the early stationary phase in the copper-supplemented medium changed from pink to green, the MDH activity in the extracts decreased and the reduced *c*-type cytochromes in the same extracts turned to oxidized form. The green-colored portions of the extracts were found to show no MDH activity, but they contained almost the same amount of MDH proteins as that present in the pink extracts according to analysis through non-denaturing PAGE technique (data not shown). The spectrum of the green portions revealed that the cytochromes *c* in those portions were in completely oxidized forms.

The dark-green extracts prepared from cells harvested at the late stationary phase in the copper-supplemented medium were also found to contain MDH proteins with no enzyme activity and *c*-type cytochromes that were completely oxidized. The extracts showed a broad absorption peak around 620 nm, suggesting a possible association of greenish color with the oxidized form of blue copper protein (15).

Stability of MDH

When cell-free extracts prepared from cells growing at the mid-exponential phase in the presence or absence of copper were stored at 4℃ under air, the MDH in cell extracts from cells grown in the copper-deficient medium was found to be more stable than the MDH from cells grown in the copper-supplemented medium (Fig. 3A). It was also found that there were no changes in color (data not shown) and MDH activity for over a week when the pink extracts from cells growing at the mid-exponential phase in the copper-supplemented medium were incubated at 4℃ under nitrogen gas soon after

preparation (Fig. 3B). The pink color of the extracts prepared from cells harvested at early stationary phase in copper-supplemented medium was also unchanged under nitrogen gas. The MDH in the extracts was found to be more stable under nitrogen gas than under air. MDHs in crude cell extracts prepared from cells growing at the mid-exponential phase were found to be more stable than those in extracts of cells growing at the early stationary phase, regardless of the presence of copper in the cultivation medium, when those extracts were stored under air or nitrogen gas (Fig. 3A and B). It was also found that there were no differences in the stability of MDHs when the cell extracts prepared from cells harvested at the early stationary phase in the presence or absence of copper were left under air or nitrogen gas (Fig. 3A and B).

When cell extracts were stored at 4℃ under air with methanol (10 mM), MDH in crude extracts from cells growing at the mid-exponential or early stationary phase in the copper-supplemented medium showed stable enzyme activity for over a week, which is comparable to that in cell extracts prepared from cells growing in the copper deficient medium (Fig. 3C). Incubation of cell extracts with methanol under air was also found to enhance the stability of MDH in extracts from cells harvested at the early stationary phase, regardless of the presence of copper in the medium, to the level of that in extracts from cells growing at the mid-exponential phase. The inactive MDH in the dark-green cell-free extracts prepared from cells harvested at the late stationary phase in the copper-supplemented medium, however, was not reactivated by the addition of methanol.

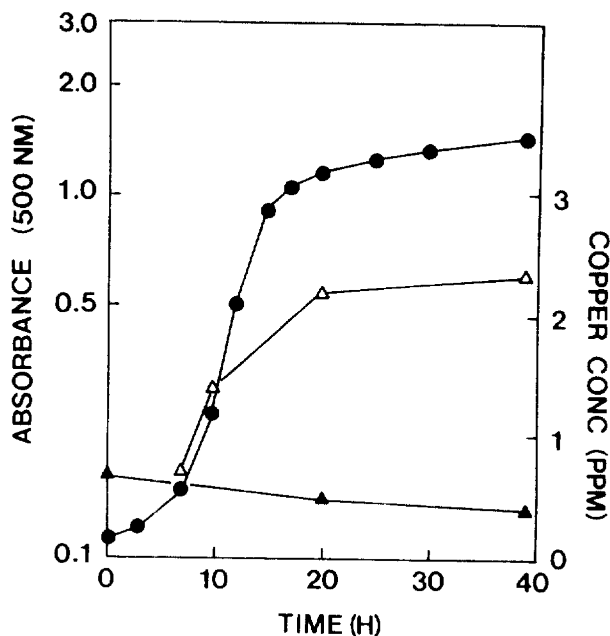

Fig. 4. Accumulation of copper in the cell during growth of *Methylobacillus* sp. SK1. Copper contents in the cell (-△-) and culture medium (-▲-) were determined during growth of the cell in the copper-supplemented medium using Inductively Coupled Plasma Spectrophotometer as described in Materials and Methods. The medium prepared with no added copper was found to contain less than 1×10^{-4} ppm of copper. The closed circle (-●-) designates the cell density at the stage of culture growth. Data represents the mean values of three tests

Accumulation of copper in the cell

It was found during cultivation of *Methylobacillus* sp. SK1 that copper concentration in cells growing in the presence of added copper increased as the cells grew, whereas that in the medium decreased (Fig. 4). The MDH activity in cells growing in copper-supplemented medium was found to decrease after the exponential phase as the concentration of copper within the cell increases.

Antigenic specificity

Double immunodiffusion revealed that the cell extracts, exhibiting strong or no MDH activity, prepared from cells of *Methylobacillus* sp. SK1 harvested at the mid-exponential or late stationary phase, respectively, in the presence of copper cross-react with antiserum raised against the MDH purified from cells of the bacterium growing at the mid-exponential phase in the absence of copper (data not shown). The pattern of immunoprecipitates indicated that MDHs, regardless of the presence of activity, in cells grown in the presence of copper have antigenic sites identical to those of the cells grown in the absence of copper. It was also found that the inactive MDH in the dark-green colored portions of extracts from cells grown with copper shares common antigenic determinants with the active MDH from cells grown without added copper (data not shown).

Discussion

It has been reported that organism 4025, an obligate methylotroph able to grow on methanol or methylamine, requires copper for maximum growth on methanol or methylamine (5, 20). The bacterium was found to contain no *a*-type cytochrome and uses *o*-type cytochrome as a terminal oxidase (15). Cells grown on methanol in the presence of copper (1.0 mg $CuSO_4\cdot5H_2O$ per liter) were pale pink (15). Addition of copper to the growth medium increased the rate and extent of growth of the cell on methanol or methylamine.

It was found in this experiment that copper added into the medium does not stimulate the growth of *Methy-*

lobacillus sp. SK1 on methanol; copper was found to be inhibitory to growth after the exponential phase, indicating that utilization of methanol by *Methylobacillus* sp. SK1 does not require copper-containing enzyme and that copper may be directly or indirectly involved in the retardation of growth of the cell on methanol after exponential growth phase. A previous experiment that *Methylobacillus* sp. SK1 grown in the presence of copper has no *a*-type cytochromes and uses *o*-type cytochrome as a terminal electron acceptor (10). This supports the present result that the bacterium does not require copper to use methanol as a source of carbon and energy. We assume that organism 4025, which also has no *a*-type cytochromes, grows on methanol better in the presence of added copper since the organism synthesizes a special electron acceptor, azurin (3), in the presence of copper which may replace cytochrome *c* in electron transport chains of the organism since it may be a better electron acceptor than the *c*-type cytochromes. It seems that *Methylobacillus* sp. SK1 does not produce this kind of novel electron carrier during growth in copper-supplemented medium and that the effects of copper on the growth on methylotrophic substrates are not identical in methylotrophic bacteria.

The rapid decrease in the activity of MDH after exponential growth phase and color changes in cells growing in the presence of copper, together with the active accumulation of copper during exponential phase, imply that copper may be responsible for these consequences. The changes in color of crude cell extracts from pink to dark-green under air, disappearance of MDH activity and oxidation of *c*-type cytochromes followed by the color changes in the extracts, and stabilization under nitrogen gas of MDH activity and color of extracts from cells grown with copper, however, indicate that oxygen, not only the copper, may also be involved in all the novel phenomena that occurred in the extracts or in the cells grown in the presence of copper.

The data clearly indicate that both copper and oxygen are responsible for the lower MDH activity and growth rate of cells grown in copper-supplemented medium than those of cells cultivated in copper-deficient medium. First of all, the growth rate and dissolved oxygen concentration decreased rapidly after late exponential phase, but there was a sudden increase in dissolved oxygen in early stationary phase. Also, cellular pastes of *Methylobacillus* sp. SK1 grown in copper-supplemented medium changed color from pink to pale green after early stationary phase. In addition, MDH activity in cells grown with copper dropped off sharply between the exponential and stationary phases. Finally, the dark green portions of crude extracts of cells grown with copper showed no MDH activity. It seems likely that methanol, the electron donor of MDH, may prevent the decrease in enzyme activity caused by copper and oxygen since the enzyme activity in crude cell extracts prepared from cells grown in the presence of coppper was found to be as stable under air in the presence of methanol as that in cell extracts from cells grown in the absence of copper There is other evidences supporting this assumption. The concentration of methanol in the medium decreased sharply during the exponential phase and became very low between the exponential and stationary phases. The growth rate and MDH activity of the cells growing in the presence of copper decreased from the late exponential phase more rapidly than those of the cells growing in the copper-deficient medium.

In the presence of copper, cells grown after the exponential phase that exhibited low or no MDH activity contained the same amount of MDH proteins as that in cells showing high MDH activity. This suggests that the enzyme in cells growing after the exponential phase with copper may be inactivated irreversibly, but not simply inhibited, by the coupled action of oxygen and copper. The dark-green cell extracts exhibiting no MDH activity prepared from cells growing at the late stationary phase did not show any MDH activities when the extracts were incubated under nitrogen gas or under air in the presence of methanol supports this assumption (data not shown). The assumption may be further supported by the observations that the dark-greenish portions of cell extracts from the early stationary phase cells that were exposed to air also contained inactive MDH, the concentration of MDH protein in that portions was not different from that in pink portions, and that the greenish portions exhibited no MDH activities, even after incubation under nitrogen gas or under air with methanol.

It may be that the inactivation of MDH in cells or cell extracts prepared from the cells grown in copper-supplemented medium is not caused by modification in the structure of the enzyme. There are several facts that suppore this assumption. The inactive enzymes in the cell or cell extracts were shown to share identical subunit structure and antifgenic sites with the active MDH prepared from cells grown in copper-deficient medium. The *c*-type cytochromes in the green portions of cell extracts and dark-green colored extracts prepared from cells grown in the presence of copper were present in completely oxdized forms. These extracts showed a broad absorption peak around 620 nm. All these suggest that the MDH in cells grown in the presence of copper may be inactivated in the presence of copper and oxygen and the absence of the electron donating methanol. This may be through the acceleration of oxidative mod-

ification of the electron transport complex between MDH and cytochrome *c* by certain kinds of blue copper protein synthesized in *Methylobacillus* sp. SK1 during growth in the presence of copper. This could result in the irreversible changes in the redox state of MDH. The blue protein, however, may not be the well-known azurin, since azurin is known to act as a normal electron carrier supporting the growth of organism 4025 under high copper concentrations (3).

Acknowledgement

This work was supported by the Non-directed Research Fund (Project No. 01-D-0585) from the Korea Research Foundation in 1994.

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