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Physiological Role of Mercury-methylation in *Clostridium cochlearium* T-2C

Hidemitsu S. Pan-Hou and Nobumasa Imura

School of Pharmaceutical Sciences, Kitasato University, 9-1, Shirokane 5 Chome, Minato-Ku, Tokyo 108, Japan

It is well known that microorganisms can subject mercury to a range of biotransformation including degradation of organomercury and methylation of inorganic mercury. The enzymatic degradation of organomercurials has been considered to be an essential process for many species of bacteria to acquire organomercury resistance (FURUKAWA & TONOMURA 1971, SCHOTTEL 1978, SUMMERS & SILVER 1978, PAN-HOU & IMURA 1981). In contrast, less information is available concerning the physiological importance of microbial mercury-methylation which has also been well documented in various microorganisms (JENSEN & JERNELÖV 1969, YAMADA & TONOMURA 1971, VONK & SIJPESTEIJN 1973, HAMDY & NOYES 1975, IMURA et al. 1977).

LANDNER (1971) found that a mercury resistant strain of Neurospora crassa had a relatively higher mercury methylating activity and speculated that the ability to methylate inorganic mercury in Neurospora might imply in the mercury detoxication. Recently, we reported a preliminary experimental result suggesting that the methylcobalamin dependent mercury-methylation acts as a detoxication mechanism for Clostridium cochlearium T-2C (PAN-HOU & IMURA 1982). This prompted us to confirm that the mercury methylation is a main function of this anaerobe to detoxify mercuric mercury.

In this paper, we present convincing evidence that the mercury-methylating activity in Clostridium cochlearium T-2C plays an important role in the detoxication of inorganic mercury ion.

MATERIALS AND METHODS

Organisms and Culture Clostridium cochlearium T-2C which is known to synthesize various types of cobalamin including methylcobalamin and has an ability to methylate inorganic mercury (YAMADA & TONOMURA 1971, PAN-HOU et al. 1980) was routinely grown at 30°C under an atmosphere of nitrogen in a vitamin B_{12} assay medium (Difco). Growth was monitored by measuring the turbidity at 540 nm.

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Isolation of vitamin B_{12} auxotrophic strain Twenty milliliters of mid log growth phase cells were harvested by centrifugation, washed two times with 30 mL of 0.2 M Tris-maleate buffer (pH 6.0), and resuspended in 10 mL of the same buffer with 200 µg/mL of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (MERGERY & GERITS 1978). The MNNG-treated cells were plated on the medium containing 2% agar and supplemented with 50 ng/mL of vitamin B_{12} (cyanocobalamin). The resulting colonies requiring vitamin B_{12} were isolated by standard procedures of replica plating techniques.

Determination of total cobalamin and methylcobalamin The supernatant obtained by centrifugation of sonicated cells in 0.1 M acetate buffer (pH 4.6) at 10000xg for 15 min was employed for determination of total cobalamin Methylcobalamin in the supernatant was isolated by paper chromatography before determination (PAN-HOU et al. 1980). Lactobacillus leichmannii ATCC-7830 was used for determination of the vitamin B_{12} analogs as reported previously (PAN-HOU et al. 1980).

Examination of methylmercury forming activity The organisms were grown in a 100 mL of the medium containing 5 µM $HgCl_2$ and supplemented with 25 ng/mL of vitamin B_{12} at 30°C in the dark. Methylmercury formed in the culture medium during 48 h incubation was determined by gas chromatography as described previously (IMURA et al. 1977).

Determination of minimum inhibitory concentration of mercurials The levels of mercury resistance were determined according to the method of CLARK et al. (1977).

Cellular uptake of labeled mercurials The washed cells (5×10^8/mL) were incubated at 30°C in the reaction mixture containing 50 mM Tris-HCl buffer (pH 7.5), 1 mM sodium thioglycolate, 1 µM $^{203}HgCl_2$ or $CH_3{}^{203}HgCl$ (Sp. Act. 1 mCi/mg Hg). After incubation, mercury bound to the cells was determined as described previously (PAN-HOU et al. 1981).

Effect of mercurials on protein biosynthesis The supernatant (S-30) obtained by centrifugation of the sonicated mid log growth phase cells at 30000xg for 30 min was used as the S-30 preparation for cell-free protein biosynthesis. The assay medium contained 50 mM Tris-HCl buffer (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 0.25 mM GTP, 1.5 mM phosphoenolpyruvate, 40 µg/mL of pyruvate kinase 50 ug/mL of each 20 amino acids including 10 uCi H-tyrosine and 2 mg/mL of S-30 or 1 mg/mL of the washed cells. After incubation at 37°C,

50 μL aliquots of the reaction mixture were placed on paper disks (1 cm^2, Toyo filter paper No. 514). Hot TCA-insoluble radioactivity on the disks in toluene scintillator was counted (NAKADA et al. 1980) with a liquid scintillation spectrometer (Aloka Co. Ltd.).

RESULTS

No significant amount of total cobalamins was detected in the vitamin B_{12} auxotrophic cells even when 25 ng/mL of cyanocobalamin was added in the medium for their normal growth (Table 1).

Table 1. Effect of vitamin B_{12} on bacterial growth and contents of cellular cobalamin

Strain	Growth[a] Vitamin B_{12} added (ng/mL)				Cobalamin[b]	
	0	10	25	50	T-Co[c] (ng/mg protein)	M-Co[d] (ng/mg protein)
Parent	0.88	0.92	0.91	0.89	1.89	0.63
Vitamin B_{12} auxotroph	0.06	0.36	0.92	0.91	0.11	N.D.[e]

a) Growth was determined by measuring the turbidity at 540 nm after 20 h of culture. b) Cellular cobalamins were determined after 20 h of culture in the vitamin B_{12} assay medium supplemented with 25 ng/mL of cyanocobalamin. c) Total cobalamin. d) Methylcobalamin. e) Not detectable.

The vitamin B_{12} auxotrophic strain isolated by the treatment with MNNG lost its ability to methylate inorganic mercury and became more sensitive to inorganic mercury compared with its parent strain. However, the minimum inhibitory concentration of methylmercury for both strains was essentially the same (Table 2).

Table 2. Mercury-methylating activity and susceptibility to mercurials

Strain	Methylmercury formed (nmol/L)	MIC[a]	
		CH_3HgCl	$HgCl_2$
Parent	6.4	10	80
Vitamin B_{12} auxotroph	N.D.[b]	10	20

a) Minimum inhibitory concentration. b) Not detectable.

When the cells were incubated with ^{203}Hg-labeled mercurials at 1 μM concentration, 2 times more methylmercury than inorganic mercury was taken up by the cells of both strains. However, no consistent difference in the uptake of mercury compounds tested was observed between the vitamin B_{12} auxotrophic and the parent cells (Fig. 1).

Next, effects of methylmercury and inorganic mercury on protein synthesis of this anaerobe was further examined. Methylmercury added in the cell suspension showed slightly stronger inhibitory effect than inorganic mercury on protein synthesis in the cells. In the cell free system, however, inorganic mercury inhibited protein synthesis more strongly than methylmercury (Fig. 2).

DISCUSSION

Although microbial methylation of inorganic mercury considered to be of general microbial nature, has been suspected to be involved in the detoxication of mercury, no direct experimental evidence is available on the physiological importance of this process. Our present experiment is designed to provide some informations which help us to understand the role of mercury methylation in microorganisms.

Clostridium cochlearium T-2C, a mercury sensitive strain, was isolated by treating its original strain, T-2P, carrying a mercury-resistant plasmid with acridine orange (Pan-Hou et al., 1980). The T-2C strain thus obtained having an ability to synthesize various types of cobalamin including methylcobalamin, can methylate inorganic mercury probably by the participation of methylcobalamin (IMURA et al.1977).

In order to know the physiological role of methylcobalamin dependent mercury-methylation in this bacterium, the first attempt was designed to isolate a mutant strain which lacks the ability to produce cobalamins. After treatment with MNNG, a vitamin B_{12} requiring strain was isolated (Table 1). Bioassay of cellular methylcobalamin using L. leichmannii ATCC-7830 demonstrated that no detectable amount of methylcobalamin was present in the vitamin B_{12} auxotrophic strain cultured for several passages in the medium supplemented with 25 ng/mL of vitamin B_{12} (Table 1). We then compared the characteristics of the parent strain (T-2C) with those of the mutant strain to clarify the role of mercury-methylation.

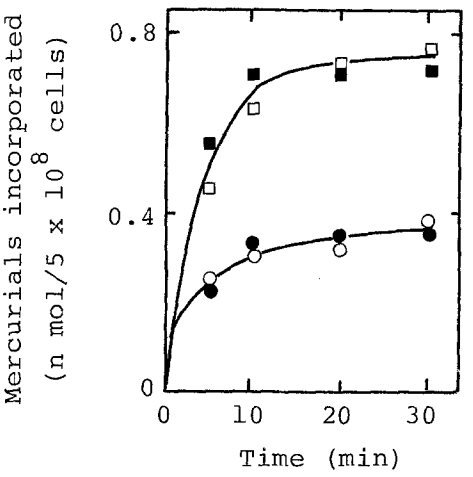

Fig. 1. Uptake of $^{203}HgCl_2$(○,●) and CH_3HgCl(□,■) by the vitamin B_{12} auxotrophic strain(●,■) and the T-2C strain(○,□) of C. cochlearium.

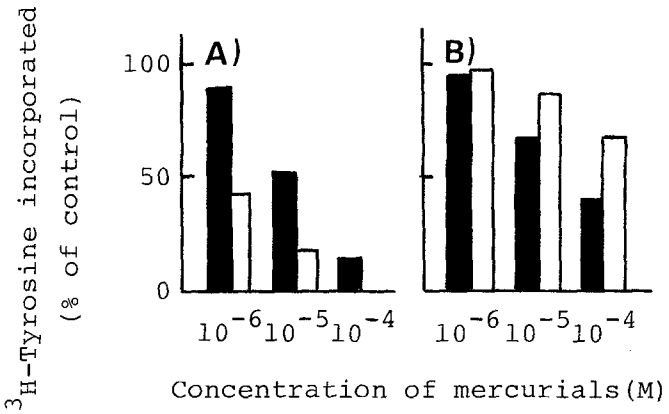

Fig. 2. Effect of mercurials on protein synthesis of C. cochlearium T-2C.

The S-30(**A**) and the suspension of intact cells(**B**) were incubated with various concentrations of CH_3HgCl(■) or $HgCl_2$(□) at 37°C for 45 min. After incubation, the radioactivity in the acid-insoluble fraction was measured as described in Materials and Methods. All values represent the averages of the duplicate samples.

Formation of methylmercury by the organisms was first examined. As shown in Table 2, no detectable amount of methylmercury could be formed by the vitamin B_{12} auxotrophic strain, while the strain T-2C produced methylmercury in the medium as reported previously when the organisms were grown in the presence of 5 μM mercuric chloride in the vitamin B_{12} assay medium supplemented with 25 ng/mL of vitamin B_{12} at 30°C for 48 h in the dark. This result supported our hypothesis that methylcobalamin might be a major factor responsible for the methylation of inorganic mercury by the bacterium (IMURA et al. 1977). The vitamin B_{12} auxotrophic strain which lost the ability to methylate inorganic mercury showed higher sensitivity to inorganic mercury than its parent strain. However, it is noteworthy that the vitamin B_{12} auxotroph did show a same extent of resistance to methylmercury as that of the parent strain (Table 2).

As shown in Fig. 1, no significant difference in the uptake (including adsorption on the cell surface) of mercurials was observed between the vitamin B_{12} auxotrophic and the parent strains. This observation indicates that the increased sensitivity to inorganic mercury shown by the vitamin B_{12} auxotroph is not due to the increase in cellular mercury levels which has been considered to be one of the factors influencing bacterial sensitivity to metals (CHOPRA 1971, KONDO et al. 1974, HORITSU & ITO 1980, PAN-HOU & IMURA 1981, SILVER et al. 1981). Further, any strain of Clostridium cochlearium T-2 so far isolated has no activity to reduce mercuric mercury to inert elemental mercury. Thus, the hypersensitivity of the vitamin B_{12} auxotrophic strain to inorganic mercury seemed to be correlated with loss of the mercury-methylating activity.

It has been known that the in vitro inhibitory effect of inorganic mercury on functional proteins of mammalian cells is stronger than that of methylmercury (IMURA et al. 1980, NAKADA et al. 1980). If cellular methylation of inorganic mercury indeed imply the detoxication of mercury, inorganic mercury is expected to show higher inhibitory effect on some of bacterial metabolisms than methylmercury. Then the effects of methylmercury and inorganic mercury on protein synthesizing activity of the T-2C strain were compared. Consistent with the hypothesis, the direct inhibitory effect of inorganic mercury on cell free protein synthesizing system was in fact stronger than that of methylmercury (Fig. 2). A similar result was obtained when the cell free protein synthesizing system of the vitamin B_{12} auxotroph was used. In addition, no significant difference in the net synthesizing activity

was observed between the two strains.(Data not shown). These results suggested that the higher sensitivity to inorganic mercury noted for the mutant was not ascribed to the change of protein synthesizing activity after the treatment with MNNG. While methylmercury added in the cell suspension showed a little stronger inhibitory effect on protein synthesis in the cells than inorganic mercury (Fig. 2). The higher depressing effect on the activity shown by methylmercury appeared to be a reflection of its relatively great affinity (probably its permeability) to the cells (Fig. 1) as in the case of mammalian cells (NAKADA et al. 1980).

The results presented above, therefore, lead us to conclude that microbial methylation of inorganic mercury in the cells to produce less toxic methylmercury, in terms of the direct inhibitory effect on functional proteins, acts as a detoxication mechanism to build up bacterial tolerance to inorganic mercury.

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