Enzymatic Reduction of Hexavalent Chromium by Hexavalent Chromium Tolerant *Pseudomonas ambigua* G-1

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Received March 23, 1987

When hexavalent chromium (Cr^{6+}) tolerant *Pseudomonas ambigua* G-1 was cultivated in nutrient broth containing 150 ppm Cr^{6+}, the Cr^{6+} content of the broth rapidly decreased. The Cr^{6+} reducing enzyme found in a cell-free extract of *P. ambigua* G-1 required NADH but not NADPH as a hydrogen donor for the reduction of Cr^{6+}. The specific activities of cell-free extracts of several Cr^{6+} sensitive mutants derived from *P. ambigua* G-1 showed decreases to one fourth to one tenth of that of *P. ambigua* G-1. Glucose protected the Cr^{6+} reducing enzyme against inactivation on dialysis.

Although chromium (Cr) is known to be essential for the growth of microorganisms, a high concentration of Cr is toxic for many bacteria. Of the two stable Cr forms as to valency, hexavalent Cr(Cr^{6+}) is more toxic than the trivalent form (Cr^{3+}). Many kinds of bacteria have been isolated which are able to grow on a medium containing a high concentration of Cr^{6+}.[1~5] The mechanisms of tolerance to Cr^{6+} of bacteria have not been yet sufficiently studied. Cr^{6+} can be reduced through an indirect microbial process in marine environments.[6]

We reported previously that Cr^{6+} tolerant *Pseudomonas ambigua* G-1 had a thick envelope which prevented the permeation of Cr^{6+} into the cells.[7]

In this paper, we describe another mechanism of Cr^{6+} tolerance in this bacterum, that is, the direct reduction of Cr^{6+} to Cr^{3+} by a cell-free extract of *P. ambigua* G-1.

MATERIALS AND METHODS

Materials. Potassium chromate (K_2CrO_4) was used as a hexavalent chromium. NAD-Linked yeast alcohol dehydrogenase and NADH were the products of Oriental Yeast Co., Ltd. NADPH was from Sigma Chemical Co.

Microorganism and growth conditions. P. ambigua G-1 was used throughout this study. The bacterium was isolated from activated sludge and tolerant to 4,000 ppm Cr^{6+}. The cells were cultivated aerobically in nutrient broth containing 150 ppm Cr^{6+}.

Preparation of a cell-free extract. Cells harvested at the late log phase were washed three times with 10 mM Tris–HCl buffer (pH 7.5). The washed cells, suspended in the same buffer, were subjected to ultrasonic oscillation (20 kHz) for five intervals of 10 min each, *i.e.*, for a total 50 min, under ice-cooling. The clear supernatant obtained on centrifugation at 15,000 × *g* for 20 min was used as a cell-free extract.

Enzyme assay. Cr^{6+} reducing activity was assayed by measuring the decrease in Cr^{6+}. The reaction mixture contained 0.4 μmol of K_2CrO_4, 0.4 μmol of NADH and a suitable amount of the enzyme solution in 2.0 ml of 10 mM phosphate buffer (pH 7.0). The reactions were started by the addition of the enzyme solution. After incubation for 30 min at 50°C, the reactions were stopped by heating for 2 min at 100°C. The denatured proteins were centrifuged off. Residual Cr^{6+} in the reaction mixture was determined. One unit of Cr^{6+} reducing activity was defined as the amount of enzyme which catalyzed the decrease of one μmol of Cr^{6+} per 30 min at 50°C.

Determination of total Cr and Cr^{6+}. Total Cr was measured by the atomic adsorption method using a Hitachi Atomic Adsorption Spectrophotometer type 170-40. Cr^{6+} was measured spectrophotometrically using diphenylcarbazide.[8]

Protein assay. The protein content was determined by the method of Folin and Ciocalteu[9] with egg white lysozyme as a standard.

RESULTS

Decrease in Cr^{6+} during cultivation of P. ambigua G-1

The time course of the Cr^{6+} decrease with *P. ambigua* G-1 is shown in Fig. 1. Cr^{6+} decreased rapidly during the early phase of growth. There was no decrease in total Cr in the culture medium. Although total Cr incorporated into the cell mass gradually increased as the cells grew, only 0.35 mg of total Cr was incorporated into 155 mg dry cells per 100 ml of broth after 36 hr cultivation. No decrease in Cr^{6+} occurred in the absence of the bacterium. These results indicate that *P. ambigua* G-1 was able to change Cr^{6+} to another form as to valency of Cr.

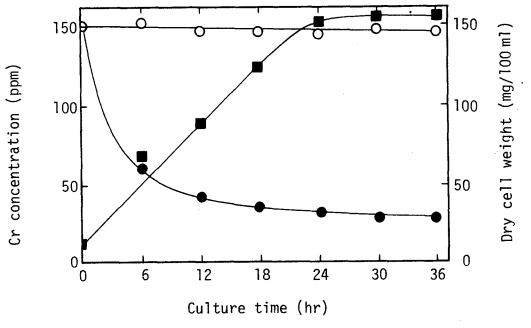

FIG. 1. Time Course of the Cr^{6+} Decrease with Cr^{6+} Tolerant *P. ambigua* G-1.

○, total Cr in medium; ●, Cr^{6+} in medium; ■, dry cell weight.

TABLE I. DECREASE IN Cr^{6+} WITH A CELL-FREE EXTRACT

The reaction mixtures (2.0 ml) contained 0.4 μmol of Cr^{6+}, 1.6 ml of a cell-free extract (100 mg protein) and 100 μmol of potassium phosphate buffer (pH 7.0). The reactions were carried out at 50°C.

Reaction time (min)	Total Cr (μM)	Cr^{6+} (μM)
0	200	200
30	198	120

Decrease in Cr^{6+} with a cell-free extract of P. ambigua G-1

To clarify the mechanism of the Cr^{6+} decrease, we investigated whether or not a cell-free extract of *P. ambigua* G-1 was able to decrease the Cr^{6+} content. The reaction mixture (2.0 ml) contained 200 μM Cr^{6+} and 100 mg (as protein) of a cell-free extract in 50 mM phosphate buffer (pH 7.0). The reaction was stopped by adding 0.5 ml of 20% trichloroacetic acid. The mixture was centrifuged to remove denatured proteins. The total Cr and Cr^{6+} in the supernatant were determined. As shown in Table I, after 30 min incubation at 50°C, Cr^{6+} was decreased to 120 μM, but no change in the total amount of Cr had occurred. A cell-free extract heated at 100°C for 5 min did not cause a decrease in Cr^{6+}. These results suggest that the cell-free extract of *P. ambigua* G-1 was able to transform Cr^{6+} to another form as to valency. The Cr^{6+} decreasing activity was located in a soluble fraction of ultracentrifugation (105,000 × *g*, 2 hr). There was no Cr^{6+} decreasing activity in the insoluble fraction. After Cr^{6+} had completely disappeared due to the action of Cr^{6+} decreasing activity in a cell-free extract, the reaction mixture was treated with ammonium persulfate in the presence of sodium hydroxide. Cr^{6+} appeared again in the oxidized reaction mixture, which indicated that the disappearance of Cr^{6+} was due to its reduction to a form of lower valency.

Requirement of NADH for Cr^{6+} reduction

As shown in Table II, the Cr^{6+} reducing activity of a cell-free extract of *P. ambigua* G-1 was found to be inactivated on dialysis. However, the activity was restored to some extent by the addition of a concentrated diffusate to the reaction mixture. This suggests that the Cr^{6+} reducing enzyme requires cofactor(s) for the reaction. So, we examined whether or not redox cofactors can restore the enzyme activity. Table II shows that NADH was required for the reduction of Cr^{6+} by the reducing enzyme. NADPH did not cause any restoration of the enzyme activity.

TABLE II. REQUIREMENT OF NADH FOR THE REDUCTION OF Cr^{6+}

A cell-free extract was dialyzed overnight against distilled water at 4°C. The diffusate was concentrated *in vacuo* under 40°C. The activity of the Cr^{6+} reducing enzyme in the cell-free extract before dialysis was taken as 100%.

Component	Cr^{6+} reducing activity (%)
Cell-free extract	100
Dialyzate	17
Diffusate	0
Dialyzate plus diffusate	70
Dialyzate plus NADH	66
Dialyzate plus NADPH	18

TABLE III. EQUIVALENT FORMATION OF Cr^{3+} TO Cr^{6+} REDUCTION

After incubation, the reaction mixture (2 ml) was acidified by the addition of 2 ml of 2 N HCl. Separation of Cr^{6+} from Cr^{3+} was performed by the tributylphosphate extraction method described by Hikime and Yoshida.[10] Cr^{6+} was completely extracted with tributylphosphate. The aqueous phase only contains Cr^{3+}.

Reaction time (min)	Cr^{6+} (μM)	Cr^{3+} (μM)	Total Cr (μM)
0	200	0	200
30	122	71	193
60	103	92	195

Reduced products on Cr^{6+} reduction

Hikime and Yoshida[10] reported that Cr^{6+} was easily extracted with tributylphosphate, but that Cr^{3+} was not. The reduced product of Cr^{6+} with the Cr^{6+} reducing enzyme could not be extracted with tributylphosphate. Since the naturally occurring stable forms as to valency of Cr are the trivalent and hexavalent forms, this suggests that the reduced product of Cr^{6+} may be the trivalent form of Cr (Cr^{3+}). The time course of Cr^{6+} reduction by the enzyme is shown in Table III. The decrease in Cr^{6+} corresponded with the increase in Cr^{3+}. To elucidate whether or not the oxidation of NADH to NAD occurs during the Cr^{6} reduction, the following experiments were performed. On the addition of the reducing enzyme, the absorbance at 340 nm rapidly decreased. After the enzyme had been completely inactivated by treatment at 80°C for 5 min, NAD-linked yeast alcohol dehydrogenase (5.2 units) and an excess amount of ethanol were added. The addition of ethanol increased the absorbance to about 70% of the initial level. These results indicate that the reduction of Cr^{6+} by the reducing enzyme was accompanied by the oxidation of NADH to NAD.

TABLE IV. Cr^{6+} REDUCING ENZYMES OF Cr^{6+} SENSITIVE MUTANTS DERIVED FROM *P. ambigua* G-1

The cells were cultivated aerobically at 30°C in nutrient broth. The details are given under MATERIALS AND METHODS.

Strain	Specific activity (units/mg protein)
P. ambigua G-1	0.086
Cr^{6+} sensitive mutant	
S-1	0.023
S-2	0.027
S-3	0.007
S-4	0.020
S-5	0.016
S-6	0.020
S-7	0.015

Cr^{6+} reducing enzyme activities of Cr^{6+} sensitive mutants derived from P. ambigua G-1

The Cr^{6+} sensitive mutants were derived from *P. ambigua* G-1 by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, as described previously.[7] Table IV shows the activities of the reducing enzymes of several Cr^{6+} sensitive mutants. The specific activities of the reducing enzymes in cell-free extracts of all the sensitive mutants tested showed decreases to one fourth to one tenth of that of the parent strain.

Effect of glucose

Shimada and Matsushima[12] showed that a Cr^{6+} tolerant bacterium, *Pseudomonas* K-21, was capable of reducing Cr^{6+} in the presence of glucose. So, we examined the effect of

glucose on the induction of the Cr^{6+} reducing enzyme in *P. ambigua* G-1. Glucose was added to the nutrient broth at a concentration of 0.5%. However, glucose did not affect the induction of the enzyme. On the other hand, glucose (20 mM) completely protected the enzyme from inactivation on dialysis. Fructose, galactose and sucrose did not show any protective effect at up to 50 mM.

DISCUSSION

In a previous paper,[7] we reported that Cr^{6+} tolerant *P. ambigua* G-1 exhibited a membrane barrier as to the penetration of Cr^{6+} into the cells, as a mechanism of Cr^{6+} tolerance in the bacterium. However, since some Cr accumulation in *P. ambigua* G-1 was observed when cells were cultivated in nutrient broth containing Cr^{6+}, some of the Cr^{6+} penetrated into the cells through the membrane barrier. This implied that *P. ambigua* G-1 might have a mechanism for the detoxification of Cr^{6+}. In this study, we demonstrated that *P. ambigua* G-1 could reduce Cr^{6+} to Cr^{3+}, *i.e.*, it has a Cr^{6+} reducing enzyme for Cr^{6+} reduction. This reduction may be a second mechanism of Cr^{6+} tolerance in *P. ambigua* G-1.

The reducing enzyme of *P. ambigua* G-1 requires NADH as a hyrogen donor, but not NADPH, in the reduction of Cr^{6+}. Gruber and Jennette[11] showed that the incubation of Cr^{6+} with rat liver microsomes in the presence of NADPH resulted in reduction of the Cr^{6+}, suggesting that the Cr^{6+} reducing enzyme in rat liver microsomes required NADPH as a hydrogen donor.

Shimada and Matsushima[12] pointed out that glucose might play an important part in the Cr^{6+} reduction by Cr^{6+} tolerant *Pseudomonas* K-21, which was isolated from Cr sludge. On the other hand, *P. ambigua* G-1 did not require glucose for the reduction of Cr^{6+}. However, glucose protected the Cr^{6+} reducing enzyme from inactivation on dialysis. Thus, glucose acted as a stabilizer for the reducing enzyme. At present, we have no idea as to the mechanism of the protection.

Although Cr^{3+} and NAD were found in the reaction mixture as products after reduction of Cr^{6+} by the reducing enzyme, the detailed mechanism of the reaction was not clarified. We are now attempting the purification and characterization of the Cr^{6+} reducing enzyme of *P. ambigua* G-1.

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