NAD(P)H-Dependent Chromium(VI) Reductase of *Pseudomonas ambigua* G-1: a Cr(V) Intermediate Is Formed during the Reduction of Cr(VI) to Cr(III)

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An NAD(P)H-dependent Cr(VI) reductase (molecular weight = 65,000) was purified from a Cr(VI)-resistant bacterium, *Pseudomonas ambigua* G-1. Stoichiometric analysis of the enzymatic reaction showed that the enzyme catalyzed the reduction of 1 mol of Cr(VI) to Cr(III) while consuming 3 mol of NADH as an electron donor. Chromium(VI) was reduced to Cr(V) by one equivalent NADH molecule in the absence of the enzyme. Electron spin resonance analysis showed that Cr(V) species (g = 1.979) was formed during the enzymatic reduction. The amount of Cr(V) species formed was about 10 times larger than that of the nonenzymatic reduction. These findings show that the Cr(VI) reductase reduced Cr(VI) to Cr(III) with at least two reaction steps via Cr(V) as an intermediate.

Hexavalent chromium [Cr(VI)] compounds are extremely toxic, mutagenic, and carcinogenic (5, 10, 18, 29). They have strong oxidizing activities and cause various biological damages. Some bacteria have Cr(VI)-reducing activities. We reported previously that *Pseudomonas ambigua* G-1 showed NADH-dependent Cr(VI)-reducing activity in the cell extract (11–13). Ishibashi et al. (14) found NAD(P)H-dependent Cr(VI)-reducing activity in the cell-free supernatant fluids from *Pseudomonas putida*. Das and Chandra (6) have reported that Cr(VI) was reduced to Cr(III) in the presence of NAD(P)H in the cell extract of *Streptomyces* species. A membrane-associated chromate reduction system was also reported. Ohtake and coworkers (20) and Wang and coworkers (26–28) have shown that Cr(VI) was reduced to Cr(III) under anaerobic conditions by *Enterobacter cloacae* and its reduction was caused by the respiratory chain system of the cell membrane. We considered that these bacterial Cr(VI) reduction systems contribute to its detoxification by Cr(VI)-resistant bacteria. However, the Cr(VI) reductase has not yet been purified, and the details of the reaction mechanism are still unclear.

P. ambigua G-1 was able to grow in medium containing up to 20 mM K_2CrO_4 (13). The Cr(VI)-reducing activity (11) of this strain was more thermostable than that of other pseudomonads (14). The Cr(VI)-sensitive mutants of this strain lost the reducing activity (12). These results indicate that this strain is a good source to investigate the Cr(VI) reduction mechanism which contributes to Cr(VI) detoxication.

In the present paper, we report the purification of NAD(P)H-dependent Cr(VI) reductases of *P. ambigua* G-1 and the analysis of the catalytic mechanism of Cr(VI) reduction by the enzymes.

MATERIALS AND METHODS

Chemicals. NADH, NADPH, and NAD were from Oriental Yeast Co. (Tokyo, Japan). K_2CrO_4, $CrCl_3$, and 3-(*N*-morpholino)propanesulfonic acid (MOPS) were from Wako Pure Chemical Industry Ltd. (Tokyo, Japan). Tris was from Sigma Chemical Co. (St. Louis, Mo.), and 1,5-diphenylcarbazide was from Nakarai Chemicals Ltd. (Tokyo, Japan).

Preparation of Cr(VI) reductase. Cells grown in 4 liters of L broth at 37°C were harvested at the late logarithmic phase, washed twice with 20 mM Tris-HCl, pH 7.2, suspended in 100 ml of the same buffer, and disrupted by sonication. After removal of the unbroken cells by centrifugation (1,500 × *g*, 30 min), the supernatant fluid was heat treated at 60°C for 2 min and centrifuged (1,500 × *g*, 30 min). The supernatant fluid was separated on a DE32 column (3.5 by 6 cm; Whatman Ltd., Maidstone, England) previously equilibrated with buffer A (50 mM glucose in 20 mM Tris-HCl, pH 7.2), and proteins were eluted with 0.4 M NaCl in buffer A at a flow rate of 100 ml/h. The fractions carrying the Cr(VI)-reducing activity were concentrated and separated by Cu^{2+} chelate affinity chromatography using an AF-chelate Toyopearl 650M column (2.6 by 4 cm; Toso Ltd., Tokyo, Japan) which was sequentially equilibrated with 200 μM $CuSO_4$ solution and buffer B (0.5 M NaCl in buffer A). Proteins were eluted with a 0 to 60 mM linear gradient of imidazole in buffer B at a flow rate of 80 ml/h. The active fractions were concentrated, dialyzed against buffer A, then applied to a DEAE-Toyopearl 650M column (1.5 by 7 cm; Toso Ltd.) previously equilibrated with buffer A, and eluted with a 0 to 0.4 M linear gradient of NaCl in buffer A at a flow rate of 70 ml/h. In the next step, the active fractions were concentrated and separated on a Sephadex G-100 column (1.5 by 68 cm; Pharmacia Co., Uppsala, Sweden) with 0.3 M NaCl in buffer A at a flow rate of 14 ml/h. Finally, the active fractions were concentrated, dialyzed against buffer A, and purified by high-performance liquid chromatography (HPLC) using a DEAE-5PW column (Toso Ltd.; equilibrated with buffer A) with a 0 to 0.6 M linear gradient of NaCl in buffer A at a flow rate of 0.5 ml/min. All of the procedures described above were carried out at 8°C, but HPLC purification was done at room temperature.

Assay method for Cr(VI) reductase. The Cr(VI)-reducing activity was assayed by measuring the decrease of Cr(VI) as
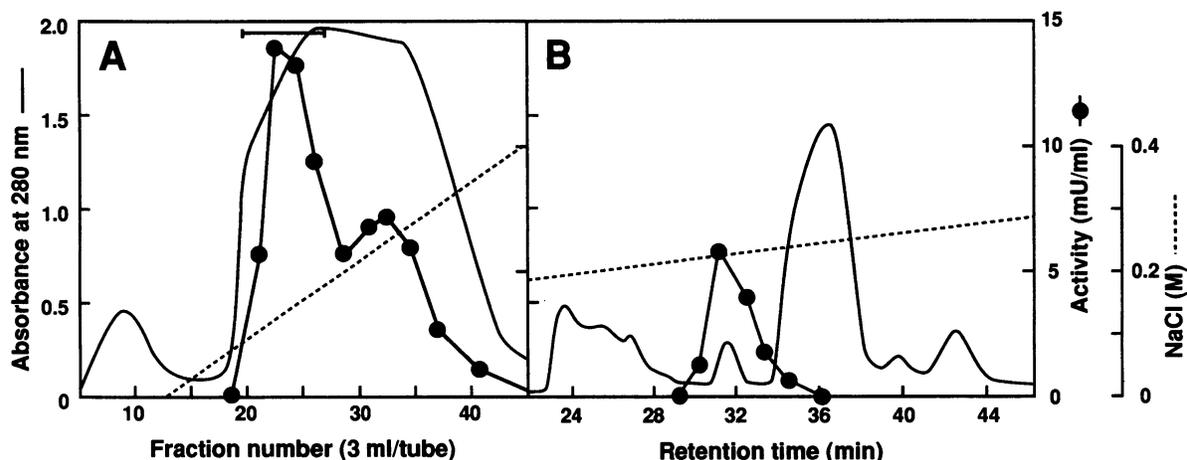
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FIG. 1. (A) DEAE-Toyopearl 650M column chromatography. Active fractions from Cu chelate affinity chromatography were pooled, dialyzed against buffer A, applied to a DEAE-Toyopearl column for chromatography, and eluted with a 0 to 0.4 M gradient of NaCl. Activity, Cr(VI) reductase activity. Fractions 21 to 27, indicated by a bar, were pooled and applied to a Sephadex G-100 column for chromatography. (B) DEAE-5PW column chromatography. The active fractions from Sephadex G-100 chromatography were dialyzed against buffer A, applied to a DEAE-5PW column for HPLC, and eluted with a 0 to 0.6 M gradient of NaCl in buffer A.

described previously (11). The amount of residual Cr(VI) in the reaction mixture was determined with 1,5-diphenylcarbazide. One unit of the Cr(VI)-reducing activity was defined as the amount of the enzyme which decreased 1 μmol of Cr(VI) per min at 50°C.

Estimation of molecular weight of Cr(VI) reductase. Purified Cr(VI) reductase was applied to a HPLC gel filtration column (DIOL-300; Shimadzu, Kyoto, Japan) which showed higher resolution and reproducibility than Sephadex G-100. The enzyme was separated with 0.3 M NaCl in 20 mM Tris-HCl (pH 7.2) at a flow rate of 0.5 ml/min. Glutamate dehydrogenase (290,000), lactate dehydrogenase (142,000), enolase (67,000), adenylate kinase (32,000), and cytochrome *c* (12,400) were used as molecular weight standards. The same sample was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis after heat denaturation in the presence of 75 mM dithiothreitol (17) by using cross-linked cytochrome *c* (Oriental Yeast Co., Tokyo, Japan) as a molecular weight standard.

Stoichiometric analysis of Cr(VI) reduction. One milliliter of reaction mixture containing 0.66 mU of enzyme, 0.2 mM K_2CrO_4, and 0.2 mM NADH in 20 mM Tris-HCl buffer (pH 7.0) was incubated for 0, 0.5, 1.0, 1.5, 2.0, and 2.5 h at 50°C. The components (NADH, NAD, and CrO_4^{2-}) in 0.2 ml of the reaction mixture were quantitatively analyzed immediately by HPLC. Residual CrO_4^{2-} ion was analyzed on a mono Q HR 5/5 column (Pharmacia Co.) equilibrated with 20 mM Tris-HCl buffer (pH 8.6). Chromate ion was eluted with a 0 to 0.5 M gradient of NaCl in the same buffer at a flow rate of 2.0 ml/min (24) and detected at 240 nm. Coenzymes (NADH and NAD) were separated on a Shim-pack CLC octyldecyl silane column (6 mm by 15 cm; Shimadzu) with 10 mM phosphate buffer (pH 2.6) at a flow rate of 1.5 ml/min and detected at 260 nm as described by the manufacturer.

ESR measurement. Reduced form of chromium was characterized by electron spin resonance (ESR) spectrometry. The reaction mixture contained 4.3 mU of enzyme per ml, 1.0 mM K_2CrO_4, and 1.0 mM NADH. ESR spectra (X band) were obtained with a JEOL JES-FE1XG spectrometer with a Teflon capillary tube (0.5 mm [inside diameter] by 20 cm) as a cell. The g value was calibrated with a standard sample

(2,2,6,6-tetramethyl-4-piperidinol-1-oxyl, g = 2.0055). All spectra were measured at room temperature.

RESULTS

We purified the Cr(VI) reductase of *P. ambigua* G-1 by heat treatment, anion-exchange chromatography, Cu^{2+} chelate affinity chromatography, gel filtration, and HPLC with an anion-exchanger column. On DEAE-Toyopearl, two active peaks were obtained (Fig. 1A). The first peak, showing higher activity, was pooled and applied to the next step of purification. The purified sample gave a single band on polyacrylamide gel electrophoresis (data not shown). Overall 38-fold purification relative to that of the crude extract was realized, as shown in Table 1. The crude extract contained a large amount of an unidentified low-molecular-weight compound which had Cr(VI)-reducing activity. It was removed by DE32 column chromatography. Thus, the apparent enzyme yield (1.3%) seemed to be extraordinarily low.

The estimated molecular mass of the purified enzyme was 65,000 Da (intact) on gel filtration and 25,000 Da (denatured) on SDS-polyacrylamide gel electrophoresis. We also inves-

TABLE 1. Purification of Cr(VI) reductase

Step	Vol (ml)	Total protein (mg)	Total activity (mU)[a]	Sp act (mU/mg)	Yield (%)	Purification (fold)
Crude extract	119	4,200	2,900	0.7	100	1
Heat treatment	97	1,518	5,830	4.0	201	6.0
DE32	25.5	538.1	810	1.5	27.9	2.3
AF-chelate-Toyopearl	11.7	234.0	297	1.3	10.2	1.9
DEAE-Toyopearl	1.9	93.2	97	1.0	3.3	1.6
Sephadex G-100	1.8	30.5	90	3.0	3.1	4.5
HPLC, DEAE-5PW	5.7	1.5	38	25.3	1.3	38.0

[a] An aliquot of enzyme solution was added to 1 ml of 20 mM Tris-HCl (pH 7.2) containing 0.2 mM K_2CrO_4 and 0.2 mM NADH and incubated at 50°C for 30 min. The amount of residual Cr(VI) in the reaction mixture was determined by the 1,5-diphenylcarbazide method. Milliunits are nanomoles of Cr(VI) decreased per minute.
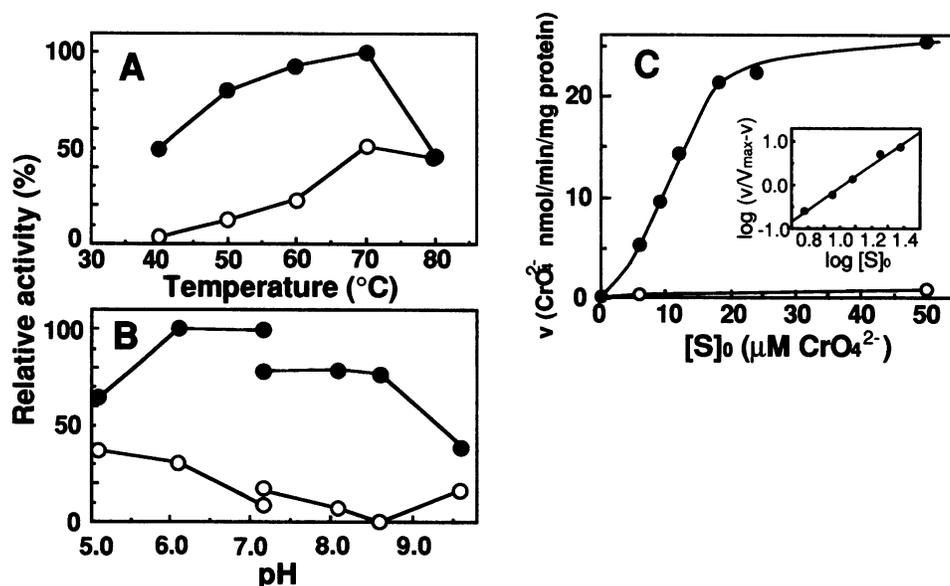
FIG. 2. Enzymatic properties of purified Cr(VI) reductase. Symbols: ●, in the presence of enzyme; ○, in the absence of enzyme. (A) Effect of temperature. Reaction mixtures (0.66 mU of enzyme, 0.2 mM NADH, and 0.2 mM K_2CrO_4 in 1 ml of 20 mM Tris-HCl [pH 7.2]) were incubated for 30 min at 40 to 80°C. Residual Cr(VI) in the reaction mixture was determined by HPLC using a mono Q column. (B) Effect of pH. Phosphate buffer (100 mM) was used for pHs 5.1, 6.1, and 7.2; 100 mM Tris-HCl buffer was used for pHs 7.2, 8.1, 8.6, and 9.6. Reactions were carried out at 50°C for 30 min. Other conditions and symbols were the same as for panel A. (C) Rate-versus-substrate plot. The same mixture as for panel A, but containing 0 to 50 μM K_2CrO_4, was incubated at 50°C for 30 min. (Inset) Hill's plot.

tigated the second active peak on DEAE-Toyopearl chromatography but have not yet succeeded in its purification to homogeneity. However, the molecular mass was estimated to be 130,000 Da on gel filtration, and we could not detect a visible band of 25,000 Da (identical to this purified enzyme) on SDS-polyacrylamide gel electrophoresis. This suggested that this strain also produces another Cr(VI) reductase.

We examined some enzymatic properties of purified Cr(VI) reductase. The optimal temperature and pH were determined on the basis of the difference in Cr(VI)-reducing activities in the presence and absence of the enzyme (because the nonenzymatic reductions were not insignificant at high temperature and low pH). It was found that the optimal conditions were 50°C and pH 8.6 (Fig. 2A and B). In these conditions, nonenzymatic reduction was at a relatively low level. However, this enzyme showed high activity at a wide range of temperatures (40 to 70°C) and pHs (6 to 9). In phosphate buffer, the enzyme was more active (25% higher) than in Tris-HCl at pH 7.2. The rate-versus-substrate plot gave a sigmoidal curve (Fig. 2C). An apparent Hill's coefficient (n) was 2.6 (Fig. 2C, inset). These data indicated that the enzyme is allosteric. Kinetic analysis showed a K_m value of 13 μM CrO_4^{2-} and a V_{max} of 27 nmol/min/mg of protein.

In our previous paper, it was reported that the enzymatic reduction of Cr(VI) by a cell extract of *P. ambigua* G-1 required NADH as an electron donor (11). We tested the effects of other electron donors on the Cr(VI) enzymatic reduction (Table 2). This enzyme was active in the presence of NADH or NADPH. NADPH gave 63% as much activity as NADH. The enzyme was not active in the absence of these coenzymes. Glutathione (4), ascorbic acid (25), D-glucose (4), and D-fructose (21), which were reported as the nonenzymatic reductants of Cr(VI), did not support the Cr(VI)-reducing activity of this enzyme.

In the reaction mixture, Cr(VI) reduction and NADH consumption were analyzed stoichiometrically (Fig. 3). Dur-

ing incubation of K_2CrO_4 with NADH, both CrO_4^{2-} and NADH levels were slightly decreased nonenzymatically (Fig. 3A). Since each decreased amount was equivalent, the nonenzymatic reduction of Cr(VI) by NADH was possibly a one-electron reduction of Cr(VI) to Cr(V). In the presence of the enzyme, the decrease of CrO_4^{2-} proceeded strongly (Fig. 3B). The enzymatic reduction was equilibrated during a 2.5-h incubation. At the early phase, in a 0.5-h reaction, the ratio between the amount of decreased NADH and that of CrO_4^{2-} was 2.0 and gradually increased to 2.8 at equilibrated phase. These results indicated that the enzyme catalyzed a three-electron reduction of Cr(VI) to Cr(III), consuming three NADH molecules. In addition, the molar ratio at the early phase was different from that of the three-electron

TABLE 2. Effects of electron donor on Cr(VI) reductase activity

Electron donor (0.2 mM)	Activity (nmol/30 min)[a]		Ratio[b]	Relative enzymatic activity[c] (%)
	+Enzyme	−Enzyme		
None	<0.1	<0.1	—	0
NADH	10.7	2.8	3.8	100
NADPH	6.0	1.0	6.0	63
Glutathione	5.6	5.8	0.97	0[d]
Ascorbic acid[e]	7.0	7.2	0.96	0[d]
D-Glucose	<0.1	<0.1	—	0
D-Fructose	<0.1	<0.1	—	0

[a] Average from duplicate assays. Reaction mixtures (0.33 mU of enzyme, 0.2 mM electron donor, and 0.2 mM K_2CrO_4 in 1 ml of 20 mM Tris-HCl [pH 7.2]) were incubated for 30 min at 50°C. The amount of residual Cr(VI) in the reaction mixture was determined by the HPLC method using the mono Q column.
[b] (+Enzyme)/(−Enzyme). —, not determined.
[c] [(+Enzyme) − (−Enzyme)]/[(+Enzyme)$_{NADH}$ − (−Enzyme)$_{NADH}$].
[d] Negative value was regarded as 0.
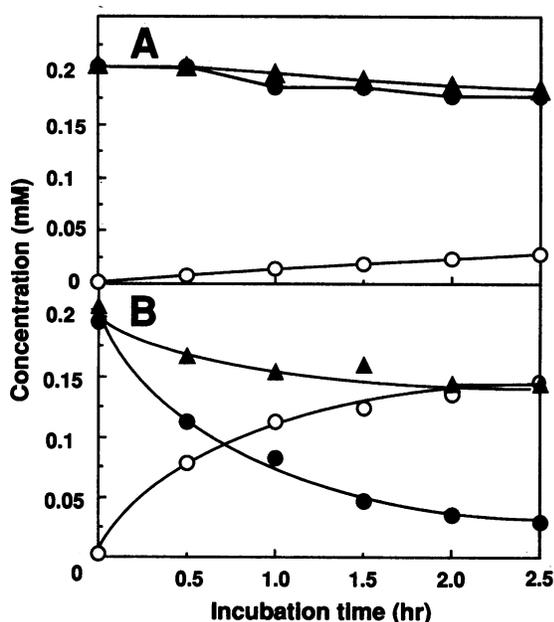[e] 0.04 mM ascorbic acid.

FIG. 3. Stoichiometric analysis of the enzymatic Cr(VI) reduction. The initial composition of the reaction mixture was 0.2 mM K_2CrO_4 and 0.2 mM NADH in 20 mM Tris-HCl (pH 7.2). (A) In the absence of the enzyme; (B) in the presence of the enzyme (0.66 mU/ml). Each reaction mixture (0.2 ml) was subjected to mono Q and CLC octyldecyl silane column chromatography to analyze the concentrations of CrO_4^{2-} (▲), NADH (●), and NAD (○).

FIG. 4. ESR analysis of the enzymatic reduction of Cr(VI). The initial compositions of the reaction mixture (total, 1.0 ml) were 1.0 mM K_2CrO_4 in 20 mM MOPS buffer (pH 7.0) (a); 1.0 mM K_2CrO_4, 4.3 mU of enzyme per ml, and 1.0 mM NADH in the same buffer (b); and the same as in line b but without enzyme and NADH (d and e, respectively). Reaction mixtures were incubated at 50°C for 2 h. Modulation amplitude, 20 G; field, 3,350 ± 500 G; scan time, 8 min; time constant, 0.3 s; receiver gain, 5,000; analysis temperature, 25°C. (c) The same as line b, but modulation amplitude, 0.25 G; field, 3,405 ± 25 G; receiver gain, 7,900.

reduction. This result suggested that the enzymatic event proceeded by two (or more) steps. In this condition, 75% of the CrO_4^{2-} ions remained at the equilibrated phase because NADH was consumed. When the enzyme reacted with 20 μM K_2CrO_4, six of seven parts of the CrO_4^{2-} were reduced within 30 min (data not shown).

We performed an ESR analysis to identify the intermediate and product of the enzymatic reduction of Cr(VI). When K_2CrO_4 solution was incubated with NADH and the enzyme, a signal due to Cr(V) (g value = 1.979) (21) was observed (Fig. 4b). The signal showed a complex structure on narrow-range scanning (Fig. 4c). This spectrum corresponded to the superhyperfine structure which is identical to the complexes of Cr(V) and diol compounds such as NAD(P)H, flavin adenine dinucleotide, and some sugars (21). The Cr(V) signal decreased by the prolongation of reaction time (data not shown). During the incubation in the absence of enzyme, the signal due to Cr(V) also appeared but was weaker (about 1/10) than that observed in the presence of the enzyme (Fig. 4d). These results suggested that the enzyme formed one Cr(V) molecule as an intermediate by using one NADH molecule as an electron donor.

However, the signal due to Cr(III) was not observed. When the enzyme was added to 0.2 mM $CrCl_3$, the ESR signal of Cr(III) (Fig. 5a) was completely masked (Fig. 5b). When the excess amount of $CrCl_3$ (1.0 mM) was incubated with the enzyme, the signal of Cr(III) reappeared (Fig. 5c) but its peak to peak line width (ΔH_{pp}) was two times wider than that in the absence of enzyme. These data suggested that the enzyme interacted with Cr(III). Thus, the enzymatically produced Cr(III) also interacted with the enzyme, and its signal intensity was too weak to be detected by our ESR system.
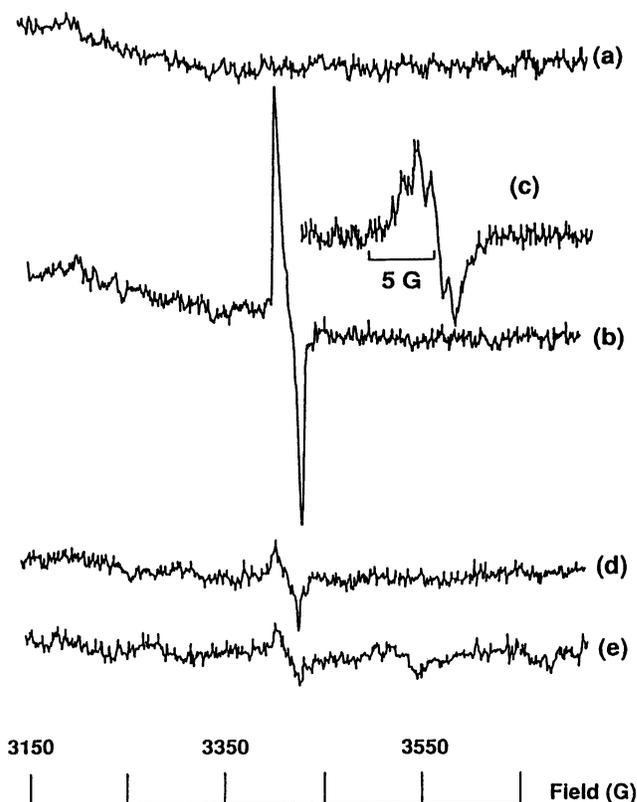
DISCUSSION

An NAD(P)H-dependent Cr(VI) reductase was purified to homogeneity from cell extracts of *P. ambigua* G-1. The estimated molecular weight for the intact enzyme was 65,000 on gel filtration, and that for the denatured subunits was 25,000 on SDS-polyacrylamide gel electrophoresis. The ratio of these observed molecular weights was 2.6:1. These data suggested that this enzyme has a dimeric or trimeric structure. Further study, such as a study with a laser scattering method or mass spectrometry, is necessary to determine the correct oligomeric structure. Kinetic analysis showed that this enzyme is homotropically allosteric. The enzyme was active within a wide range of temperatures (40 to 70°C) and pHs (6 to 9). These data suggested that this enzyme is active in physiological intracellular conditions, such as 37°C and pH 7.

P. ambigua G-1 has another NAD(P)H-dependent Cr(VI)-reducing activity, the minor active fraction on DEAE-Toyopearl chromatography with a molecular mass of 130,000 Da as estimated by gel filtration. However, we have not yet succeeded in its purification. Ishibashi et al. (14) reported that *P. putida* has an NAD(P)H-dependent Cr(VI)-reducing activity. It showed a K_m of 40 μM CrO_4^{2-}, which is an affinity similar to that of *P. ambigua* G-1 (K_m = 13 μM). It
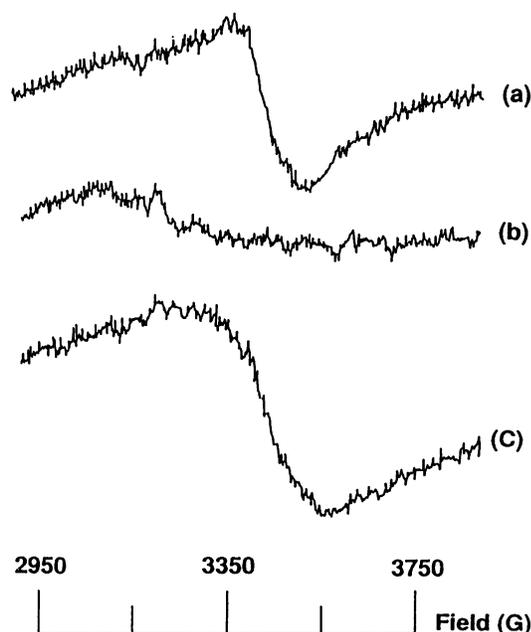
FIG. 5. Effect of the enzyme on the ESR spectrum of Cr(III). The compositions of the reaction mixtures (total, 1 ml) were 0.2 mM $CrCl_3$ and 0.2 mM NADH in 20 mM MOPS buffer, pH 7.0 (a), the same as in line a but with 4.3 mU of enzyme per ml added (b), and the same as in line b but with 1.0 mM $CrCl_3$ (c). The mixtures were incubated at 50°C for 30 min and analyzed under the conditions described in the legend to Fig. 3.

may be a system identical to that of *P. ambigua* G-1, even though it seemed different on thermostability. However, it is quite different from the anaerobic membrane-associated system of *E. cloacae* (20, 26–28).

We have described the catalytic mechanism of purified Cr(VI) reductase. The evidence demonstrates that this enzyme catalyzes the reduction of Cr(VI) to Cr(III) by two (or more) steps. First, Cr(VI) accepts one electron from one NADH molecule and generates Cr(V) as an intermediate. Second, the Cr(V) intermediate accepts two electrons from two molecules of the same coenzyme. The first step proceeds more rapidly than the second one.

Shi and Dalal (21) proposed a model of Cr(VI) reduction to Cr(III) by diol compounds in which diols are decomposed to aldehydes. Our data showed that NADH was converted to NAD during Cr(VI) reduction in either the presence or absence of enzyme. It shows that NADH acts as an electron donor by its oxidation to NAD. The model for diols is not applicable to the case of NADH.

Recent reports showed that Cr(VI) is reduced nonenzymatically by some bioreductants, such as NAD(P)H, ascorbic acid, glutathione, and reducing sugars (1, 4, 9, 21, 23, 25). ESR analysis revealed that the reduction of Cr(VI) caused by these reagents proceeded up to Cr(V). It was also suggested that Cr(V) is actually a toxic and mutagenic agent (2, 15, 16). Some reports showed that glutathione reductase (22), DT-diaphorase (7), aldehyde oxidase (3), and cytochrome P-450 (8, 19, 23) have Cr(VI)-reducing activity. However, it is still unclear whether these enzymes contribute to the detoxication or rather to the toxification. It is necessary to elucidate the behavior of the intermediates formed during reduction in order to achieve a good understanding of the overall mechanism of Cr(VI) toxicity and its detoxication.

We propose the two-step reduction model as mentioned above. We believe that this model is applicable for the other biological reduction system of Cr(VI), even though it should be different from that of other organisms in which these steps proceed by the nonenzymatic way, by distinct enzymes, or by a single enzyme, as in this case.

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