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CHROMATE REDUCTION BY RABBIT LIVER ALDEHYDE OXIDASE

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SUMMARY. Chromate was reduced during the oxidation of 1-methylnicotinamide chloride by partially purified rabbit liver aldehyde oxidase. In addition to 1-methylnicotinamide, several other electron donor substrates for aldehyde oxidase were able to support the enzymatic chromate reduction. The reduction required the presence of both enzyme and the electron donor substrate. The rate of the chromate reduction was retarded by inhibitors of aldehyde oxidase but was not affected by substrates or inhibitors of xanthine oxidase. These results are consistent with the involvement of aldehyde oxidase in the reduction of chromate by rabbit liver cytosolic enzyme preparations.

Chromates and other chromium(VI) compounds are toxic and carcinogenic in experimental animals and are mutagenic in the Ames test (1,2). Compounds containing chromium(VI) have also been implicated as factors in the high incidence of lung cancer among chromate workers (3). In contrast with chromates, most chromium(III) compounds are relatively nontoxic, noncarcinogenic, and nonmutagenic. The two oxidation states of chromium also differ in their metabolic fates. Chromate ions are rapidly transported across cellular membranes and are reduced to chromium(III) by both intracellular and extracellular molecules. However, chromium(III) does not easily penetrate cells and is not oxidized by cellular constituents (4).

Jennette has proposed an "uptake-reduction" model to account for the differing toxicities of chromium(VI) and chromium(III) compounds (5). According to this model, the greater toxicity exhibited by chromate is the result of its more efficient uptake into cells and its ability to undergo subsequent intracellular reduction. Chromium(III) or other labile oxidation states of chromium formed during the reduction are then capable of reacting

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directly with cellular proteins and nucleic acids. The direct oxidation of certain critical molecules by intracellular chromate may also occur.

Although reductive metabolism is believed to play a key role in the mechanism of chromate toxicity, relatively few compounds have been identified which reduce chromate at physiological pH. Among low molecular weight compounds which have been studied, only ascorbate and thiols reduce chromate at significant rates in vitro (6). Certain enzymes of the cytoplasm, endoplasmic reticulum and mitochondria of mammalian cells have been reported to catalyze chromate reduction (7,8). The few chromate reductases which have been characterized include microsomal cytochrome P-450 systems (9,10), rat liver DT-diaphorase (11), and human erythrocyte glutathione reductase (12). All of these enzymes depend on NADPH or NADH as electron donor cofactors.

In this communication, we report evidence that chromate is reduced in vitro by rabbit liver cytosolic aldehyde oxidase. Aldehyde oxidase (E.C.1.2.3.1) is a molybdenum-containing flavoenzyme which occurs in the liver and other tissues of many mammals, including humans, and which is capable of oxidizing or reducing a variety of xenobiotics (13). In contrast with other reported chromate reductases, this enzyme does not require pyridine nucleotide cofactors for its catalytic activity, but uses aldehydes or heterocyclic imines (e.g., purines, pyrimidines, pyridinium salts) as electron donor substrates. Aldehyde oxidase-catalyzed reduction appears to represent an additional pathway of cytoplasmic chromate metabolism which could have possible significance in vivo.

MATERIALS AND METHODS

Reagents. 1-Methylnicotinamide chloride, 6-methylpurine, menadione, allopurinol and ethylenediamine tetraacetic acid ferric-sodium salt were purchased from Sigma; benzaldehyde, potassium dichromate, potassium monohydrogen phosphate, and potassium cyanide from Fisher; xanthine from Aldrich; and potassium ferricyanide from Mallinkrodt.

Enzyme Preparation. Frozen livers from mature rabbits (mixed sexes) were obtained from Pel-Freez Biologicals and were stored at -20° prior to use. Aldehyde oxidase was partially purified by ammonium sulfate fractionation of heat treated rabbit liver cytosol according to the procedure of Rajagopalan and coworkers (14). Control aldehyde oxidase activity was measured by the method of Rajagopalan and Handler using 1-methylnicotinamide chloride as substrate and

potassium ferricyanide as electron acceptor (15). Protein concentrations were determined by the Lowry method (16).

Chromate Reduction Assays. Reduction was determined spectrophotometrically by measuring the rate of disappearance of the 373nm band ($\varepsilon = 4.7 \times 10^3\ M^{-1}cm^{-1}$) of chromate. Sample cuvettes contained 2.0 ml of 0.05M phosphate buffer (pH 7.8, 0.1mM EDTA present), 0.1-0.2 ml of aldehyde oxidate preparation (2 mg/ml final protein concentration), 0.1 ml of 3mM potassium dichromate (giving 0.2mM final concentration of chromate, the predominant form of hexavalent chromium at pH 7.8), 0.1 ml of 0.3 M 1-methylnicotinamide chloride (10mM final concentration) and deionized water to give 3.0 ml final volume. Final concentrations of alternative reducing substrates were as listed in Table 3. Reference cuvettes contained deionized water instead of reducing substrate. Reactions were initiated by addition of substrate after preincubation of cuvettes at 37° for five minutes. Initial rates of chromate reduction were measured over the first two minutes of reaction. Inhibitors, when present, were included in the preincubations and had final concentrations as listed in Table 2. All spectroscopic and kinetic measurements were recorded on a Hitachi 100-80 model spectrophotometer equipped with thermostatted cell holders and program mode for enzyme kinetic analysis. Chromate determinations by the diphenylcarbazide method were performed as described by DeFlora et al (11).

RESULTS AND DISCUSSION

Chromate was reduced when incubated with partially purified rabbit liver aldehyde oxidase and 1-methylnicotinamide chloride, a substrate for oxidation by the enzyme. The reduction was accompanied by a time-dependent decrease in the 373-nm visible absorption band of chromate. No significant reduction occurred when 1-methylnicotinamide chloride or aldehyde oxidase was incubated with chromate in the absence of other reaction components (Table 1). The rate of chromate reduction was dependent on the concentrations of 1-methylnicotinamide chloride and aldehyde oxidase preparation added to the incubations (data

Table 1. Chromate Reductase Activity of Rabbit Liver Aldehyde Oxidase

Incubation Conditions	Chromate Reductase Activity (Nmol chromate reduced/min/mg protein)
Enzyme + 1-methylnicotinamide + chromate	6.0 ± 0.3
Enzyme + chromate	0.1 ± 0.2
1-Methylnicotinamide + chromate	0

Incubation and reduction assays were carried out as described in Materials and Methods. Each value represents the mean ± SD of four experiments. The control aldehyde oxidase, measured with 1 mM potassium ferricyanide, was 120 ± 2 nmol ferricyanide reduced/min/mg protein (mean of two assays).

Table 2. Inhibition of Rabbit Liver Chromate Reductase

Compound	Percent Inhibition
None	0
Menadione (3.3 μM)	78
KCN (0.2 mM)	59
Allopurinol (0.2 mM)	0

Incubations and assays were carried out as described in Materials and Methods. Results are expressed as percent inhibition of chromate reductase activity with 1-methylnicotinamide chloride (10 mM) as electron donor substrate. Each value represents the mean of four experiments, each done in triplicate. Control chromate reductase activity was 5.6 ± 0.5 nmol chromate reduced/min/mg protein (mean ± SE; N = 4). Control aldehyde oxidase activity was 75.8 ± 8.9 nmol ferricyanide reduced/min/mg protein (mean ± SE; N = 4).

not presented). Decreased chromate reductase activity was observed when inhibitors of aldehyde oxidase, such as menadione or potassium cyanide, were present in the incubation mixtures (Table 2).

Reduction of chromate could also be monitored by treating aliquots of incubation mixtures with diphenylcarbazide, a reagent which forms a colored complex with chromium(VI), but not with chromium(III) (17). This technique was used to determine the time course of the chromate reduction (Figure 1). The concentration of chromate was decreased to approximately 24% of its original value during a two-hour incubation with aldehyde oxidase and 1-methylnicotinamide chloride; no significant nonenzymatic reduction of chromate by protein or 1-methylnicotinamide chloride occurred during this time.

In addition to 1-methylnicotinamide chloride, a variety of other aromatic nitrogen heterocycles and aldehydes are known to act as electron donor substrates for aldehyde oxidase (13). All of the alternative substrates which we examined were effective in supporting chromate reduction (Table 3). Furthermore, those compounds which were the most active substrates for aldehyde oxidase, as measured by ferricyanide reduction, were also the most active electron donor substrates for the chromate reductase.

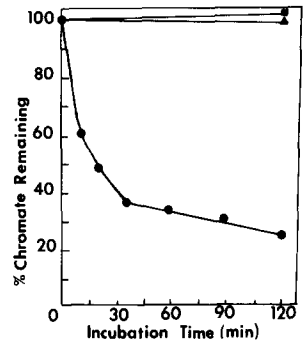

Figure 1. Time course for reduction of chromate by rabbit liver aldehyde oxidase preparations. All incubations were carried out with shaking at 37° and contained phosphate buffer (0.033M, pH 7.8), chromate (0.2mM), 1-methylnicotinamide chloride (10mM) and aldehyde oxidase preparation (2 mg/ml). Control experiments were carried out in the absence of aldehyde oxidase (■) or of 1-methylnicotinamide (▲). Each value represents the mean of two experiments, each in triplicate. Chromate concentrations were assayed by the diphenylcarbazide method (11).

Partially purified preparations of rabbit liver aldehyde oxidase were found to contain small amounts of xanthine oxidase, a molybdenum-containing enzyme which shares many of the catalytic properties of aldehyde oxidase, including its ability to reduce a large number of electron acceptors (13). Therefore, we investigated the possible involvement of this enzyme in the observed chromate reduction. No significant chromate reduction was detected when xanthine, a substrate for xanthine oxidase, was incubated with chromate

Table 3. Electron Donor Dependence of Rabbit Liver Chromate Reductase

Electron Donor	Chromate Reductase Activity (Nmol chromate reduced/min/mg protein)	
1-Methylnicotinamide (10 mM)	4.2 ± 0.2	(65.3)
Quinoline (1mM)	0.9 ± 0.1	(9.1)
Benzaldehyde (1 mM)	5.6 ± 0.4	(192)
6-Methylpurine (3.5 μM)	3.3 ± 0.5	(60.0)
Xanthine (0.3 mM)	0.1 ± 0.1	(5.3)

Incubations and reduction assays were carried out as described in Materials and methods. Values of chromate reductase activity represent mean ± SD of triplicate experiments. Values of aldehyde oxidase activities with these electron donors are given in parentheses; each value is expressed as nmol ferricyanide reduced/min/mg protein (mean of duplicate assays).

and partially purified aldehyde oxidase (Table 3). Furthermore, allopurinol, a specific inhibitor of xanthine oxidase, did not decrease the rate of enzymatic chromate reduction when N-methylnicotinamide served as the electron donor substrate (Table 2).

The data presented in Tables 1-3 demonstrate that the enzymatic reduction of chromate was dependent on the presence of compounds which act as electron donors for aldehyde oxidase and was diminished by inhibitors of aldehyde oxidase. Chromate reduction was not affected by substrates or inhibitors of xanthine oxidase. These results strongly suggest that aldehyde oxidase, rather than xanthine oxidase or some other oxido-reductase present in the enzyme preparation, is responsible for catalyzing the observed chromate reduction.

Chromium(VI) reductions usually result in the formation of chromium(III); intermediate oxidation states are unstable and readily undergo disproportionation unless they are complexed with stabilizing ligands. Products with absorbance at 550-650nm were formed during the aldehyde oxidase-catalyzed chromate reduction, and such absorption is consistent with the accumulation of chromium(III) species in solution. However, since the incubation media contained protein and other ligands (e.g., EDTA) which could complex with chromium(III), the interpretation of these very weak absorption bands is difficult. Furthermore, we cannot rule out the formation of long-lived chromium(V) species during the reductions. Chromium(V) complexes have been detected by ESR during reductions of chromate by glutathione, ribonucleotides, and microsomal systems (18,19,20).

The chromate reducing ability of aldehyde oxidase is consistent with the broad specificity of the enzyme towards electron acceptors, which include oxygen, nitroaromatic compounds, sulfoxides, silicomolybdate, ferricyanide and others (13). The catalytic molybdenum, iron-sulfur, and flavin centers of reduced aldehyde oxidase are each possible sites for the transfer of electrons to acceptors (21). Although the redox center responsible for chromate reduction has not yet been established, it is noteworthy that aldehyde oxidase

and several other enzymes with chromate reductase activity (e.g., NADPH cytochrome P-450 reductase, DT-diaphorase, glutathione reductase) are flavoproteins (7).

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