Purification and Characterization of Chlordecone Reductase from Human Liver

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The toxic organochlorine pesticide, chlordecone (Kepone), is excreted in human bile primarily as a stable, reduced monoalcohol metabolite. This bioreduction is catalyzed by a hepatic cytosolic enzyme activity termed chlordecone reductase. We purified this enzyme from human liver and found that chlordecone reductase resembles the family of xenobiotic metabolizing enzymes referred to as the aldo-keto reductases based on its biochemical characteristics, including its ability to catalyze the reduction of a carbonyl-containing substrate. However, analyses of liver cytosolic samples on immunoblots developed with anti-chlordecone reductase antibodies revealed that immunoreactive proteins were present only in those mammalian species that convert chlordecone to chlordecone alcohol in vivo (man, gerbil, and rabbit) and not in those species unable to reduce chlordecone (rat, mouse, and hamster). Hence, chlordecone reductase is unique among aldo-keto reductases in being species-specific. Quantitative immunoblot analyses of seven human liver specimens disclosed two immunoreactive proteins whose total concentration varied over a 6-fold range. Moreover, the amount of immunoreactive protein was directly proportional to chlordecone reductase activity in each sample. We conclude that chlordecone reductase is a unique aldo-keto reductase of potential clinical importance whose expression varies markedly among individuals.

The aldo-keto reductases are a family of curious cytoplasmic enzymes that convert many xenobiotic aldehydes and ketones to their corresponding reduced alcohol derivatives (1). These enzymes have broad substrate specificities, are present in abundance in most animal tissues, and are not inducible by their substrates (2). Despite their ubiquitous distribution, no physiological role for the aldo-keto reductases has yet been determined (3). Nevertheless, these enzymes are of clinical importance because they metabolize such drugs as daunorubicin (4), warfarin (5), and naloxone (6) and have been implicated in the pathogenesis of diabetic cataracts (7) and muscular dystrophy (8).

We have proposed that aldo-keto reductases play a key role in human metabolism of the toxic, organochlorine pesticide, chlordecone (CD) (9-11). Studies of blood, bile, and stool obtained from industrial workers heavily exposed to this organochlorine ketone suggested that CD undergoes bioreduction, primarily in the liver, to chlordecone alcohol (CDOH), a more readily excretable, monoalcohol derivative. However, administration of CD to rats, guinea pigs, hamsters, mice, and gerbils revealed that only in the last species did CDOH appear in the stool, bile, and liver (9). Subsequent tests of gerbil liver extracts disclosed that conversion of CD to CDOH was catalyzed in vitro by a cytosolic enzyme activity termed chlordecone reductase (CD reductase) (10). CD reductase activity shares many of the biochemical characteristics of the aldo-keto reductases. However, CD reductase activity is unusual in being species-specific and in being inducible by its substrate, CD (10). In the present study, we have definitively identified this enzyme activity by purifying CD reductase from human liver. We also provide immunoochemical evidence that CD reductase is a species-specific carbonyl reductase whose concentration in human liver varies markedly among individuals.

**EXPERIMENTAL PROCEDURES

Materials

CD (>99% pure as judged by GLC-mass spectroscopy) was a gift of the Environmental Protection Agency (Research Triangle Park, NC). CDOH and monohydrochlordecone alcohol (monohydro-CDOH) were prepared as described elsewhere (11). Pyridine 4-aldehyde, 4-benzoylpyridine, 9,10-phenanthrenequinone, NADP+, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, pyrazole, HgCl₂, CuSO₄, quercetin, indomethacin, diphenylhydantoin, ethacrynic acid, and reactive red 120-Agarose were purchased from Sigma; sodium phenobarbital from Amend Drug and Chemical Co. (Irvington, NJ); hydroxylapalatite and nitricellulose from Bio-Rad; DAE-E-cellulose from Whatman; goat anti-rabbit IgG and rabbit peroxidase anti-peroxidase from Miles Laboratories; and 3,3′-diaminobenzidine from Pfaltz & Bauer (Stamford, CT).

Mongolian gerbils were purchased from Tumblebrook Farms (West Brookfield, MA); Sprague-Dawley rats and New Zealand white rabbits from Flow Laboratories (McLean, VA); Hartley guinea pigs, Golden Syrian hamsters, and CD-1 mice from Charles River Breeding Laboratories (Wilmington, MA). Human liver specimens were obtained at surgery under protocols approved by the Committee for the Conduct of Human Research at the Medical College of Virginia. All patients have been described in detail elsewhere (12, 13).

Methods

Purification of Human Liver Chlordecone Reductase—All purification procedures were performed at 4 °C. Human liver (120 g) from

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The abbreviations used are: CD, chlordecone (1,1a,3,3a,4,4,5,5a,6-deca chlorrooctahydro-1,3,4-metheno-2H-cyclobuta[c]phenalen-2-one); CDOH, chlordecone alcohol (1,1a,3,3a,4,4,5,5a,6-deca chlorrooctahydro-1,3,4-metheno-2H-cyclobuta[c]phenalen-2-ol).
patient 16 (see Ref. 13) was minced and then homogenized in 2 volumes of 0.1 M potassium phosphate buffer, pH 6.5, in a Potter-Elvehjem homogenizer. A cytosolic fraction was prepared by centrifugation of the homogenate at 9,000 \( \times g \) for 15 min, followed by centrifugation of the supernatant at 105,000 \( \times g \) for 60 min. The supernatant, defined as cytosol, was fractionated by adding ammonium sulfate with constant stirring and adjusting the pH to 7.5 with 3 M ammonium hydroxide. The proteins which precipitated between 25% and 60% saturation were collected by centrifugation at 12,000 \( \times g \) for 20 min, dissolved in a minimal volume of buffer A (10 mM Tris-HCl, 5 mM dithiothreitol, 0.5 mM EDTA, pH 7.5), and dialyzed against 4 liters of buffer A with one change of buffer, and then were clarified by centrifugation at 12,000 \( \times g \) for 30 min. The supernatant was applied to two DEAE-cellulose columns (2.6 \( \times 32 \) cm), in parallel, equilibrated with 2 liters of buffer A. The columns were washed with the same buffer until the 3,000 of the effluent was less than 0.05 A. An 800-ml linear salt gradient of 0–200 mM NaCl in buffer A was passed through the column at a flow rate of 30 ml/h to elute the bound proteins. The peak CD reductase activity appeared in the eluate at approximately 25 mM NaCl. The enzyme-rich fractions were combined, extensively dialyzed against buffer A, and applied at a flow rate of 10 ml/h to a Reactive Red-Agarose column (2.5 \( \times 9 \) cm) previously equilibrated with 500 ml of buffer A. The column was initially washed with buffer A, followed by 100 ml of 0.5 M NaCl in buffer A, and then a 400-ml linear salt gradient of 0.5–3.5 M NaCl to elute the bound proteins. The peak CD reductase activity eluted at approximately 2.5 M NaCl. Enzyme-rich fractions were combined, extensively dialyzed against buffer A, and further purified by chromatography on a hydroxylapatite column (1 \( \times 5.5 \) cm) previously equilibrated with 40 ml of buffer A and then eluted with a 50-ml linear gradient of 0–200 mM potassium phosphate, pH 6.5. Fractions containing only CD reductase, as indicated by a single, silver-stained band when analyzed by electrophoresis on SDS-polyacrylamide gels, were concentrated by ultrafiltration and stored at -70 °C in 50% glycerol.

**Biochemical Methods**—CD reductase activity was assayed by incubating the protein sample in the presence of CD and an NADPH generating system in 100 mM potassium phosphate, pH 7.5, for 60 min at 37 °C (10). The product formed (CDOH) was extracted from the reaction mixture and measured by gas-liquid chromatography with the use of an internal standard (10). Other buffers tested (each containing only CD reductase, as indicated by a single, silver-stained band when analyzed by electrophoresis on SDS-polyacrylamide gels, were concentrated by ultrafiltration and stored at -70 °C in 50% glycerol.

**Purification of Human Liver Chlordecone Reductase**

**Results**

**Purification**—CD reductase was purified to apparent homogeneity from human liver by sequential column chromatography on DEAE-cellulose, Reactive Red-Agarose, and hydroxylapatite (Table I). Enzyme activity appeared as a single peak after each chromatographic step. In a typical purification sequence, 8% of the initial enzyme activity was recovered, representing a 348-fold enrichment in activity (Table I). When analyzed by electrophoresis on SDS-polyacrylamide gels, the purified CD reductase migrated as a single silver-stained band with an apparent Mr of 37,400 (Fig. 1). Application of purified CD reductase to an isoelectric focusing tube gel produced a single Coomassie Blue-stained band having an isoelectric point of pH 7.0 (data not shown).

**Table I**

| Protein | Activity* | Specific activity | Recovery | Purification |
|--------|-----------|-----------------|----------|-------------|
| Cytosol | 8580      | 197             | 0.023    | 100         | 1            |
| DEAE-cellulose | 2992 | 123             | 0.041    | 62          | 2            |
| Reactive Red-Agarose | 364 | 60               | 0.163    | 30          | 7            |
| Hydroxylapatite | 22 | 50               | 2.26     | 25          | 98           |

* Activity was measured as described under "Methods."

**FIG. 1. Biochemical and immunochemical characterization of human liver CD reductase.** Purified CD reductase was subjected to electrophoresis in 10% polyacrylamide gels and then either silver-stained or electrophoretically transferred to nitrocellulose and reacted with anti-CD reductase antiserum as described under "Methods." The indicated molecular weight was estimated from the mobilities of standard proteins.

**Catalytic Properties**—Optimal reduction of CD by purified CD reductase was detected at pH 7.5 in a phosphate buffer. All further characterizations of CD reductase were performed at the more physiologically relevant value of pH 7.5. The \( K_m \) value for oxidation of NADPH by CD reductase in the presence of CD was 5 \( \mu \)M, whereas no activity was detected (<0.005 nmol/min/mg) when NADH was substituted as a cofactor (not shown). CD reductase was unable to catalyze the oxidation of chlordecone alcohol with either NADP* or NAD* as cofactor over a wide pH range. CD reductase catalyzed the reduction of CD (\( K_m \) of 3 \( \mu \)M and a \( V_{max} \) of 87 pmol of CD reduced/min), but did not reduce other substrates that contained a carbonyl moiety such as pyridine 4-aldehyde, 4-benzoylpyridine, and 9,10-phenanthrenequinone (<0.005 unit/mg of protein/min) (not shown).

Of the agents known to inhibit the "classic" human liver carbonyl reductases (17), quercetin and p-chloromercuriben-
zoate inhibited CD reductase by greater than 90%, whereas HgCl₂ and CuSO₄ (17) inhibited human CD reductase only moderately (Table II). Established inhibitors of human liver aldehyde reductase (diphenylhydantoin and phenobarbital) (17) and of alcohol dehydrogenase (pyrazole) were without effect on CD reductase activity (Table II).

**Immunological Studies**—A high titer of anti-CD reductase antibodies was readily raised by injecting rabbits with purified CD reductase. When added to a reaction mixture purified CD reductase was not recognized on similar immunoblot. A single protein with an estimated molecular weight of 37,400 inhibited CD reductase activity (Table II). Analyses of purified CD reductase on immunoblots developed with anti-human CD reductase antiserum produced a concentration-dependent inhibition of CD reduction reaching a maximum value of greater than 80% inhibition (Fig. 2). This immunological reactivity was specific inasmuch as purified CD reductase was not recognized on similar immunoblots developed with antibodies that react with "classic" hepatic aldehyde reductase (diphenylhydantoin and phenobarbital) (17) and of alcohol dehydrogenase (pyrazole) were without effect on CD reductase activity (Fig. 2). Analyses of purified CD reductase on immunoblots developed with anti-human CD reductase revealed a single protein with an estimated Mᵋ = 37,400 (Fig. 1). This immunological reactivity was specific inasmuch as purified CD reductase was not recognized on similar immunoblots developed with antibody that acts with "classic" hepatic aldehyde reductase in man and in other mammals (18) (data not shown). We tested samples of liver cytosol isolated from various mammalian species with the use of immunoblots developed with anti-CD reductase antiserum and discovered that immunoreactive proteins were present in the rabbit, gerbil, and man, species that exhibit measurable CD reductase activity (Fig. 3). Liver cytosol from species that lack CD reductase activity (rat, mouse, and hamster) contained no detectable anti-CD reductase-reactive proteins (Fig. 3). The sole exception to this correspondence was guinea pig liver cytosol, which lacks detectable CD reductase activity, but did exhibit weak immunoreactivity on the immunoblot (Fig. 3). The gerbil contains three CD reductase-immunoreactive proteins, whereas only two were recognized in man and in the rabbit (Fig. 3). This difference may explain why gerbil liver cytosol contains 2 or 3 times higher CD reductase activity when compared with rabbit or human liver cytosol, respectively (10).

To determine the interindividual variability of CD reductase, we developed immunoblots of human liver cytosol from seven patients with anti-CD reductase antiserum. We also measured CD reductase activity in the same samples. In three of the patients, there was only a single immunoreactive protein having an electrophoretic mobility the same as that of purified CD reductase (Fig. 4). In the other four patients, there was a second immunologically related protein which

**TABLE II**

| Inhibitor         | Concentration (mM) | Inhibition (%) |
|-------------------|--------------------|----------------|
| HgCl₂             | 0.001              | 32             |
| CuSO₄             | 0.01               | 47             |
| Quercetin         | 0.01               | 38             |
| Indomethacin      | 0.1                | 73             |
| Ethacrynic acid   | 0.1                | 81             |
| p-Chloromercurbenzoate | 0.1            | 91             |
| Phenobarbital     | 1.0                | 4              |
| Diphenylhydantoin | 1.0                | 18             |
| Pyrazole          | 1.0                | 9              |

**FIG. 2**. Inhibition by anti-CD reductase antiserum of the reduction of CD by purified human liver CD reductase. Purified CD reductase (10 µg) was incubated with anti-CD reductase antiserum for 5 min at 23°C prior to measurement of CD reductase activity as described under "Methods." The results have been corrected by subtracting the values determined in parallel incubations containing equal amounts of nonimmune serum.

**FIG. 3**. Identification of anti-CD reductase immunoreactive hepatic proteins in various mammalian species. Hepatic cytosolic protein (10 µg) was analyzed on immunoblots developed with anti-CD reductase antiserum as described in Fig. 1. In a similar immunoblot developed with nonimmune control serum, no immunoreactive proteins were visible. Abbreviations used are: MO, mouse; CDR, CD reductase; RT, rat; HA, hamster; GP, guinea pig; GE, gerbil; RB, rabbit; HU, human.

**FIG. 4**. Interindividual variability of human liver CD reductase. Human cytosolic protein (10 µg) and purified CD reductase (STD) (0.1, 0.5, and 1.0 µg) were analyzed on immunoblots developed with anti-CD reductase antiserum as described in Fig. 1. Relative density was determined by scanning densitometry of the immunoblot in Fig. 4. CD reductase activity was measured as described under "Methods." The data are given as the percentage of the respective values for patient 16 which were as follows: immunoblots, arbitrary units; CD reductase activity, 0.043 nmol/min/mg.

| Patient number | Relative density | Chlordecone reductase activity |
|----------------|-----------------|-------------------------------|
| 1              | 16              | 17                            |
| 11             | 45              | 56                            |
| 15             | 76              | 66                            |
| 16             | 100             | 100                           |
| 17             | 47              | 39                            |
| 18             | 44              | 44                            |
| 19             | 49              | 39                            |
migrated slightly faster than did CD reductase (Fig. 4). The relative density of the total immunoreactive proteins as determined by scanning densitometry varied as much as 6-fold among the seven patient samples. This value was consistent with the 6-fold variability we detected in CD reductase activity among these seven samples (Table III) \( r = 0.83 \). In most cases, CD reductase activity mirrored the relative density of the immunoreactive proteins, suggesting that both immunoreactive proteins may catalyze reduction of CD reductase.

**DISCUSSION**

It is difficult to classify CD reductase using classical biochemical criteria. CD reductase resembles previously discussed human liver carbonyl reductases (17) in being a monomeric cytosolic enzyme which requires NADPH to catalyze the reduction of a xenobiotic ketone to the corresponding alcohol product. CD reductase also resembles these enzymes in being sensitive to quercetin and \( p \)-chloromercuribenzoate, in being widely distributed among tissues, and in having molecular weights in the 30,000–40,000 range (17). However, unlike carbonyl reductases, CD reductase is inactive in reducing common ketone substrates. Also, CD reductase appears to be inducible by its substrate (10). Finally, CD reductase is species-specific, unlike carbonyl reductase which are present in most of the mammalian species examined (1).

In the past, immunochemical characterizations of aldo-keto reductases have been restricted to double diffusion analysis (19–21) and immunotitration experiments (22–24). The advent of immunochemical analysis offers a powerful and sensitive technique for making inter- and intra-species comparisons of closely related proteins such as the "classical" aldo-keto reductases (18) and CD reductase. We have assembled strong evidence that CD reductase is species-specific: (a) when rats, hamsters, guinea pigs, or gerbils were treated with a single injection of CD, only gerbils produced CDOH (9); (b) incubation of gerbil liver cytosol with NADPH and CD produced CDOH, whereas liver cytosol from other species was inactive (10); and (c) immunoblot analyses revealed that only those species capable of reducing CD contain significant amounts of hepatic cytosolic proteins immunochemically related to CD reductase (Fig. 3). This last finding suggests that the inability to reduce CD is due to the absence of CD reductase protein rather than altered substrate specificity of an immunochemically related enzyme. From these results, we conclude that CD reductase represents a previously undetected form of human liver carbonyl reductase.

There are many isozymic forms of carbonyl reductase ubiquitously distributed throughout mammalian tissues and apparently expressed constitutively (1). Therefore, it has been proposed that the carbonyl reductases serve only in general detoxication rather than in physiological pathways. However, unlike the classic carbonyl reductases, CD reductase displays a highly restricted specificity for exogenous ketone substrates and a low turnover rate for CD. These characteristics raise the possibility that there may be endogenous substrates for CD reductase. Even if general detoxication is the major function of carbonyl reductases, the present results suggest that interindividual variability in the liver concentration of CD reductase may have a dramatic effect on a given patient's susceptibility to a toxic chemical for which metabolic elimination is controlled by CD reductase-catalyzed reduction. Indeed, we found that the half-time for the disappearance of CD from 22 exposed workers varied as much as 6-fold (25) and was independent of the amount of CD initially detected in the blood (25). In the present study of seven patients, none of whom was exposed to CD, anti-CD reductase immunoreactive proteins and CD reductase activity also varied by 6-fold, whereas in the same human liver samples, the amounts of "classic" immunoreactive carbonyl reductase varied less than 2-fold (18). Unanswered is whether there is variability among individuals in the inducibility of CD reductase in addition to the variability in the amount of enzyme spontaneously expressed in unexposed patients. Although the molecular basis for the regulation of this enzyme is totally unknown at present, the availability of purified CD reductase and of anti-CD reductase antibodies will make it possible now to investigate this question.

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