Biochemical Pharmacology, Vol. 31, No. 23, pp. 3885–3890, 1982.
Printed in Great Britain.

0006-2952/82/233885-06 $03.00/0

GLUTATHIONE-DEPENDENT REDUCTIVE DEHALOGENATION OF 2,2′,4′-TRICHLOROACETOPHENONE TO 2′,4′-DICHLOROACETOPHENONE*

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(*Received* 9 *January* 1982; *accepted* 23 *April* 1982)

Abstract—α-Haloketones are highly reactive compounds, which are known to undergo enzymatic reduction to methyl ketones. The objective of this research was to characterize the enzymes involved in this reaction and to investigate the mechanism of the reaction. 2,2′,4′-Trichloroacetophenone was reduced to 2′,4′-dichloroacetophenone by glutathione-dependent cytosolic enzymes present in the liver, kidney, and brain. The actual substrate for the enzyme was *S*-(2,4-dichlorophenacyl)glutathione, which is formed by the nonenzymic reaction of 2,2′,4′-trichloroacetophenone and glutathione. The reaction mechanism may involve an enzyme-catalyzed nucleophilic attack of glutathione on the sulfur atom of *S*-(2,4-dichlorophenacyl)glutathione to yield a carbanion and glutathione disulfide; protonation of the carbanion would yield 2′,4′-dichloroacetophenone. Stoichiometry studies showed that the glutathione disulfide/2′,4′-dichloroacetophenone ratio was 1.25 ± 0.13.

α-Haloketones, such as α-chloroacetophenone, are employed as temporary incapacitating agents [1] and have been identified as metabolites of chlorfenvinphos, dimethylvinphos and tetrachlorvinphos [2–4]. In addition, 1,3-dichloroacetone has been suggested as a possible metabolite of the flame retardant, *tris*-(1,3-dichloro-2-propyl)phosphate [5], and chloroacetones are mutagenic components of chlorinated effluents [6]. α-Haloketones may also serve as active site-directed inhibitors of enzymes such as chymotrypsin [7]. Earlier studies showed that glutathione (GSH) is involved in the reductive dehalogenation of 2,2′4′-trichloroacetophenone (TCAP) to 2′,4′-dichloroacetophenone (DCAP) by hepatic cytosolic enzymes, and preliminary reaction mechanism studies suggested that *S*-(2,4-dichlorophenacyl)glutathione (SDPG) served as the substrate [8].

The objective of the studies reported herein was to characterize the enzymes involved in the metabolism of α-haloketones to methyl ketones and to study the mechanism of the reaction.

MATERIALS AND METHODS

Chemicals. 2,2′4′-Trichloroacetophenone, 2′,4′-dichloroacetophenone, 4′-chloroacetophenone, D-(-)-penicillamine (99+%), and deuterium oxide (99.7 atom% ^2H) were obtained from the Aldrich Chemical Co., Milwaukee, WI. Glutathione, L-cysteine hydrochloride hydrate, Sephadex QAE-25, and cysteamine hydrochloride were obtained from the Sigma Chemical Co., St. Louis, MO; Bio-Beads SM-2 were purchased from Bio-Rad Laboratories, Richmond, CA. *S*-(2,4-Dichlorophenacyl) glutathione was prepared by a modification of the method of Hutson *et al.* [8]. The reaction mixture was prepared and incubated as described previously [8]. The solution was then concentrated to about 50 ml, extracted twice with an equal volume of ether, and lyophilized; the residue was suspended in 1 ml of water and applied to a 1 cm × 10 cm column of Bio-Beads SM-2 (20–50 mesh). The column was washed with about 300 ml of water, the SDPG was eluted with methanol, and 10 ml fractions were collected. The SDPG content of the fractions collected was measured by high performance liquid chromatography (HPLC), and the fractions containing SDPG were pooled and evaporated to dryness. No TCAP was detectable by HPLC analysis, and the SDPG showed a single ninhydrin positive spot (R_f 0.38) when analyzed by TLC (silica gel; *n*-butanol–formic acid–water, 10/1/1, by vol.). The yield was 2.5 to 3.5%. All other chemicals were of reagent grade and were obtained from commercial sources.

Enzyme preparation. Male Long–Evans rats weighing 200–300 g were used. Subcellular fractions of hepatic and extrahepatic tissues were isolated as described previously [9]; cytosolic fractions, except those used in the experiments reported in Table 1, were dialyzed overnight against 0.1 M phosphate buffer (pH 7.4) at 4° before use.

Incubation mixtures. Unless otherwise indicated, incubation mixtures contained 200 μmoles of phosphate buffer (pH 7.4), 30 μmoles of GSH, 3 μmoles

* This work was supported by National Institutes of Health Grant ES 01082. A preliminary report of this work was presented at the Fall Meeting of the American Society for Pharmacology and Experimental Therapeutics, Portland, OR, August 19–23 [*Pharmacologist* **21**, 215 (1979)].

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of TCAP, added in 30 μl of dioxane, and 5.6 mg of cytosolic protein in a total volume of 3 ml; reaction mixtures were incubated at 37° for 15 min in an atmosphere of air. In some experiments (data shown in Tables 1–3 and 5 and Fig. 1), the reaction was started by addition of TCAP and, in all other experiments, the reaction was started by addition of cytosol to the other components of the reaction mixture that had been incubated for 1.0 min at 37°; both methods of starting the reaction yielded the same results (2.4 vs 2.6 nmoles DCAP per min per mg protein). The reaction was stopped by placing the flasks on ice.

Analytical methods. The amount of DCAP formed was determined by gas chromatography/mass spectrometry (GC/MS) or HPLC. A Hewlett–Packard 5992A GC/MS equipped with a 2 mm i.d. × 6 ft glass column packed with 3% OV-210 on Chromosorb W (HP), 80/100 mesh (Pierce Chemical Co., Rockford, IL), was used. The column was operated at 140° with helium as the carrier gas. 2′,4′-Dichloroacetophenone was extracted from the cold incubation mixtures by adding 2 ml of petroleum ether (b.p. 60–70°) containing 0.16 mM 4′-chloroacetophenone as the internal standard. After mixing, 1 μl of the organic layer was injected into the GC/MS. A selected ion monitoring program was used to calculate the area ratios between DCAP (*m/z* 173, base peak) and 4′-chloroacetophenone (*m/z* 139, base peak).

In some experiments, the concentration of DCAP was measured by HPLC. An Altex HPLC equipped with a reversed-phase column (Spherisorb ODS, 4.6 mm i.d. × 250 mm) was used. The UV-photometer was operated at 254 nm. At the end of the incubation period, 96 nmoles of 4′-chloroacetophenone in 5 μl of dioxane was added to the reaction mixture and a sample (20 μl) of the incubation mixture was injected directly into the column and eluted with a mixture of 50% acetonitrile in water (v/v) at a flow rate of 2 ml/min; the retention times of SDPG, 4′-chloroacetophenone, DCAP, and TCAP were 0.9, 4.5, 6.0, and 7.2 min respectively. The amount of DCAP formed was estimated from a standard curve of DCAP (peak height)/4′-chloroacetophenone (peak height) vs DCAP concentration. When SDPG and DCAP concentrations were measured simultaneously by HPLC, the eluent was 40% acetonitrile in water containing 1% acetic acid, and a 20 μl sample of the incubation mixture was injected directly onto the column; the retention times of SDPG, DCAP, and TCAP were 5.4, 11.5 and 14.7 min respectively. The amounts of SDPG and DCAP in the reaction mixture were determined by measuring the areas of the peaks with a Hewlett–Packard model 3390A Integrator and extrapolating from a standard curve of SDPG (area) or DCAP (area) vs the amount of SDPG or DCAP respectively.

The stoichiometry of the reaction was determined by measuring the formation of glutathione disulfide (GSSG) and DCAP in the same reaction mixture. For these experiments, the incubation mixtures were prepared as described above except that the reaction mixtures were incubated for 30 min and contained 15 μmoles GSH and 3 μmoles EDTA; preliminary experiments showed that the addition of EDTA reduced the nonenzymic conversion of GSH to GSSG. The amount of DCAP formed was measured by HPLC as described above, and the amount of GSSG produced was determined as described by Pace and Dixon [10]. At the end of the incubation period, the reaction flasks were chilled in ice, and 0.74 μmoles *N*-ethylmaleimide in 0.60 ml water was added to inhibit the oxidation of GSH. A 1-ml sample of the reaction mixture was applied to a Sephadex QAE-25 column and eluted with a linear gradient of 0.05 to 0.5 M potassium phosphate buffer (pH 6.2) with a total buffer volume of 300 ml and a flow rate of 19 ml/hr. Seven milliliter fractions were collected and assayed for their GSSG content as described by Hissin and Hilf [11]. To eliminate the necessity of storing samples, two QAE-25 columns were developed simultaneously, and a sample from a reaction mixture containing TCAP was applied to one column and a sample from a reaction mixture lacking TCAP (blank) was applied to the other column.

Protein concentrations were determined by the method of Lowry *et al.* [12] with bovine serum albumin as the standard, and kinetic constants were computed by the method of Wilkinson [13].

RESULTS

The tissue distribution of the enzymes catalyzing the reduction of TCAP to DCAP was studied (Table 1). Kidney, liver, and brain cytosol exhibited the highest specific activities for DCAP formation; low specific activities were observed in lung or muscle cytosol. When subcellular fractions of liver tissue were compared for their abilities to metabolize TCAP to DCAP, cytosol exhibited the highest rate of DCAP formation (Table 2). A small, but consistent, value was detected in the nuclear fraction.

The dependence of the enzymatic reduction of TCAP to DCAP on incubation time, protein concentration, temperature, and pH was studied (Fig. 1). The reaction was linear with time for 20 min and with protein concentrations up to 5.6 mg protein/ml. The temperature optimum was 45°. No distinct pH optimum was observed; rather, the reaction rate increased as the pH was increased over the range of 6.3 to 8.7.

Studies on the cofactor requirements for TCAP metabolism to DCAP showed that, of the thiols tested, GSH and, to a lesser extent, cysteamine supported DCAP formation (Table 3). Incubation of TCAP with liver cytosol and GSH in the absence of oxygen failed to alter the rate of DCAP production compared to that seen in the presence of oxygen (data not shown).

The dependency of the metabolism of TCAP to DCAP on the concentration of TCAP and glutathione was also studied (Fig. 2). The reaction rate increased as the concentrations of TCAP and glutathione were increased at fixed concentrations of glutathione (40 mM) and TCAP (1 mM) respectively. The apparent K_M and V_{max} for TCAP were 0.43 ± 0.07 mM and 8.8 ± 0.7 nmoles DCAP per min per protein, respectively, and the apparent K_M and V_{max} for glutathione were 41.6 ± 2.2 mM and 12.5 ± 0.4 nmoles DCAP per min per protein,

Table 1. Tissue distribution of the metabolism of 2,2',4'-trichloroacetophenone to 2',4'-dichloroacetophenone*

Tissue	DCAP formation† (% of control)
Liver	100
Kidney	125
Brain	99
Muscle	22
Lung	17

*TCAP and GSH were present at final concentrations of 1 and 10 mM respectively. Cytosol prepared from the various tissues was used without dialysis. DCAP formation was determined by GC/MS. Data represent the averages of two experiments and are expressed as percentages of that obtained with liver tissue as the control.

† Control rate of DCAP formation in liver was 1.4 nmoles per min per mg protein.

Table 2. Subcellular distribution of the hepatic metabolism of 2,2',4'-trichloroacetophenone to 2',4'-dichloroacetophenone*

Fraction	DCAP formation† (% of control)
Cytosol	100
Nuclear	3.5
Mitochondrial	ND‡
Microsomal	ND

* TCAP and GSH were present at final concentrations of 1 and 10 mM respectively. Dialyzed cytosol and undialyzed nuclear, mitochondrial, and microsomal fractions were used as the enzyme sources. DCAP formation was measured by GC/MS. Data represent the averages of two experiments and are expressed as percentages of that obtained with cytosol as the control.

† Control rate of DCAP formation in cytosol was 2.3 nmoles per min per mg protein.

‡ Not detectable.

respectively. Previous studies have shown that TCAP reacts nonenzymically with GSH to form SDPG [8], indicating that SDPG may be the actual substrate for the enzyme. To test this hypothesis, synthetic SDPG was incubated in the presence of GSH and cytosol. Under these conditions, SDPG disappeared from the incubation mixture and DCAP was formed concomitantly, but the amount of SDPG lost exceeded the amount of DCAP formed (Table 4). No SDPG disappeared when incubated in the absence of GSH or in the presence of both GSH and heat-denatured cytosol (data not shown). Furthermore, when the stoichiometry of the reaction was studied, it was found that the ratio of the amount of glutathione disulfide formed to the amount of DCAP formed was 1.25 ± 0.13 (N = 3).

The effects of compounds known to react with

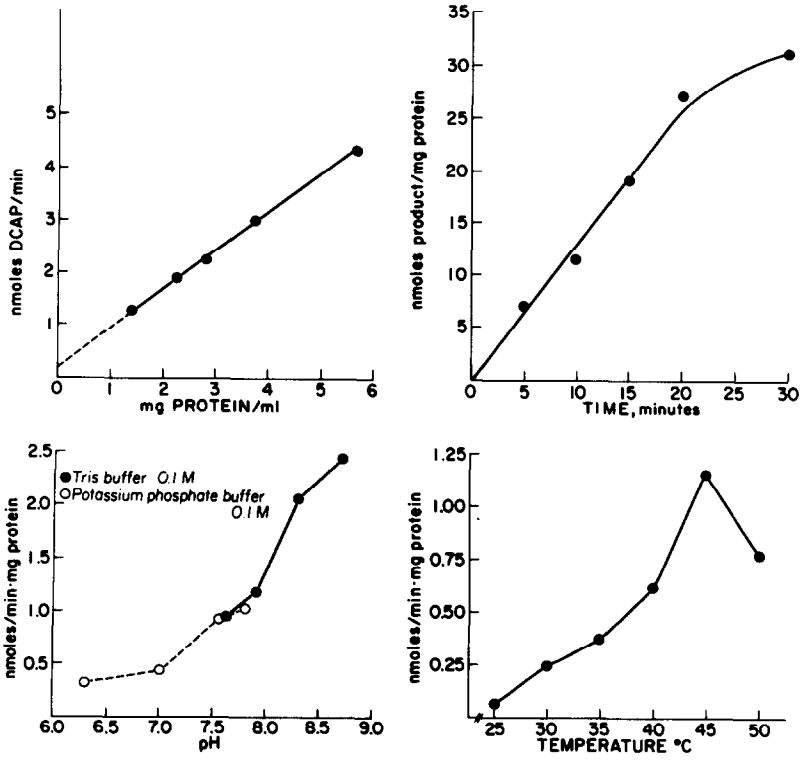

Fig. 1. Dependence of the metabolism of 2,2',4'-trichloroacetophenone to 2',4'-dichloroacetophenone on time (upper right), protein concentration (upper left), pH (lower left), and temperature (lower right). Reaction mixtures contained 10 mM GSH, 1 mM TCAP, and dialyzed hepatic cytosol and were incubated at 37° for 20 min except that the conditions were varied as indicated on the abscissa of each panel. DCAP formation was measured by GC/MS. Values are the averages of two experiments.

Table 3. Cofactor requirements for the hepatic metabolism of 2,2′,4′-trichloroacetophenone to 2′,4′-dichloroacetophenone*

Cofactor	DCAP formation† (% of control)
Glutathione	100
L-Cysteine · HCl	2.4
D-(-)-Penicillamine · HCl	2.9
Cysteamine · HCl	21.7

* Substrate and cofactors were added to final concentrations of 1 and 10 mM respectively. Dialyzed cytosol was used as the enzyme source. DCAP formation was determined by GC/MS. Data represent the averages of two experiments and are expressed as percentages of that obtained with glutathione as the control.

† Control rate of DCAP formation with GSH was 2.0 nmoles per min per mg protein.

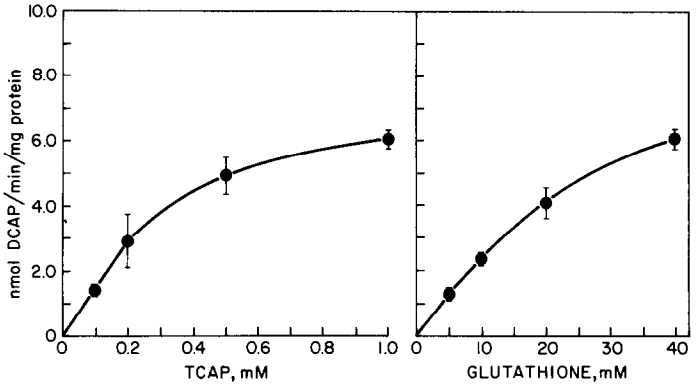

Fig. 2. Effect of 2,2′,4′-trichloroacetophenone (left) and glutathione (right) concentration on the metabolism of 2,2′,4′-trichloroacetophenone (TCAP) to 2′,4′-dichloroacetophenone (DCAP). Reaction mixtures contained dialyzed hepatic cytosol and either various concentrations of TCAP and 40 mM glutathione or various concentrations of glutathione and 1 mM TCAP and were incubated for 15 min. The amount of DCAP formed was measured by HPLC. Values are shown as means ± S.D. for three to four experiments.

Table 4. Metabolism of *S*-(2,4-dichlorophenacyl)glutathione to 2′,4′-dichloroacetophenone*

Incubation time (min)	SDPC lost (μmoles/ml)	DCAP formed (μmoles/ml)
15	0.18 ± 0.03	0.16 ± 0.02
30	0.42 ± 0.03	0.29 ± 0.02
45	0.62 ± 0.04	0.41 ± 0.05

* SDPG and GSH were present at initial concentrations of 1 and 40 mM respectively; the incubation mixtures contained 1.9 mg dialyzed rat hepatic cytosol per ml and were incubated at 37° for the times shown. SDPG and DCAP concentrations were measured by HPLC. Data are shown as means ± S.D. for three experiments.

Table 5. Effect of enzyme inhibitors on the metabolism of 2,2′,4′-trichloroacetophenone to 2′,4′-dichloroacetophenone by rat hepatic cytosol*

Addition	DCAP formation† (% of control)
p-Chloromercuribenzoic acid	0.4
Diethyl maleate	7.0
Methyl iodide	67.0
1,2-Dichloro-4-nitrobenzene	84.0
1,2-Epoxy-3-(4-nitrophenoxy)-propane	73.4

* TCAP and GSH were present in final concentrations of 1 mM and 10 mM respectively; inhibitors were added to a final concentration of 5 mM. Dialyzed cytosol was used as the enzyme source. DCAP formation was measured by GC/MS. Values are shown as the averages of two experiments.

† Control rate of DCAP formation was 2.0 nmoles per min per mg protein.

sulfhydryl groups or to serve as substrates for GSH *S*-transferases on the metabolism of TCAP or DCAP were also studied (Table 5). Only *p*-chloromercuribenzoate and dimethyl maleate produced substantial inhibition.

To determine the source of the hydrogen atom in the DCAP formed during the enzymatic reduction of TCAP, the incubation was carried out in the presence of 50% deuterium oxide (v/v). The production of [2-^{2}H]2′,4′-dichloroacetophenone was determined by GC/MS with a single ion monitoring program, which measured the relative abundances of *m/z* 188 and 189 corresponding to DCAP and [2-^{2}H]2′,4′-dichloroacetophenone respectively. The incorporation of deuterium was found to be 49.0% (47.8, 50.3). Since α-hydrogen atoms of ketones may undergo exchange reactions, DCAP was incubated with 50% deuterium oxide at pH 7.4. About 7% enrichment in deuterium in the DCAP was observed; this value has been subtracted from those value obtained in the enzymic system.

DISCUSSION

The results presented above show that α-haloketones are reduced to methyl ketones by GSH-dependent cytosolic enzymes, confirming the observation of Hutson *et al.* [8]. The enzymes catalyzing this biotransformation show properties similar to other cytosolic enzymes involved in the metabolism of halogenated compounds. The failure to observe a pH optimum has been observed previously in the GSH-dependent metabolism of methyl iodide [14], dihalomethanes [15], and 1,2-dihalomethanes [9]. This may be attributed to the increased ionization of the thiol group at alkaline pH values, which may be expected to increase the reactivity of GSH. The finding of a rather high (45°) temperature optimum is similar to that observed for the conversion of 1,2-dihaloethanes to ethylene [9].

The cofactor requirements and effects of inhibitors suggest the involvement of a GSH *S*-transferase in the reductive halogenation of α-haloketones. However, the finding that SDPG, the adduct formed nonenzymically from GSH and TCAP, is the actual substrate for the enzyme may indicate that enzymes other that GSH *S*-transferases are involved since GSH *S*-transferases are known to bind, but not metabolize, a variety of GSH conjugates [16, 17]. It should be noted that *S*-crotonyl *N*-acetylcysteamine, a thiol ester, is thought to be metabolized by a GSH *S*-transferase [18], showing that *S*-substituted thiols may serve as substrates for GSH *S*-transferases. Preliminary studies suggest that the enzyme involved in the reductive dehalogenation of α-haloketones in an anionic protein of about 29,000–37,000 daltons molecular weight and is distinct from glutathione *S*-transferase B, C. or E (M. Kitada and M. W. Anders, unpublished observations).

At least two reaction mechanisms may be considered for the GSH-dependent reduction of α-haloketones. One mechanism may involve attack of the thiol on the halogen atom of the α-haloketone to yield glutathione sulfenyl halide (GSX) and a carbanion, which would undergo rapid protonation to give the methyl ketone. Although such a mechanism has been proposed for reduction of benzyl halides and halomethyl ketones by thiol and selenol nucleophiles [19, 20], it does not appear to be applicable to the current results since SDPG, rather than TCAP itself, is the actual substrate for the enzyme. α-Hal-

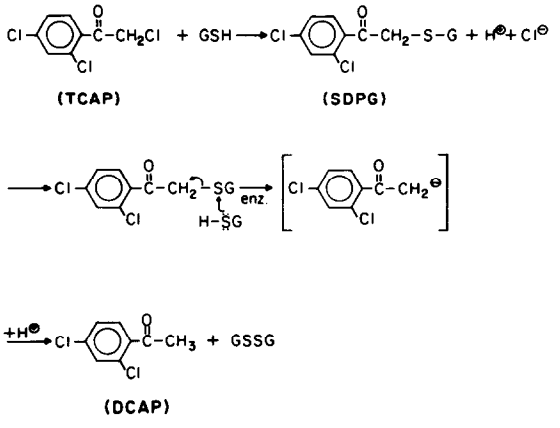

Fig. 3. Proposed reaction mechanism for the glutathione-dependent reduction of 2,2′,4′-trichloroacetophenone to 2′,4′-dichloroacetophenone.

oketones are reduced to carbonyl compounds by thiolates, probably via the intermediate formation of α-carbonyl sulfides [21]. This mechanism is consistent with the observation that SDPG is the actual substrate for the enzyme and that glutathione disulfide is formed in stoichiometric amounts with DCAP. The enzymic reaction mechanism may be visualized as a nucleophilic attack of GSH on the sulfur atom of SDPG to yield glutathione disulfide and a carbanion; the carbanion could undergo rapid protonation to give the methyl ketone (Fig. 3). The observation that [2-^{2}H]2′,4′-dichloroacetophenone is produced when the reaction is carried out in the presence of deuterium oxide supports this mechanism.

This metabolic pathway is of toxicological significance in that is provides a mechanism for the conversion of highly reactive α-haloketones to methyl ketones, which are less toxic and are subject to oxidative metabolism. These results are also of interest in that they show that glutathione conjugates may be converted to metabolic products other than mercapturic acids.

Acknowledgement—The authors thank Mr. Adrian R. Swanson for his excellent technical assistance.

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