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Inactivation of Purified Rat Liver Cytochrome *P*-450 by Chloramphenicol

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SUMMARY

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Incubation with chloramphenicol in the presence of NADPH leads to the irreversible inhibition of the 7-ethoxycoumarin deethylase and benzphetamine demethylase activities of the cytochrome *P*-450 of a reconstituted monooxygenase system purified from liver microsomes of phenobarbital-treated rats. The inactivation is accompanied by the covalent binding of 1.5 nmol ^{14}C from [1,2-^{14}C]chloramphenicol per nmol cytochrome *P*-450 but not by a decrease in cytochrome *P*-450 detectable as its carbon monoxide complex or by a decrease in heme detectable as pyridine hemachromagen. The data suggest that chloramphenicol acts as a suicide substrate of cytochrome *P*-450 and may prove to be a valuable tool for identifying amino acid residues at or near the active site of the enzyme.

INTRODUCTION

A number of compounds are known which inactivate cytochrome *P*-450 when administered to experimental animals *in vivo* or incubated *in vitro* with intact microsomes or a reconstituted monooxygenase system in the presence of NADPH (for review, see Ref. 1). In most cases the inactivation can be ascribed to destruction of the heme moiety of the cytochrome *P*-450. In the case of thiono-sulfur compounds such as carbon disulfide or parathion, modification of the protein moiety resulting from covalent binding of the atomic sulfur released during the oxidative metabolism of the compounds appears to be a major cause of the inactivation of the cytochrome *P*-450. However, there is some evidence that the inactivation is due to the aggregation of the protein resulting from attack of atomic sulfur on cysteine residues rather than to the derivatization of amino acid residues important for catalysis per se (2, 3). Furthermore, at least with parathion, destruction of the heme moiety may also contribute to the inactivation of the cytochrome *P*-450 (3). To our knowledge no compounds have been reported to inactivate cytochrome *P*-450 exclusively by virtue of covalent binding of a reactive intermediate to essential amino acid residues in the protein.

Recently it was reported that the antibiotic chloramphenicol causes irreversible inhibition of *o*-nitroanisole metabolism in rat liver microsomes, and it was suggested that an active site-directed mechanism might be involved (4). Furthermore, it has been shown that liver microsomes from phenobarbital-treated rats are effective in activating chloramphenicol to a reactive intermediate which binds covalently to the microsomal proteins (5). These findings prompted us to examine the effect of chloramphenicol on the major form of liver microsomal cytochrome *P*-450 from phenobarbital-treated rats using a reconstituted monooxygenase system.

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METHODS

Preparation of microsomes. Adult male Sprague–Dawley rats (100–200 g) were given phenobarbital (0.1% in the drinking water) for 5 days prior to sacrifice. Microsomes were prepared as described previously (6), with the final centrifugation performed at 105,000*g* for 60 min. Microsomes were stored frozen at −70°C in 10 mM Tris–acetate, pH 7.4, containing 20% glycerol and 1 mM EDTA.

Preparation of enzymes. The major form of cytochrome *P*-450 from liver microsomes of phenobarbital-treated rats was purified as described by Guengerich (7). The method involves chromatography of cholate-lysed microsomes on octylamino-Sepharose 4B (8) in the cold followed by chromatography on DEAE-cellulose at room temperature. The NADPH–cytochrome *P*-450 reductase-containing fractions from the octylamino-Sepharose column were further purified by affinity chromatography on ADP 2′5′-Sepharose (9). Both enzyme preparations were ≥95% pure as judged by SDS–polyacrylamide gel electrophoresis, performed by the method of Laemmli (10) using double-strength cathode buffer as described by Guengerich (11). The specific content of the cytochrome *P*-450 preparation used in this investigation was

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15 nmol/mg protein based on the protein concentration determined by the method of Lowry *et al.* (12) using bovine serum albumin as the standard.

Assays. The enzymatic activity of the purified cytochrome *P*-450 before and after incubation of a reconstituted system with chloramphenicol was assayed using benzphetamine and 7-ethoxycoumarin as substrates. The 1-ml incubation mixture contained 0.1 nmol cytochrome *P*-450, 0.3 unit NADPH–cytochrome *P*-450 reductase, 30 μg dilauryl L-3-phosphatidylcholine, 100 μg sodium deoxycholate, 0.05 M Hepes buffer (pH 7.5), 15 mM $MgCl_2$, 0.1 mM EDTA, 1 mM benzphetamine or 0.3 mM 7-ethoxycoumarin (added in 10 μl methanol), and 0.2 mM NADPH. Incubations were carried out for 5 min at 37°C. Under these conditions the reactions are linear with time, and cytochrome *P*-450 is the rate-limiting component. Benzphetamine metabolism was monitored by NADPH oxidation (13) using a Cary 219 spectrophotometer equipped with an automatic sample changer. Ethoxycoumarin metabolism was monitored by the method of Greenlee and Poland (14) as described by Guengerich (15). Formation of 7-hydroxycoumarin was determined with an Aminco–Bowman spectrofluorometer (excitation at 368 nm, emission at 450 nm). NADPH–cytochrome *P*-450 reductase was assayed at 30°C in 0.3 M phosphate buffer (pH 7.7). One unit is defined as the amount of enzyme catalyzing the reduction of 1 μmol cytochrome *c*/min under these conditions.

Cytochrome *P*-450 was determined by the method of Omura and Sato (16), and heme using the pyridine hemachromagen assay (16).

Incubations with chloramphenicol were carried out for 30 min at 37°C using 2 nmol cytochrome *P*-450, 1 unit reductase, 30 μg dilauryl L-3-phosphatidylcholine, 100 μg sodium deoxycholate, 0.05 M Hepes buffer (pH 7.5), 15 mM $MgCl_2$, 0.1 mM EDTA, 1 mM chloramphenicol or 0.2 mM [^{14}C]chloramphenicol (1.2 mCi/mmol) added in 10 μl absolute ethanol, and 0.2 mM NADPH.

The samples were dialyzed for 48 h at 4°C against 4 × 1-liter portions of 50 mM Tris–acetate, pH 7.4, containing 20% glycerol and 0.1 mM EDTA, prior to assay of suitable aliquots for heme, cytochrome *P*-450, and enzymatic activity.

Protein-bound radioactivity was determined by liquid scintillation counting of aliquots of the cytochrome *P*-450-containing reconstituted system incubated with [^{14}C]chloramphenicol and dialyzed as described previously, and was corrected for any radioactivity present in control samples incubated in the absence of NADPH. The dialysis procedure reduced the radioactivity in the controls to virtual background levels. That any remaining radioactivity in the incubations lacking NADPH represented residual noncovalently bound material was confirmed by chromatography on a Sephadex G-25 column (0.9 × 30 cm) equilibrated with the same buffer used in the dialysis supplemented with 0.5% sodium cholate.

Free sulfhydryl groups were determined by spectrophotometric titration with 4,4′-dipyridine disulfide (PDS).[1] To a 100-μl aliquot of the dialyzed reconstituted system was added 5 μl 10% SDS. A spectrum was run from 380 to 300 nm vs a blank (also containing SDS) which prior to dialysis had contained all the components of the reconstituted system with the exception of the cytochrome *P*-450. This was to correct for the contribution of the free sulfhydryl groups on the reductase. PDS (5 μl) was added from a 1 mM stock solution in 10 mM potassium phosphate buffer, pH 7.4, to give a threefold excess of reagent over protein sulfhydryl groups. After 10 min at 30°C, a new spectrum was recorded. An extinction coefficient of 19.8 mM^{-1} cm^{-1} at 324 nm was used to quantitate the 4-thiopyridone released (17). An extinction coefficient of 16.3 mM^{-1} cm^{-1} at 247 nm was used to determine the PDS concentration (17).

Materials. [^{14}C]Chloramphenicol [(1R,2R)-(+)1-*p*-nitrophenyl-2-[1,2-^{14}C]dichloroacetamido-1,3-propanediol], 43.2 mCi/mmol, was purchased from New England Nuclear. The compound was found to be radiochemically pure (>98%) by thin-layer chromatography on silica gel using the solvent system chloroform–methanol (100:15) or benzene–methanol–acetic acid (45:8:4). Unlabeled chloramphenicol and dilauryl L-3-phosphatidylcholine were from Sigma. 7-Ethoxycoumarin, 7-hydroxycoumarin, and PDS were from Aldrich. Benzphetamine was from the Upjohn Co.

RESULTS

Chloramphenicol has been shown to be a competitive inhibitor of *o*-nitroanisole metabolism in intact microsomes with a K_i of 0.05 mM (4). In the case of the reconstituted system, the presence of 0.05 mM chloramphenicol during assays of ethoxycoumarin metabolism was found to decrease the activity by 50%. This inhibition was completely reversible upon removal of the chloramphenicol by dialysis. However, if the cytochrome *P*-450 was preincubated with chloramphenicol in the presence of a complete system, irreversible inhibition of the ethoxycoumarin deethylase activity was found to occur relative to controls incubated in the absence of NADPH or chloramphenicol (Table 1). In a separate experiment the

TABLE 1

Chloramphenicol-mediated loss of ethoxycoumarin deethylase activity in a reconstituted system

A reconstituted system containing 2 nmol cytochrome *P*-450 was incubated with 1 mM chloramphenicol for 30 min at 37°C and dialyzed at 4°C for 48 h against buffer as described in Methods. Aliquots corresponding to 0.1 nmol cytochrome *P*-450 were then taken for assay of 7-ethoxycoumarin deethylase activity by a complete reconstituted system containing a saturating amount of the reductase, also as described in Methods. Results represent the mean ± standard deviation of duplicate or triplicate determinations.

System[a]	Cytochrome *P*-450	Heme	Ethoxycoumarin deethylase activity	
	nmol/ml	*nmol/ml*	*nmol/min/ml*	*nmol/min/nmol P-450*
Complete	1.58 ± 0.15	1.62 ± 0.00	23.9 ± 0.7	15.1
−NADPH	1.51 ± 0.18	1.55 ± 0.03	33.8 ± 0.6	22.4
−Chloramphenicol	1.73 ± 0.03	1.71[b]	37.7 ± 1.1	21.8

[a] The conditions of the incubation with chloramphenicol.

[b] Single determination.

[1] Abbreviation used: PDS, 4,4′-dipyridine disulfide.

loss of benzphetamine demethylase activity was found to parallel the loss of 7-ethoxycoumarin deethylase activity. Since the loss of enzymatic activity did not appear to be due to loss of heme or of cytochrome *P*-450, it was of interest to determine whether a metabolite of chloramphenicol had become covalently bound to the cytochrome *P*-450.

As seen in Table 2, incubation of a reconstituted system with [^{14}C]chloramphenicol in the presence of NADPH led to the covalent binding of a ^{14}C-containing product to the proteins of the reconstituted system. The fact that the stoichiometry of the inhibition of the ethoxycoumarin deethylase activity exceeded 1 nmol ^{14}C/nmol *P*-450 suggested that some of the radioactivity might be associated with the reductase. However, SDS–polyacrylamide gel electrophoresis of the ^{14}C-labeled proteins of the reconstituted system showed that only about 5% of the covalently bound radioactivity was associated with the reductase (Fig. 1). In subsequent calculations, no correction was made for the low amount of radioactivity bound to the reductase. As seen in Fig. 2, the stoichiometry of the inactivation remained constant over a range of 22–62% inactivation. The extrapolated value for 100% inactivation was 1.5 nmol ^{14}C/nmol *P*-450.

The ability of 1 mM dithiothreitol to protect against the inactivation of the cytochrome *P*-450 by chloramphenicol was also investigated. No protection was accomplished, although the amount of covalently bound radioactivity was decreased by 17% (Table 2). Since this could reflect either true protection against covalent binding or removal of already bound material, an experiment was performed in which the dithiothreitol was added subsequent to the incubation with chloramphenicol. In this case 19% of the radioactivity could be removed with no regeneration of the enzymatic activity. These two experiments suggested that although complete inactivation is accompanied by binding of 1.5 nmol ^{14}C/nmol *P*-450,

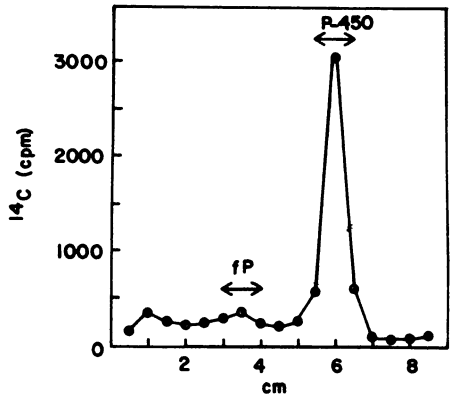

FIG. 1. *SDS-polyacrylamide gel electrophoresis of the ^{14}C-labeled proteins of a reconstituted system incubated with [^{14}C]chloramphenicol and dialyzed essentially as described in Methods with the exceptions that 0.05 mM [^{14}C]chloramphenicol (43.2 mCi/mmol) was used and the final dialysis was carried out against 25 mM Tris-HCl, pH 6.8, containing 20% glycerol and 0.1 mM EDTA*

The sample was not treated with β-mercaptoethanol prior to application. Electrophoresis was carried out using two identical 7.5% gel slabs (0.75 mm × 10 cm) prepared according to Laemmli, and using double-strength cathode buffer as described by Guengerich (11). One gel was stained for protein according to Fairbanks *et al.* (19). The other gel was dried and cut into 0.5-cm strips which were assayed for radioactivity by combustion in a Packard 306 sample oxidizer, followed by liquid scintillation counting. fP = NADPH–cytochrome *P*-450 reductase.

TABLE 2

^{14}C Binding and loss of ethoxycoumarin deethylase activity in a reconstituted system incubated with [^{14}C]chloramphenicol

A reconstituted system containing 2 nmol cytochrome *P*-450 was incubated with 0.2 mM [^{14}C]chloramphenicol for 30 min at 37°C and dialyzed for 48 h at 4°C as described in Methods. Aliquots corresponding to 0.1 nmol cytochrome *P*-450 were then taken for assay of 7-ethoxycoumarin deethylase activity by a complete reconstituted system containing a saturating amount of the reductase, also as described in Methods. All results represent the mean ± standard deviation of duplicate or triplicate determinations.

System[a]	^{14}C Binding		Ethoxycoumarin deethylase
	nmol/ml	*nmol/nmol P-450*[b]	*nmol/min/nmol P-450*
Complete	0.98 ± 0.05	0.49	22.6 ± 1.2
+1 mM DTT[c]	0.81 ± 0.02	0.40	22.8 ± 1.6
−NADPH	0.05 ± 0.01	—	31.6 ± 1.1

	$\frac{100 \times \text{nmol } ^{14}\text{C/nmol P-450}}{\%\text{ loss of activity}}$
Complete	1.65
+1 mM DTT	1.42
−NADPH	—

[a] The conditions of the incubation with chloramphenicol.

[b] Corrected for the radioactivity present in the sample incubated with chloramphenicol in the absence of NADPH. That the radioactivity present in this sample represented residual noncovalently bound material was demonstrated by gel filtering an identical sample on a Sephadex G-25 column as described in Methods.

[c] DTT = dithiothreitol.

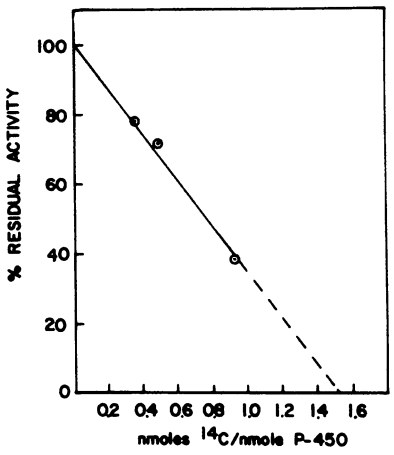

FIG. 2. *Relationship between the ethoxycoumarin deethylase activity (nmol/min/nmol P-450) and the ^{14}C labeling of the cytochrome P-450 of a reconstituted system incubated with [^{14}C]chloramphenicol and dialyzed as described in Methods*

Total protein-bound radioactivity is equated with radioactivity bound to cytochrome *P*-450, no correction being made for the low amount of radioactivity associated with the NADPH–cytochrome *P*-450 reductase (Fig. 1). The sample exhibiting 38% residual activity was prepared by adding an additional aliquot of NADPH 10 min after the incubation with chloramphenicol had been started.

perhaps only 1.2 nmol ^{14}C plays any direct role in the loss of activity.

The reactive intermediate of chloramphenicol has been suggested to be an oxamyl chloride, which would be expected to acylate hydroxyl, sulfhydryl, and amino groups in the protein (18). Rat liver cytochrome *P*-450 has previously been shown to contain seven sulfhydryl groups which are readily titratable with PDS in the presence of 0.5% SDS (3). Titration with PDS of the reconstituted system proteins after incubation with chloramphenicol indicated a loss of free sulfhydryl groups on the cytochrome *P*-450 roughly paralleling the extent of ^{14}C binding. However, structural work now in progress suggests that a significant portion of the bound ^{14}C material resides in a linkage which is stable to neutral 1 M hydroxylamine or to performic acid, treatments which would be expected to cleave thiol esters.

DISCUSSION

The data presented in this communication suggest that chloramphenicol causes inactivation of the cytochrome *P*-450 of a reconstituted system by virtue of covalent binding of an active intermediate to the protein moiety. First, both inactivation and covalent binding require the presence of NADPH. Second, the inactivation occurs without detectable heme destruction or loss of cytochrome *P*-450. Finally, the covalent binding of ^{14}C from [^{14}C]chloramphenicol to cytochrome *P*-450 appears to be rather specific, complete inactivation being accompanied by the binding of 1.5 nmol ^{14}C/nmol *P*-450. Experiments where the chloramphenicol incubations are carried out in the presence of 1 mM dithiothreitol or where the labeled protein is treated with dithiothreitol subsequent to the completion of chloramphenicol metabolism suggest that up to 20% of the bound label may not be involved in the inactivation.

The reasons for the greater than 1:1 stoichiometry of ^{14}C binding:loss of enzyme activity and for the lack of protection or reactivation of the cytochrome *P*-450 with dithiothreitol despite a 20% reduction in the amount of covalent binding to the protein remain to be elucidated. One explanation is the binding of an active intermediate of chloramphenicol to more than one amino acid residue in the cytochrome *P*-450, the derivatization of only one of which is involved in the loss of enzymatic activity. Preliminary structural data suggest the presence of two chemically different classes of amino acid adducts of chloramphenicol, one of which is readily cleaved by treatment with dithiothreitol, neutral hydroxylamine, or performic acid, suggestive of a thiol ester. An alternative explanation which could account for some of the deviation from 1:1 stoichiometry is the binding of an active intermediate to already enzymatically inactive protein, such as cytochrome *P*-420, which accounts for approximately 10% of the heme protein in the preparation used in the present investigation. On the other hand, the almost negligible amount of ^{14}C binding to the NADPH–cytochrome *P*-450 reductase suggests that only those protein molecules actively involved in metabolizing chloramphenicol become labeled.

Finally, it is important to point out that the approach used in conventional chemical modification of proteins whereby substrate protection against inactivation is used as a criterion to decide whether a particular modification is active site directed may not be valid in the case of a suicide substrate. For example, it has previously been suggested that the irreversible inhibition of microsomal monooxygenase activity caused by chloramphenicol might reflect an active site-directed mechanism (4). Part of the support for this hypothesis was the finding that *o*-nitroanisole significantly decreased the covalent binding to microsomes of ^{14}C from radiolabeled chloramphenicol. However, if the two substrates are competitive inhibitors of each other, then less reactive intermediate will be produced from the suicide substrate in the presence than in the absence of the other substrate. One would then expect less covalent binding and hence inactivation, not because the protecting substrate prevented binding of the active agent to the protein (as in conventional chemical modification), but because less active intermediate was formed. In the absence of data on the degree of both covalent binding and metabolism of the suicide substrate in the presence and absence of the putative protecting substrate, conclusions such as in Ref. 4 as to the mechanism of the apparent protection may be premature.

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