Oxidative Metabolism of Spironolactone: Evidence for the Involvement of Electrophilic Thiosteroid Species in Drug-Mediated Destruction of Rat Hepatic Cytochrome P450†

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ABSTRACT: In a preliminary paper [Decker et al. (1986) *Biochem. Biophys. Res. Commun. 136*, 1162] we have shown that the antimineralocorticoid spironolactone (SPL) preferentially inactivates dexamethasone (DEX) inducible rat hepatic cytochrome P450p isozymes in a suicidal manner. These findings are now confirmed, and the kinetic characteristics of such a process are detailed. In an effort to elucidate the mechanism of SPL-mediated inactivation of cytochrome P450, we have examined the metabolism of SPL in vitro. Incubation of [^{14}C]SPL and NADPH with liver microsomes prepared from DEX-pretreated rats results in the formation of several polar metabolites separable by HPLC with UV detection. This process is found to be dependent on NADPH, O_2, SPL, and enzyme concentration, as well as temperature. Furthermore, metabolite formation was significantly attenuated by P450 inhibitors CO and *n*-octylamine. Mass spectral analysis (thermospray LC/MS, FAB/MS, and FAB/MS/MS) of the two most prominent polar metabolites indicated that these compounds had molecular weights that corresponded to the sulfinic and sulfonic acid derivatives of deacetyl-SPL (SPL-SH). These findings document the formation of previously unreported polar metabolites of SPL by rat liver microsomes enriched in cytochrome P450p and implicate a role for this isozyme in the oxidation of the thiol moiety of deacetyl-SPL. The detection of such metabolites also implicates a catalytic trajectory that includes the thiyl radical and/or sulfenic acid species as a plausible protagonist in drug-mediated inactivation of cytochrome P450p.

Extensive studies by Menard, Gillette, and co-workers (Menard et al., 1979a,b) have established that spironolactone (SPL, Figure 1) inactivates adrenal and testicular microsomal cytochrome P450[1] in a variety of animal species, essentially in a mechanism-based suicidal process. Deacetylation of SPL to its thiol species was critical for cytochrome P450 destruction (Menard et al., 1979a). Although the guinea pig adrenal and testicular microsomal P450 appeared the most susceptible to SPL-mediated inactivation, a 10–20% loss of guinea pig hepatic microsomal P450 was also reported (Menard et al., 1979b). Colby and co-workers (Sherry et al., 1981, 1986) confirmed the earlier reports of P450 destruction in guinea pig adrenal and testicular microsomes, but failed to observe any hepatic P450 destruction by SPL-SH, thereby concluding that this species was not metabolized by guinea pig hepatic microsomal P450. However, as reported below, we also observe a 20% loss of hepatic P450 content in untreated rats, a finding that concurs with the earlier paper.

These observations and the report that SPL induced immunochemically detectable levels of the steroid-inducible P450p isozymes, without increasing the total microsomal P450 content in rats (Heuman et al., 1982), led us to examine whether SPL is a suicide substrate/inducer of P450p.[2] Indeed, we found that SPL reduced hepatic P450 content and activity in untreated rats, an effect greatly exacerbated by pretreatment with P450p inducers such as dexamethasone (DEX) (Decker et al., 1986). Furthermore, such SPL-mediated loss of rat liver microsomal P450 could be reproduced in vitro, was enhanced by rat pretreatment with DEX and phenobarbital (PB), was blocked by the so-called specific P450p inhibitor, troleandomycin (TAO), and subscribed to all the criteria of mechanism-based suicidal inactivation that had been examined (Decker et al., 1986). However, we were intrigued that such P450p loss, although accompanied by stoichiometric loss of prosthetic heme, involved a novel form of suicide inactivation, wherein the prosthetic heme [or its metabolic derivative(s)] was found to be covalently bound to the apocytochrome, apparently at the active site (Decker et al., 1986; Correia et al., 1987). Such prosthetic heme destruction appears to be associated with P450-mediated one-electron oxidations of several substrates (Davies et al., 1986; Decker et al., 1986; Guengerich, 1986; Correia et al., 1987) and is particularly overt when P450p is the catalyst (Decker et al., 1986; Correia et al., 1987). The mechanism of such SPL-mediated P450 inactivation, however, remains unclear. In an effort to elucidate the mechanism of this process, we have examined the metabolism of SPL by DEX-inducible P450 isozymes. Our findings, described herein, implicate a reactive thiyl radical and/or sulfenic

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[1] The terms cytochrome P450 and P450 will be used interchangeably throughout the text.

[2] The term P450p will be used to collectively denote all P450p isozymes (constitutive or DEX-inducible) since SPL appears to inactivate 6β-, 2β-, and 15β-testosterone hydroxylases in untreated, PB-pretreated, or DEX-pretreated rats to the extent of ≥95%. These enzymes belong to the P450IIIA gene subfamily (Nebert et al., 1987).

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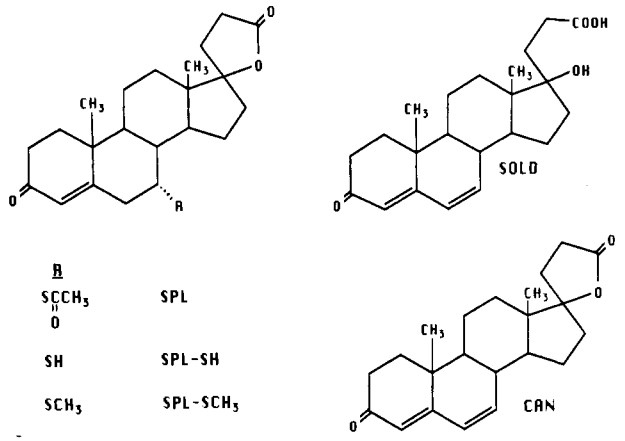

FIGURE 1: Chemical structures of spironolactone (SPL) and its analogues: SPL-SH, deacetyl SPL; SPL-SCH_3, thiomethylspironolactone; CAN, canrenone; SOLD (canrenoic acid), soldactone.

acid species as a key protagonist in the catalytic trajectory leading to SPL-mediated inactivation of P450.

Experimental Procedures

Materials. DEX and SPL were purchased from Sigma Chemical Co., St Louis, MO, and Calbiochem Inc., San Diego, CA, respectively. The SPL analogues and the [^{14}C]SPL, [^{14}C]CAN, and [^{14}C]SOLD (specific activity, 57.7, 70.5, and 60.7 mCi/mg, respectively) were generously donated by Searle Chemical Co., Skokie, IL. TAO was a gift from Dr. J. J. Korst, Pfizer Inc. All other chemicals were of reagent grade and obtained from commercial sources.

Animal Treatment. Male Sprague-Dawley rats (200–220 g) were injected intraperitoneally with DEX (100 mg/kg, dissolved in corn oil) daily for 3–4 days. Twenty-four hours later, they were given a single intraperitoneal injection of SPL (150 mg/kg) suspended in water with Tween 80 (3 drops/10 mL) and killed 3 h later. When TAO pretreatment was also included in the protocol, DEX-pretreated animals were given TAO (500 mg/kg, ip) 3 h before sacrifice. In some experiments, rats were also pretreated with either sodium phenobarbital (PB, 80 mg/kg, ip) or β-naphthoflavone (80 mg/kg, ip) daily for 4–5 days.

Enzyme Preparations. Liver microsomes were prepared as described before (Bornheim et al., 1985), from livers that were perfused in situ with ice-cold 0.15 M KCl solution, removed, and homogenized. Protein concentration and P450 content were determined by the method of Lowry et al. (1951) and Omura and Sato (1964), respectively.

Incubation Systems. A typical incubation mixture (final volume, 1.5, 3, or 6 mL) contained hepatic microsomes (2–3 μM P450), NADPH (2 mM), EDTA (1.5 mM), SPL (0.5 or 1 mM), dissolved in PC buffer containing phosphatidylcholine (25 mg/100 mL sonicated in 0.1 M phosphate buffer, pH 7.4), and 0.1 M phosphate buffer, pH 7.4. Appropriate control incubations that excluded SPL or NADPH were always included in parallel. Reactions were started by addition of NADPH and terminated at designated times by gassing with CO and chilling in ice. In some experiments, superoxide dismutase (0.1 mg/mL), catalase (0.5 mg/mL) bis(*p*-nitrophenyl) phosphate (BNPP, 0.4 mM), *n*-octylamine (3 or 5 mM), or GSH (1 or 5 mM) were also included in the reaction mixtures.

When SPL-mediated destruction of hepatic P450h was examined, the enzyme was isolated and purified from untreated rats as described (Sugiyama et al., unpublished experiments). Liver microsomal NADPH–P450 reductase was isolated and purified from PB-pretreated rats by the method of Shephard et al. (1983). The mixed-function oxidase activity of P450h was reconstituted by a 10-min preincubation of P450h (0.6 nmol/mL), NADPH–P450 reductase (≈600 units/0.1 nmol of P450h), and 20 μg of dilaurylphosphatidylcholine (sonicated in phosphate buffer, 0.1 M, pH 7.4) at 37 °C. The complete reaction mixture (final volume, 2 mL) contained SPL-SH (0.5 mM in PC buffer), EDTA (1 mM), and catalase (0.5 mg/mL). The reaction was initiated with NADPH and allowed to proceed at 37 °C for 30 min before termination as described above. Control incubations that excluded either NADPH or SPL-SH were also run in parallel.

Enzyme Assays. Cytochrome P450 destruction assays were conducted as detailed previously (Correia et al., 1987), and P450 content was monitored in the incubates as described (Estabrook et al., 1972). Erythromycin *N*-demethylase activity and regio- and stereoselective testosterone hydroxylase activities were monitored by HPLC by the method reported previously (Bornheim et al., 1987; Correia et al., 1987).

Irreversible Binding of SPL or Analogues to Rat Liver Microsomal Protein. Reaction mixtures were similar to the prototype incubation described above, except that [^{14}C]SPL (0.015–0.5 μCi), [^{14}C]CAN (0.5 μCi), or [^{14}C]SOLD (0.5 μCi) was included per reaction along with a nominal concentration of 0.5 or 1 mM of the corresponding cold steroid, and the incubations were carried out at 37 °C for 30 min before termination with either 25% trichloroacetic acid or 5% H_2SO_4 in MeOH (v/v). The protein precipitates were then exhaustively and sequentially washed with various organic solvents (acetone, ethanol, ether, ethyl acetate) to remove residual drug. The precipitates were then dissolved in 1 N NaOH, the protein concentration was determined in aliquots, and the remainder was quantitated by liquid scintillation counting. Parallel incubations that excluded NADPH, but were identical in every other respect, were always included as controls.

In Vitro Generation and Isolation of SPL Metabolites. Reaction mixtures were identical with the prototype described above, except that they also included [^{14}C]SPL (≈0.015 μCi). Incubations were carried out at 37 °C for 30 min, chilled, and then centrifuged at 100000*g* for 30 min. The supernatants were collected, filtered through a 0.45-μm Rainin nylon 66 filter, and subjected to HPLC or passed through a Waters C_{18} SepPak and the metabolites eluted with MeOH/H_2O (50:50 v/v). The HPLC system used for separation of metabolites was equipped with a Rainin Dynamax C_{18} column (5 μm, 4.6 mm × 20 cm) connected to a Hewlett-Packard diode array (UV) detector set at 240 nm. In some experiments, when radiolabeled SPL was omitted, soldactone (42.5 μM) was included as an internal standard. The mobile phase used was 0.02 M NaOAc (pH 4.0)/MeOH (50:50 v/v), at a flow rate of 1 mL/min, and the metabolite peaks (a and b) eluting at 15 and 17 min, respectively, were collected individually and subjected to scintillation counting and mass spectrometric analyses as described below. Metabolites could also be quantitated by integration of the area under the peaks. Such values, calculated by using the extinction coefficient of SPL at 240 nm, agreed reasonably well with those quantitated by inclusion of either [^{14}C]SPL or the internal standard.

Syntheses of Authentic SPL Sulfinic and Sulfonic Acid Derivatives. These compounds were chemically synthesized by the procedure described (Filby et al., 1973). SPL (0.05 mol) dissolved in CH_2Cl_2 was cooled to −30 °C before addition of 10-mL aliquots of *m*-chloroperbenzoic acid (mCPBA, 0.1 mol, dissolved in 200 mL of CH_2Cl_2 and also cooled at −30

°C) at half-hour intervals. The mixture was left standing overnight at −30 °C before further cooling at −70 °C, rapid filtration to remove mCPBA, and evaporation under N_2. The residue was then dissolved in MeOH/H_2O (50:50 v/v) before HPLC to separate the sulfinic and sulfonic acid derivatives as described above.

Mass Spectrometric Analyses of SPL Metabolites: LC/MS. Thermospray (TSP) LC/MS spectra were generated by using an LKB Model 2150 HPLC pump with LKB Model 2152 LC controlled (LKB, Bromma, Sweden) coupled to a Vestec thermospray LC-MS Model 201 (Vestec Corp., Houston, TX) dedicated LC/MS system. The column used was an Ultrasphere C_{18} (5 μm, 4.6 mm × 15 cm) column protected with a 5-μm Ultrasphere precolumn. The mobile phase consisted of 0.2 M ammonium acetate/methanol/glacial acetic acid (50:50:1, v/v/v) pumped isocratically at a flow rate of 1.0 mL/min. Operating temperatures for the TSP interface were T_1 (vaporizer) = 135 °C, T_2(tip) = 174 °C, jet(vapor) = 258 °C, and source block = 290 °C. The system was operated in the positive ion mode with a repeller voltage of 40 V and a filament current of 300 μA.

FAB/MS and FAB/MS/MS. FAB/MS analyses were performed on a VG 70 SEQ hybrid tandem instrument of EBQQ geometry (VG Analytical Ltd., Manchester, U.K.), equipped with an Ion Tech fast atom gun and VG 11/250 data system. The system was operated in the negative ion mode, and a solution of sodium iodide in water (0.1 M) was used for calibration. Ionization was achieved following bombardment with xenon (7 keV) as the primary beam, and conventional FAB spectra were recorded via the data system at an accelerating potential of 8 kV. Samples of metabolites (1–2 μg) purified by HPLC were redissolved in deionized water (3 μL) and added to glycerol (3 μL) on a FAB target.

Collisionally activated dissociation (CAD) was performed in the first (radio frequency only) quadrupole region by using argon as the collision gas. The pressure in the quadrupole analyzer housing was maintained at 1 × 10^{-6} Torr, and daughter ion spectra were obtained by selection of the appropriate $(M - H)^-$ parent by adjusting the magnetic field and scanning the quadrupole mass analyzer from m/z 1000 to 50 over a period of 5 s. Daughter ion spectra were obtained with collision energies ranging from 20 to 235 eV to identify conditions yielding the maximum fragmentation of selected parent ions.

Results and Discussion

Effects of in Vivo SPL Administration to DEX-Pretreated Rats on Hepatic Microsomal P450 Content and Function. SPL administration to DEX-pretreated rats results in nearly stoichiometric losses (≈50%) in hepatic microsomal P450 and heme content with concomitant loss of >95% of P450p-selective enzyme activities within 3 h of treatment (Decker et al., 1986). A corresponding loss of 21% in hepatic microsomal P450 content and corresponding 75 and 58% losses in ethylmorphine and benzphetamine *N*-demethylases, coupled with nearly 80% loss in 2α-testosterone hydroxylase activity in untreated rats, attest to inactivation of P450h[3] by SPL as well (Decker et al., 1986). On the other hand, a mere 12% SPL-mediated reduction in 16β-testosterone hydroxylase activity in PB-pretreated rats indicated that P450b is not a major target of the drug (M. C. Underwood and M. A. Correia, unpublished observations).

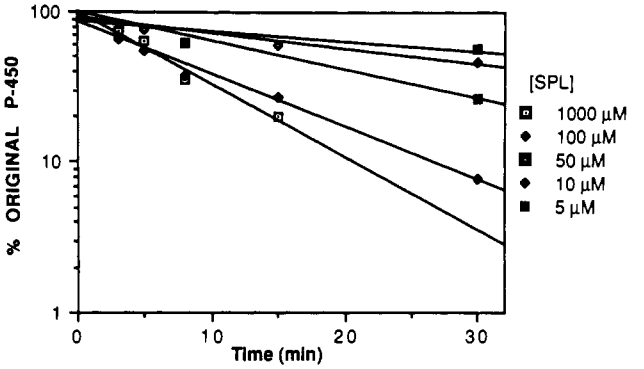

FIGURE 2: In vitro destruction of hepatic microsomal P450 by SPL. Concentration-dependent destruction of P450 content in liver microsomes from DEX-pretreated rats. The maximum possible spectrally detectable P450 loss observed after incubation with SPL (1 mM), in the presence of NADPH, at 37 °C for 30 min is depicted as 100% "original" SPL-susceptible P450. It is noted that at SPL concentrations of 5–100 μM complete deacetylation to SPL-SH is observed, whereas at the higher concentration 40% of SPL is converted to SPL-SH at the start of the reaction.

Table I: In Vitro Effects of SPL and Analogues on Rat Hepatic Microsomal Cytochrome P450 Content[a]

rat pretreatment	steroids	P450 loss (% of original) −NADPH	P450 loss (% of original) +NADPH
none	SPL	0	21
DEX	SPL	0	56
	SPL-SH	0	51
	none	0	4
	SPL/BNPP (0.4 mM)	0	7
	CAN	0	1
	SOLD	0	2
	SPL-SCH_3	0	6
DEX/TAO	SPL	0	23

[a] All values are means of two individual experiments. Basal (100%) values for microsomal P450 content (nmol/mg of protein) were 1.62 ± 0.21 (DEX), 1.74 ± 0.20 (DEX/TAO), and 0.98 ± 0.07 (none). For details please see Experimental Procedures.

SPL-mediated hepatic microsomal P450 loss and functional inactivation in DEX-pretreated rats appear to be critically dependent on its thiol functionality since the compound devoid of the thioester moiety of SPL, canrenone [CAN, an in vivo metabolite (Cook et al., 1988)] failed to lower either hepatic P450 content or a P450p-selective functional marker, erythromycin *N*-demethylase (not shown).

In Vitro Inactivation of Rat Hepatic Microsomal P450 by SPL and/or Its Analogues: Kinetics and Features. The kinetics of spectrophotometrically determined P450 destruction by SPL (5–1000 μM) are depicted in Figure 2. When $t_{1/2}$ (min) obtained from this figure is plotted against 1/[SPL] mM^{-1}, values for K_{inact} and the dissociation constant of the inactivator for the enzyme (K_I) could be calculated to be 0.08 min^{-1} and ≈17 μM, respectively. We have used three different methods to calculate the partition ratios for SPL-mediated destruction of P450 in DEX-pretreated rat liver microsomes. The substrate exhaustion technique with SPL (2.5 and 5.0 μM) yields a partition ratio of 22.4 ± 3.9 (N = 4), whereas by the substrate disappearance method with SPL-SH (50 μM) the partition ratio was 24.9. On the other hand, if the ratios of the polar metabolites formed to the P450 loss are computed

[3] Indeed, this possibility has been confirmed by our observation that SPL-SH inactivates isolated purified rat liver P450h by ≈50% in a system reconstituted with NADPH–P450 reductase. In addition, incubation of SPL (0.5 mM) with liver microsomes from PB-pretreated rats also resulted in a 50–80% inactivation of 2α-testosterone hydroxylase activity, thereby lending further support for such a possibility (M. Underwood and M. A. Correia, unpublished observations).

Table II: Irreversible Binding of SPL and Its Structural Analogues to Liver Microsomal Protein from DEX-Pretreated and Untreated Rats[a]

rat pretreatment	analogue	addition (mM)	irreversible binding (nmol/mg of protein)		
			−NADPH	+NADPH	Δ
none	[^{14}C]SPL*		11.5	13.7	2.2
	[^{14}C]SOLD*		5.57	5.99	0.4
	[^{14}C]CAN*		2.75	4.51	1.8
DEX	[^{14}C]SPL*		12.3 ± 1.53	18.7 ± 1.62	6.3 ± 4.3
	[^{14}C]SOLD*		4.47	6.93	2.5
	[^{14}C]CAN*		3.27 ± 0.47	14.8 ± 2.47	11.5 ± 2.1
DEX	[^{14}C]SPL		12.6 ± 4.12	20.0 ± 5.57	7.5 ± 4.2
	[^{14}C]SPL	BNPP (0.4)	5.75		
	[^{14}C]SPL	GSH (1)	6.51	10.9	4.4
	[^{14}C]SPL	GSH (1) DTT (1)	3.61	5.39	1.8
	[^{14}C]SPL	GSH (5.0)	6.72 ± 2.17[b]	12.3 ± 4.04[c]	5.5 ± 2.7
	[^{14}C]SPL	GSH (5.0) DTT (5.0)	1.68	2.14	0.5
	[^{14}C]CAN		4.01 ± 2.94	14.2 ± 7.04	10.2 ± 4.2
	[^{14}C]CAN	GSH (5)	3.23 ± 1.98	5.64 ± 3.66	2.4 ± 1.8

[a] For experimental details please see Experimental Procedures. The concentration of SPL or analogue was 0.5 mM, except where indicated by an asterisk, when the concentration was 1 mM. Values are mean ± SD of at least three separate experiments, except where mean of two individual experiments is given as a single value. [b] Statistically significant at $p < 0.01$ when compared with corresponding [^{14}C]SPL controls incubated in the absence of GSH. [c] Statistically significant at $p < 0.05$ when compared with corresponding [^{14}C]SPL controls incubated in the absence of GSH.

at the time of maximal P450 inactivation (30 min), then a value of 19.5 ± 7.5 ($N = 7$) is obtained. Thus, the partition ratios obtained with three separate methods of determination are in close agreement with each other.

The enhancement of SPL-mediated P450 loss in liver microsomes enriched in P450p by DEX pretreatment of rats, and its attenuation by in vivo complexation of P450p with its specific inhibitor TAO, may be seen in Table I. These findings support our previous results that P450p is a target for SPL-mediated inactivation (Decker et al., 1986). Furthermore, when aliquots of SPL/NADPH-incubated liver microsomes from DEX-pretreated rats were assayed in a fresh incubation system, P450p-dependent 2β-, 6β-, 15β-, and 18-testosterone hydroxylase activities were found to be inhibited by ≈90% (M. C. Underwood and M. A. Correia, unpublished observations). These findings confirm the in vivo observation that P450p is indeed a target for SPL inactivation.

In addition, the involvement of the SPL-SH species in the destruction is verified not only by the finding that this species is equipotent to SPL in its destruction of liver microsomal P450 but also by the marked attenuation observed when BNPP, a microsomal esterase inhibitor, is included along with SPL in the reaction mixture (Table I). These findings thus confirm that, in analogy to the adrenal and testicular microsomal systems (Menard et al., 1979a; Sherry et al., 1986), SPL-SH plays a key role in the destruction of hepatic microsomal P450 in rats. Furthermore, the structural requirement of an SH functionality for SPL-mediated inactivation in vitro may also be appreciated by the negligible P450 losses observed (Table I) when SPL was replaced in the incubation system by the desulfurylated analogues [CAN and soldactone (SOLD), Figure 1] or thiomethyl-SPL (a significant in vivo metabolite in humans; Overdiek et al., 1985). These findings again suggest that an oxidizable thiol moiety is critical for P450 destruction.

From studies employing either [^{14}C]- or [^{35}S]SPL, Menard and co-workers (Menard et al., 1979b) have suggested that SPL-mediated destruction of adrenal or testicular microsomal P450 was associated with P450-dependent oxidative activation of the free SH moiety and subsequent covalent binding of the S atom to an adrenal microsomal 50-kDa protein (with the electrophoretic characteristics of adrenal cytochrome P450). Such binding with the accompanying loss of P450 heme was postulated to account for the irreversible inactivation of the enzyme. They also suggested that SPL-mediated destruction of adrenal P450 did not mimic that of its hepatic counterpart caused by compounds such as CS_2 or parathion. P450 destruction by these substrates is associated with binding of atomic sulfur to protein and retention of intact heme, albeit as P420 (Dalvi et al., 1974, 1975; De Matteis, 1974). In contrast, during SPL-mediated destruction of adrenal and testicular microsomal P450, heme was lost concomitantly (Menard et al., 1979b).

To determine whether a similar process occurs during SPL-mediated destruction of hepatic P450, we have monitored the NADPH-dependent irreversible binding to microsomal protein of [^{14}C]SPL, [^{14}C]CAN, or [^{14}C]SOLD catalyzed by liver microsomes from untreated or DEX-pretreated rats. As can be seen (Table II), NADPH significantly increased the binding of [^{14}C]SPL to liver microsomal protein from untreated rats. DEX pretreatment of rats greatly magnified such an NADPH-dependent increase[4] (Table II), consistent with the observed enhancement of P450 destruction observed in these liver microsomes (Table I). We were intrigued, however, that not only was a disproportionately high ratio (≈12:1) of NADPH-dependent covalent steroid binding (nmol/mg of microsomal protein) to corresponding microsomal P450 loss (nmol/mg of protein) observed but also substantial binding of SPL could be detected even in the absence of NADPH. Inspection of the previously reported data in the adrenal system (Menard et al., 1979b) indicates similar NADPH-independent irreversible drug binding, which apparently is concentration dependent and quenched to a large extent by inclusion of GSH and dithiothreitol (DTT) in the incubation system (Menard et al., 1979b). Such binding most likely is due to interaction of the protein sulfhydryls with the SPL-SH resulting in "SPL–protein mixed disulfides". This possibility is strengthened by the finding that inclusion of BNPP in the incubation system considerably attenuated SPL binding in the absence of NADPH (Table II). Since the microsomal esterases independent of NADPH would deacetylate SPL to SPL-SH, such an occurrence in the absence of NADPH is not that surprising. Furthermore, as expected, inclusion of GSH and/or DTT in the incubation largely attenuated the NADPH-in-

[4] Similar findings were obtained when [^{14}C]SPL was replaced with [^{35}S]SPL as the substrate in preliminary studies that were unfortunately limited by the availability of [^{35}S]SPL.

dependent binding, indicating not only that protein alkylation occurs external to the P450 active site but that it may involve nonspecific interaction of SPL-SH with microsomal protein sulfhydryls to form mixed disulfides (Table II). In addition to decreasing mixed disulfides, inclusion of DTT further lowered SPL binding to protein in the +NADPH + GSH system, possibly because of its binding to P450 heme and competitive inhibition of SPL metabolism within the P450 active site and/or quenching of reactive SPL-derived metabolites after their escape to the exterior (Table II). Thus, although the above findings indicated that SPL-mediated P450 inactivation is accompanied by covalent binding of SPL to microsomal protein, the lack of stoichiometry between these parameters, particularly after attenuation of the nonspecific SPL binding, raises doubts about the role of such binding in SPL-mediated destruction of P450 heme.

Such skepticism is enhanced by the finding that the NADPH-dependent irreversible binding of [^{14}C]CAN to liver microsomal protein from untreated or DEX-pretreated rats is even greater than that of [^{14}C]SPL in corresponding systems (Table II). Attenuation of [^{14}C]CAN binding by inclusion of GSH in the incubation system (Table II) suggests that it might be due to an electrophilic metabolite (6–7 epoxide?) which escapes the P450 active site and reacts with nucleophiles external to that site. The binding of [^{14}C]SOLD, on the other hand, was not as pronounced (Table II), but this may be largely due to the relatively greater polarity and consequently lesser accessibility of this analogue to the P450 active site than of its closed-ring lactone analogue CAN.

More importantly, since [^{14}C]CAN binds to a greater extent than [^{14}C]SPL (Table II), the question remains whether the observed binding of the latter is due to activation of its thiol moiety per se or activation of its putative metabolite, CAN. Since CAN does not destroy P450 in vivo or in vitro (Table I), the relevance of irreversible steroid binding in P450 inactivation remains questionable.

Additional evidence that tends to exonerate apoprotein alkylation as a significant factor in SPL-mediated P450 inactivation is the finding that not only is stoichiometric loss of spectrally detectable heme observed in vitro but also no release of intact heme is detected in vivo after SPL-mediated inactivation of P450. If the spectrally detectable P450 loss were due to discharge of its prosthetic heme after SPL-mediated apocytochrome alkylation, then release of intact heme into the hepatic "free" heme pool would have been indicated by the two reliable reporters of such an event: saturation of tryptophan pyrrolase and stimulation of microsomal heme oxygenase. These hepatic indices have signaled the loss of intact P450 heme after either apoprotein akylation by CS_2 or parathion (De Matteis, 1974) or endotoxin-enhanced P450 turnover (Bissell & Hammaker, 1977). However, they were not altered in the manner expected if intact heme were to be released into the free heme pool in SPL-treated DEX-pretreated rats (Decker et al., 1986). Such findings with SPL are not at all surprising, in view of our report that a significant fraction of P450 prosthetic heme destroyed by SPL apparently undergoes fragmentation (or electronic disruption of its porphyrin skeleton) and can be found irreversibly bound to the microsomal protein, presumably at the active site of the cytochrome (Decker et al., 1986).

Characterization of in Vitro Metabolism of SPL by Hepatic Microsomal P450 from DEX-Pretreated Rats. To gain some insight into the mechanism of this unusual mode of drug-mediated suicidal inactivation of P450, we have examined the metabolism of SPL in systems catalyzed by "P450p-enriched" liver microsomes from DEX-pretreated rats. Supernatants (100000*g*) of liver microsomes incubated with SPL in the presence or absence of NADPH were passed through C_{18} SepPak and metabolites eluted with aqueous 50% MeOH and subjected to HPLC as described (Experimental Procedures). The HPLC profiles of the NADPH-supplemented systems revealed the presence of two relatively polar metabolite peaks (a and b) eluting at 14.9 and 16.5 min, respectively. These particular peaks were absent in the corresponding control incubations containing SPL but devoid of NADPH. Furthermore, their formation was dependent on O_2, NADPH, SPL, and P450 concentration as well as temperature (Table III), indicating that they were generated enzymatically. Inclusion of P450 inhibitors CO or *n*-octylamine significantly attenuated the yield of these metabolites, implicating P450 in their formation (Table III). Inhibition by *n*-octylamine also excluded any contributions to the formation of these metabolites by the microsomal flavin monooxygenase system, which is believed to convert thiocarbamides to their corresponding sulfenic acids (Krieter et al., 1986). Comparable incubations containing liver microsomes from PB-pretreated, β-naphthoflavone-pretreated, or untreated rats yielded relatively lower amounts of these metabolites, consistent with the lower P450p content of these preparations (Table III).

Inclusion of GSH in the system not only reduced the generation of these metabolites but also significantly attenuated P450 loss (Table III). Such attenuation apparently is not due to conversion of SPL-SH to its GSH adduct and thus to effective reduction of the active (inactivating) substrate species in the system, since GSH failed to reduce the HPLC/UV-detectable levels of SPL-SH (results not shown). Inclusion

Table III: In Vitro Conversion of SPL to Polar Metabolites by Rat Liver Microsomes

rat pretreatment	incubation system[a]	P450 loss (% of original)	polar metabolites (nmol formed/incubation)
DEX	complete	48.5 ± 3.50	88.8 ± 21.9
	−NADPH	0.0	5.01 ± 10.0
	+CO	0.0	0.0
	0 °C	1.6	19.0
	P450 [1 μM]	50.0	36.3
	SPL [0.1 mM]	39.6	48.0
	SPL [0.01 mM]	19.1	6.59
	n-octylamine [3 mM]	26.5	25.6
DEX	complete	48.5 ± 3.50	88.6 ± 19.2
	+catalase (0.5 mg/mL)	43.0 ± 3.68[b]	77.4 ± 39.7[b]
	+SOD (0.1 mg/mL)	52.3 ± 3.97[b]	57.8 ± 19.1[b]
	complete	43.9 ± 7.54	78.7 ± 17.8
	+catalase + SOD	43.1 ± 13.5[b]	53.7 ± 19.8[b]
DEX	complete	51.6 ± 5.49	100.5 ± 32.0
	+GSH [5 mM]	34.1 ± 11.6[c]	61.9 ± 28.8[b]
DEX	complete	42.6 ± 6.15	76.3 ± 15.7
none	complete	21.4 ± 10.2[d]	44.0 ± 22.3[e]
PB	complete	19.9 ± 3.83[f]	35.9 ± 8.82[d]
β-NF	complete	15.3 ± 5.30[f]	17.7 ± 5.26[f]

[a] SOD, superoxide dismutase. The complete incubation mixture (final volume, 3 mL) consisted of P450 (3 μM), [^{14}C]SPL (1.0 mM), EDTA (1.5 mM), NADPH (1.5 mM), and 0.1 M phosphate buffer, pH 7.4. Reactions were carried out at 37 °C for 30 min, and P450 loss was monitored and metabolites were isolated by HPLC and quantitated as described (Experimental Procedures). All values are mean ± SD of three to seven individual experiments, except where the mean of two experiments is given as a single value. [b,c] Statistical significance when compared with its corresponding complete system run in parallel: (*b*) NS; (*c*) $p < 0.02$. [d,e,f] Statistical significance when compared with the corresponding DEX/complete system: (*d*) $p < 0.005$; (*e*) $p < 0.05$; (*f*) $p < 0.001$.

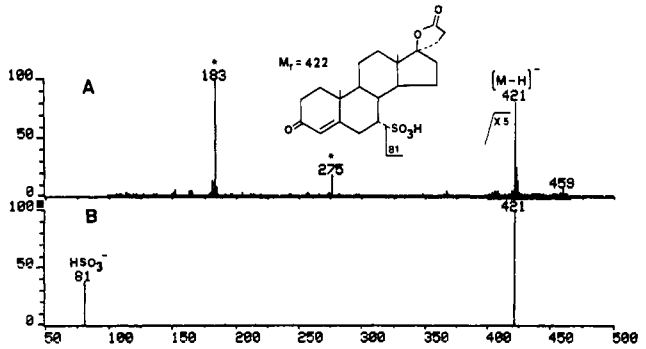

FIGURE 3: (A) Negative ion FAB mass spectrum of peak a (sulfonic acid species). *Y* and *X* axes denote relative abundance and mass/charge, respectively. Ions denoted by asterisks are from the glycerol matrix. (B) Daughter ion spectrum following collisional activation of *m/z* 421 ($[M - H]^-$), obtained from the same sample. *Y* and *X* axes denote relative abundance and mass/charge, respectively. For details see Experimental Procedures.

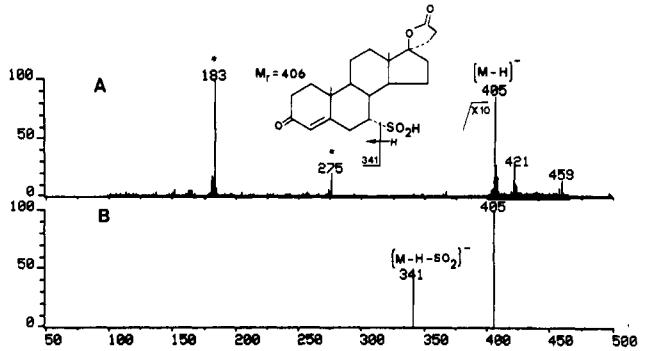

FIGURE 4: (A) Negative ion FAB mass spectrum of peak b (sulfinic acid species). *Y* and *X* axes denote relative abundance and mass/charge, respectively. Ions denoted by asterisks are from the glycerol matrix. (B) Daughter ion spectrum following collisional activation of *m/z* 405 ($[M - H]^-$), obtained from the same sample. *Y* and *X* axes denote relative abundance and mass/charge, respectively. For details see Experimental Procedures.

of catalase did not significantly affect metabolite formation, thereby excluding the contribution of any externally generated H_2O_2 to such metabolism. On the other hand, although superoxide dismutase by itself or in combination with catalase reduced the liver microsomal production of metabolites, such a reduction failed to reach statistical significance (Table III). The consistent metabolite attenuation observed with both GSH and superoxide dismutase, neither of which would be accessible to the active site of P450, supports, the possibility that the polar metabolites are generated from an oxidized SPL-SH metabolite(s) released from the active site.

Structural Characterization of the P450-Dependent Metabolites of SPL. To determine the structural identity of these metabolites, each metabolite peak was collected, purified by repeated HPLC, lyophilized, and subjected to thermospray LC/MS, as well as FAB/MS and FAB/MS/MS analyses as described (Experimental Procedures). Conditions for TSP/MS were optimized by performing multiple injections of pure SPL-SH (M_r = 374) and CAN (M_r = 340) via the direct inlet. The spectrum for SPL-SH (not shown) exhibited the protonated molecular ion at *m/z* 375 and an ammonium ion adduct at *m/z* 392. The base peak in the spectrum was at *m/z* 341, which would result from loss of the elements of H_2S from the MH^+ species or may derive from protonated CAN formed by thermal elimination of H_2S from SPL-SH in the TSP interface. The spectrum also showed an ion at *m/z* 358 that corresponds to either loss of H_2S from the ammonia adduct ion at *m/z* 392 or to an ammonium ion adduct of CAN formed thermally.

When the supernatant obtained from NADPH-supported incubations was analyzed by LC/MS, two peaks (a and b) were observed, eluting at 7.9 and 8.5 min, respectively. The mass spectra of these two components were essentially identical with each other and with that of CAN. They exhibited a base peak at *m/z* 341, accompanied by an ammonium ion adduct at *m/z* 358. Injection of supernatant from incubations that lacked NADPH showed no such peaks. Since authentic SPL and CAN elute much later in the chromatogram (>30 min) than the materials giving rise to peaks a and b, it appeared that the latter species were polar metabolites of SPL that decompose thermally under thermospray conditions to give CAN.

Further evidence for the structures of these metabolites was obtained by FAB/MS and FAB/MS/MS analyses of specimens that had been purified by HPLC. Negative ion FAB/MS of peak a (Figure 3A) showed an intense ion at *m/z* 421 $(M - H)^-$, suggesting a molecular weight of 422. This value would correspond to the molecular weight of a sulfonic acid metabolite of SPL. When the ion at *m/z* 421 was subjected to CAD (collision energy, 235 eV), only one daughter ion was observed, viz, at *m/z* 81, corresponding to HSO_3^- (Figure 3B). The formation of this daughter anion is analogous to the generation of the *m/z* 97 from steroid sulfates under FAB/MS/MS conditions (Gaskell, 1988).

Negative ion FAB/MS of peak b (Figure 4A) showed an intense ion at *m/z* 405 and two additional ions at 421 and 459. This suggested that the ion at *m/z* 405 was derived from a sulfinic acid metabolite of SPL (M_r = 406) that had undergone partial autoxidation to the above sulfonic acid derivative during the purification procedure. The ion at *m/z* 405, however, also arises from the glycerol matrix, although it is usually very weak in the background spectra. To confirm that this ion was derived primarily from the SPL metabolite, it was selected for CAD (collision energy, 25 eV) under FAB/MS/MS conditions, when it yielded only one daughter ion at *m/z* 341 (Figure 4B). This loss of 64 amu corresponds to elimination of the elements of SO_2 from the $(M - H)^-$ parent ion and is fully consistent with the proposal that the material contained in peak b is the sulfinic acid metabolite of SPL. The isobaric ion derived from the glycerol matrix, on the other hand, does not eliminate 64 amu under CAD conditions. Interestingly, the anticipated HSO_2^- daughter ion (*m/z* 65) from this metabolite was not observed on CAD of the $(M - H)^-$ parent over a broad range of collision energies.

Finally, comparison of HPLC and FAB/MS/MS profiles of the enzymatically generated metabolites with those of authentic chemically synthesized sulfinic and sulfonic acid derivatives of SPL allowed us to unequivocally confirm their structural identity (results not shown).

Mechanistic Implications of the Observed SPL Metabolism in the Destruction of P450. In this paper, we show that liver microsomes from DEX-pretreated rats convert SPL to previously unidentified polar oxidized metabolites. Inhibition of such conversion by the microsomal esterase inhibitor BNPP (results not shown), coupled by the fact that these same metabolites were identified in incubations of human adrenal microsomes when SPL-SH was the substrate (Decker, Rettie, Rashed, Baillie, and Correia, preliminary findings), implicates SPL-SH as the intermediate that is then further oxidized by P450 to SPL metabolites, which we have characterized by mass spectrometric analyses as the sulfinic and sulfonic acid derivatives of SPL-SH. Apparently in this process a reactive SPL species is also generated which destroys the enzyme during its oxidative metabolism. We have shown previously

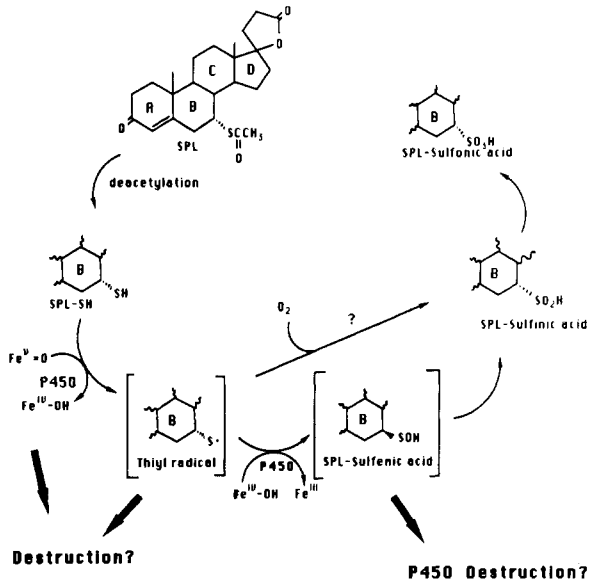

FIGURE 5: Scheme for generation of the sulfinic and sulfonic acid derivatives of SPL. For details see Results and Discussion.

that such destruction partly entails covalent linkage of the prosthetic heme to the apoprotein, apparently at the active site of the enzyme (Decker et al., 1986). Neither superoxide dismutase nor catalase (alone or in combination) attenuates SPL-mediated destruction of cytochrome P450, thereby indicating that the process occurs at the active site and thus subscribes to the criteria of mechanism-based suicide inactivation. Of the hepatic P450 isozymes examined to date, the constitutive P450h and the steroid-inducible P450p may be singled out as targets for such inactivation.

The key protagonist in their inactivation, however, remains to be identified. The unequivocal identification of the sulfinic and sulfonic acid metabolites of SPL argues for a catalytic trajectory that proceeds by way of a thiyl radical and/or sulfenic acid species as their direct precursor (Figure 5). Accordingly, one-electron oxidation of SPL by P450Fe(V)=O would result in the formation of the thiyl radical, in the process generating P450Fe(IV)=O. The thiyl radical could be further oxidized by P450Fe(IV)=O to form SPL-SOH, which could destroy the heme moiety,[5] react with protein sulfhydryls, or, in principle, on its release from the active site either be trapped by GSH (or even SPL-SH) or undergo further oxidation to the polar metabolites. On the other hand, the thiyl radicals that escape direct reaction with P450–heme or oxidation to SPL-SOH could, on leaking out of the active site, directly react with molecular O_2, ultimately resulting in the sulfinic and sulfonic acid derivatives of SPL (Schafer et al., 1978; Quintiliani et al., 1977; Harman et al., 1984). Indeed, such a possibility would rationalize the attenuation of metabolite formation by both GSH and superoxide dismutase. In view of the apparent inaccessibility of either reagent to the P450 active site, such attenuation would also appear to preclude this site as the site of generation of the sulfinic and sulfonic acid species. Theoretically, exclusive P450-catalyzed formation of these species is plausible but would require two and three catalytic cycles, respectively.

Although it is conceivable that the two processes (oxidized SPL metabolite formation and P450 destruction) may not be inextricably linked, metabolite formation not only appears to parallel P450 destruction but exhibits many similar enzymatic features and kinetic characteristics. Given this collective evidence, the attenuation of P450 destruction and metabolite formation by GSH[6] as well as the notorious reactivity of sulfenic acids in general, we find it tempting to speculate that the electrophilic thiyl radical and/or sulfenic acid species might be a key protagonist in the SPL-mediated cytochrome P450 destruction.

Alternatively, as indicated above, one-electron oxidation of SPL-SH by P450 would result not only in the formation of the thiyl radical (SPL-S$^\bullet$) but also in the concomitant generation of the catalytic intermediate P450Fe(IV)=O (Figure 5). This iron–oxo species is believed to be generated not only during one-electron oxidations of 4-alkyldihydropyridines by P450 (Augusto et al., 1982) but also during catalyses by peroxidases such as cytochrome *c* peroxidase (Finzel et al., 1984; Florence, 1985; Rice et al., 1983). This oxo species, or its protonated counterpart Fe(III)OH$^\bullet$, has been implicated in the irreversible inactivation of cytochrome *c* by H_2O_2 (Florence, 1985; Rice et al., 1983). Furthermore, we have shown that one-electron oxidation of the 4-ethyldihydropyridine (DDEP) by P450p is associated with destruction of its prosthetic heme and irreversible binding of the heme product(s) to the apoprotein, very similar to that observed after SPL-mediated destruction of P450p (Decker et al., 1986; Correia et al., 1987). Since both these substrates apparently undergo one-electron oxidation by P450, it is tempting to implicate the catalytic intermediate, P450Fe(IV)=O/Fe(III)OH$^\bullet$,[7] particularly if allowed to persist, as a common determinant in the unusual P450p–heme destruction mediated by these compounds.

Such a proposal is particularly attractive in view of the unusual active site environment of P450p. Alignment of the conserved distal helical regions in the vicinity of the O_2-binding site of the prosthetic heme of P450cam and several membrane-bound P450 isozymes (Poulos et al., 1987; Poulos, 1988) with that of P450p indicates that in the immediate vicinity of the usually conserved threonine residue [Thr_{252} in P450cam; Thr_{310}/proline (Pro_{310}) in reported rat P450p sequences] a tyrosine residue (Tyr_{308}) is positioned in all inducible and constitutive rat P450p isozymes as well as human and rifampin-induced rabbit orthologues reported to date (Gonzalez et al., 1985, 1986a,b; Molowa et al., 1986; Potenza et al., 1988). The exact functional significance of this residue is unclear. As suggested by us recently (Correia & Sugiyama, 1988; Correia et al., 1988), it is possible that it might in fact be recruited in radical-mediated P450 heme destruction, much in the same fashion that a strategically placed Tyr residue in prostaglandin H synthase appears to be. Accordingly, a tyrosyl radical is believed to critically assist in the prostaglandin H synthase mediated cooxidation of arachidonic acid to PGG_2 (Karthein et al., 1988). Such a cooxidation is also associated with radical-mediated inactivation of prostaglandin H synthase, prosthetic heme destruction, and increased proteolytic susceptibility of the enzyme (Egan et al., 1976; Hemler et al., 1978; Chen et al., 1987). The latter two events are also

[5] Indeed, modification of the prosthetic heme by electrophilic formamidine–sulfenic acid species is believed to be responsible for the mechanism-based inactivation of lactoperoxidase by thiocarbamide goitrogens (Doerge, 1986).

[6] The partial attenuation of SPL-mediated P450 destruction by GSH (Table III) could only be rationalized if the destructive SPL species were to reversibly shuttle in and out of the active site. Efforts are under way to determine if a GSH-SPL metabolite adduct or GSSG (resulting from GSH-mediated reduction of SPL-S$^\bullet$/SPL-SOH) is detectable in the incubation mixture.

[7] In the normal catalytic cycle, this intermediate is very short lived since radical recombination promptly reduces the OH$^\bullet$ and quenches this P450 species to its resting state.

consistent features of hepatic P450 destruction by not only SPL but also other one-electron oxidizable P450p substrates such as the 4-alkyldihydropyridines (Tephly et al., 1986; Correia et al., 1987).

In summary, we have shown that liver microsomal P450 isozymes (P450p and P450h) from DEX-pretreated rats convert SPL to oxidized polar metabolites, two of which we have identified as the sulfinic and sulfonic acid derivatives of SPL. In this process, P450 is inactivated with a partition ratio of ≈20. Although, to our knowledge, this is the first instance that P450-dependent metabolism of SPL has been shown to yield sulfinic and sulfonic acid products, P450-dependent oxidation of other thio compounds (such as 6-thiopurine and possibly methimazole) to the sulfenic acid species has been reported previously (Hyslop & Jardine, 1981; Abraham et al., 1983; Kedderis & Rickert, 1985). On the other hand, microsomal flavin monooxygenases are also believed to metabolize various organic thiocarbamides and thiols to the corresponding sulfenic acids (Krieter et al., 1986), whereas myeloperoxidase is apparently found to oxidize propylthiouracil to its sulfonic acid derivative (Waldhauser & Uetrecht, 1988). A sulfenic acid species has also been identified as a secondary metabolite in the urine of rats given hexachloro-1,3-butadiene (Nash et al., 1984), but the identity of the enzyme involved in its formation remains to be divulged.

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Registry No. SPL, 52-01-7; SPL-SH, 38753-76-3; SPL-SO_2H, 120577-88-0; SPL-SO_3H, 120577-89-1; NADPH, 53-57-6; O_2, 7782-44-7; cytochrome P450, 9035-51-2.

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Formation and Utilization of Formyl Phosphate by N^{10}-Formyltetrahydrofolate Synthetase: Evidence for Formyl Phosphate as an Intermediate in the Reaction†

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ABSTRACT: N^{10}-Formyltetrahydrofolate synthetase from bacteria and yeast catalyzes a slow formate-dependent ADP formation in the absence of H_4folate. The synthesis of formyl phosphate by the enzyme was detected by trapping the intermediate as formyl hydroxamate. That the "formate kinase" activity was part of the catalytic center of N^{10}-formyltetrahydrofolate synthetase was shown by demonstrating coordinate inactivation of the "kinase" and synthetase activities by heat and a sulfhydryl reagent, similar effects of monovalent cations, similar K_m values for substrates, and similar K_i values for the inhibitor phosphonoacetaldehyde for both activities. The relative rates of the kinase activities for the bacterial and yeast enzymes are about 10^{-4} and 4×10^{-6} of their respective synthetase activities. These slow rates for the kinase reaction can be explained by the slow dissociation of ADP and formyl phosphate from the enzyme. This conclusion is supported by rapid-quench studies where a "burst" of ADP formation (6.4 s^{-1}) was observed that is considerably faster than the steady-state rate (0.024 s^{-1}). The demonstration of enzyme-bound products by a micropartition assay and the lack of a significant formate-stimulated exchange between ADP and ATP provide further evidence for the slow release of the products from the enzyme. The synthesis of N^{10}-CHO-H_4folate when H_4folate was added to the E-formyl phosphate-ADP complex is also characterized by a "burst" of product formation. The rate of this burst phase at 5 °C occurs with a rate constant of 18 s^{-1} compared to 14 s^{-1} for the overall reaction at the same temperature. These results provide further evidence for formyl phosphate as an intermediate in the reaction and are consistent with the sequential mechanism of the normal catalytic pathway. Positional isotope exchange experiments using [β,γ-^{18}O]ATP showed no evidence for exchange during turnover experiments in the presence of either H_4folate or the competitive inhibitor pteroyltriglutamate. The absence of scrambling of the ^{18}O label as observed by ^{31}P NMR suggests that the central complex may impose restraints to limit free rotation of the P_β oxygens of the product ADP.

The enzyme N^{10}-formyltetrahydrofolate synthetase catalyzes the ATP-dependent formylation of H_4folate[1] at the N^{10}-position (eq 1). The procaryotic enzyme is a monofunctional

$$\text{MgATP} + \text{HCOO}^- + \text{H}_4\text{folate} \overset{M^+}{\leftrightarrow} \text{MgADP} + \text{P}_i + N^{10}\text{-HCO-H}_4\text{folate} \quad (1)$$

tetramer of four identical subunits (Himes & Harmony, 1973). In eucaryotic organisms, the N^{10}-HCO-H_4folate synthetase activity along with N^5,N^{10}-methenylH_4folate cyclohydrolase and N^5,N^{10}-methyleneH_4folate dehydrogenase activities exists as a dimeric, multifunctional, and multidomain protein called C_1-H_4folate synthase. Properties of these enzymes are described in two review articles (Himes & Harmony, 1973; MacKenzie, 1984).

Most of the previous studies on the mechanism of the reaction have been done with the bacterial enzyme. Steady-state

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[1] Abbreviations: H_4folate, tetrahydrofolate; N^{10}-HCO-H_4folate, N^{10}-formyltetrahydrofolate; $PteGlu_3$, pteroyltriglutamate; Pte, pteroic acid; TFA-Pte, (trifluoroacetyl)pteroic acid; TFA-$PteGlu_3$, [(trifluoroacetyl)pteroyl]triglutamate; *t*-Boc-Glu-γ-OBzl, *tert*-butoxycarbonyl-L-glutamic acid γ-benzyl ester; HOBt, hydroxybenzotriazole; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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