Secobarbital (SB) is a relatively selective mechanism-based inactivator of cytochrome P450 2B1, that partitions between epoxidation and heme and protein modification during its enzyme inactivation. The SB-2B1 heme adduct formed in situ in a functionally reconstituted system has been spectroscopically documented and structurally characterized as N-(5-(2-hydroxypropyl)-5-(1-methylbutyl)barbituric acid)protoporphyrin IX. The SB-protein modification has been localized to SB peptide 277–323 corresponding to the active site helix I of cytochrome P450 101. The targeting of heme and this active site peptide suggests that the 2B1 active site topology could influence the course of its inactivation. To explore this possibility, the individual SB epoxidation, heme and protein modification, and corresponding molar partition ratios of the wild type and seven structural 2B1 mutants, site-directed at specific substrate recognition sites, and known to influence 2B1 catalysis were examined after Escherichia coli expression. These studies reveal that Thr-302 is critical for SB-mediated heme N-alkylation, whereas Val-367 is a critical determinant of 2B1 protein modification, and Val-363 is important for SB epoxidation. SB docking into a refined 2B1 homology model coupled with molecular dynamics analyses provide a logical rationale for these findings.

The sedative hypnotic secobarbital (SB) is an olefinic barbiturate that selectively inactivates rat liver cytochrome P450 2B1. Such inactivation is mechanism-based, entailing the partitioning of the drug between prosthetic heme and protein modification and epoxidation (Fig. 1). We have recently isolated the SB-modified heme and using a variety of mass spectrometric techniques, structurally characterized it as the N-(5-(2-hydroxypropyl)-5-(1-methylbutyl)barbituric acid)protoporphyrin IX adduct (1). HPLC-peptide mapping of lysyl endopeptidase C (Lys-C) digests of the corresponding SB-modified protein coupled with micro Edman degradation has led to the identification of the Lys-C peptide 277–323 as the target of the modification (1). This peptide includes the peptide domain corresponding to the distal helix I of P450 101, an evolutionarily highly conserved region that brackets the heme, nestles the heme-iron-bound oxygen as well as contacts the substrates (2–4), and thus is an active site feature. The precise residue that is modified is unknown, but this peptide contains several nucleophilic residues that would be good candidates. Nevertheless, identification of the SB-modified peptide as an active site domain suggests that some residues are sufficiently close to the oxidatively activated SB-moiety to intercept it at the least, part of the time, thereby rationalizing the observed partitioning between heme and protein alkylation and epoxidation (Fig. 1) (1).

Several structural mutants of cytochromes P450 (P450s), including cytochrome P450 2B1 (P450 2B1), site-directed at putative substrate recognition sites (SRS) (4, 5) have been designed, heterologously expressed in Escherichia coli and functionally characterized with respect to several substrates and/or inhibitors (6–16). As with other P450 structural mutants (6–11), the studies with 2B1 mutants have shown that certain site-directed mutations not only profoundly alter the extent of the catalytic competence of the enzymes, but also the regio- and stereoselectivity of these metabolic reactions and the susceptibility of each structural mutant to mechanism-based inactivation by chloramphenicol and its analogs (15, 16). Such structure-function relationships suggest that specific SRS alterations can profoundly affect active-site events. However, for a given mutant, the findings markedly differed with each substrate employed, underscoring the relative importance of each individual substrate-active site fit (16). In the studies described below, we have used these site-directed mutants to examine whether mutations of certain SRS residues influence SB:cytochrome P450 2B1 active site interactions and consequently its partitioning between heme and protein modification and epoxidation.

**MATERIALS AND METHODS**

**[2-14C]SB Synthesis**

[2-14C]SB was synthesized with minor modifications and its structure was confirmed by H NMR and mass spectrometric analyses as described previously (1, 17). Its specific activity was 0.34 Ci/μmol, and its radiochemical purity was >97%.

**Expression of P450 2B1 and Its Mutants**

Selected mutants (N114V, F206L, V363A, V363L, V367A, and G478S) previously expressed in COS cells from the pBEC21B vector were constructed in the pKK 233-2 expression vector. Plasmids harboring the P450 2B1 wild type and mutant cDNAs were transformed in Topp3 cells. E. coli were grown and harvested at peak expression of each.

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**Secobarbital-mediated Inactivation of Cytochrome P450 2B1 and Its Active Site Mutants**

**PARTITIONING BETWEEN HEME AND PROTEIN ALKYLATION AND EPOXIDATION**

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SB Inactivation of P450 2B1 and Its Structural Mutants

Purification of P450 2B1 and P450 Reductase

P450 2B1 and cytochrome P450 reductase were purified from liver microsomes of phenobarbital-pretreated male Sprague-Dawley rats by the methods of Waxman and Walsh (18) and Shephard et al. (19), respectively. P450 and heme content was determined as described previously (20).

SB-meditated Inactivation of P450 2B1 and Its Mutants

P450 2B1 or a P450 2B1 mutant (0.5 nmol) was incubated with P450 reductase (1 nmol), DLPC (60 nmol), catalase (280 units), EDTA (1 μmol), [14C]SB (1 μmol), and NADPH (1 μmol) in 1 ml of 50 mM Hepes buffer (pH 7.5), containing 15 mM MgCl₂, and 20% glycerol at 37 °C for 15 min. At the end of this incubation, a 10-μl aliquot of the incubation mixture containing 10 pmol of P450 was used to determine pentoxyresorufin O-deethylase (PROD) activity, as detailed (1). In some cases, when parallel formation of SB-heme adduct or SB-epoxide was monitored, aliquots of the incubation mixture were removed at different intervals and chilled immediately in dry ice to stop the reaction.

HPLC Determination of SB-epoxide

The SB-epoxide used as the authentic standard was chemically synthesized by the method of Harvey et al. (21), purified by HPLC on a silica column using an isocratic solvent elution system consisting of hexane/ethyl acetate (3:2, v/v), and its identity was confirmed by positive liquid secondary ionization mass spectrometry analysis. The SB-epoxide formed during incubation was extracted with ethyl acetate, dried under N₂, and separated by reversed phase HPLC on a C18-column using a solvent of 40% acetonitrile/0.1% TFA/H₂O (1 ml/min) with UV detection at 210 nm. The HPLC epoxide peak was confirmed by comparison of its retention with that of the authentic chemically synthesized SB-epoxide and by its [14C]SB-derived radioactivity, when [14C]SB was included as the substrate in the reaction.

Determination of SB-Heme Adduct in the Reconstituted System by Difference Visible Absorption Spectroscopy

The incubation mixtures were identical to the one described above, except that NADPH was omitted from the control. After incubation, the control (−NADPH sample) was used to obtain a baseline between 400 and 500 nm in an SLM-Aminco 2000™ UV-Vis spectrophotometer. The contents of the sample cuvette were then replaced with aliquots of the +NADPH incubation mixture, and the two cuvettes were rescanned using the −NADPH sample as the reference.

The reversibility of the SB-heme adduct was examined as follows. The absolute spectra or the difference spectra (with the −NADPH incubation as reference) of the SB/NADPH-inactivated P450 2B1 were recorded at various intervals over 24 h. The sample was either scanned directly or scanned after removal of NADPH from the incubation by either the addition of NADP (at a 10-fold excess of the NADP present in the reconstituted system) to competitively inhibit the NADPH-dependent reaction or after dialysis against Hepes buffer containing 0.5% bovine serum albumin (which would also remove excess SB), in order to prevent potential reactivation of the enzyme after reversal of the SB-heme adduct and regeneration of the enzyme. In some instances, this was further ensured by bubbling CO into the sample. The extent of enzyme regeneration was established by monitoring the relative PROD activity of the incubations. The extent of P450 2B1 structural regeneration after reversal of the SB-heme adduct was also assessed by the relative amounts of heme and SB-heme adduct present after concurrent HPLC and/or MALDI monitoring, at the start and end of SB inactivation as well as after the procedures used to reverse the SB-heme adduct.

Determination of SB-Heme Adduct and Irreversible [14C]SB-Protein Binding

Wild type P450 2B1 or each of its mutants was incubated in the presence or absence of NADPH as detailed above. The inactivation reaction was stopped with TFA (final concentration, 10%), and 50 μl of liver microsomes (containing 1 nmol of P450) from untreated rats were added to the mixture as carrier hemoprotein. The SB-heme adducts were extracted with two equivalent volumes of TFA/2-butanol (10%), and the organic phase was removed by rotary evaporator. The residue dissolved in acetonitrile/acetic acid/H₂O (4:3:3, v/v/v) was analyzed by reversed phase HPLC on a C8 column, using a solvent system consisting of solvent A (0.1% TFA/H₂O) and solvent B (90% acetonitrile/0.1% TFA/H₂O) and a linear gradient elution from 45% B to 75% B over 30 min at a flow rate of 1 ml/min, with 415 nm detection. The fractions were collected every 2 min and subjected to scintillation counting.

Irreversible [14C]SB binding to the protein was determined in incubations similar to those described above, except that GSH (2 mM) was included to trap the reactive electrophilic epoxy that escapes the active site. The protein was precipitated by 5% H₂SO₄/MeOH, washed extensively with organic solvents, as described previously (16), dissolved in NaOH, and aliquots were subjected to scintillation counting and protein determination.

Mass Spectrometric Analysis of the SB-Heme Adduct

The reaction mixture containing the inactivated P450 2B1 (100 pmol) was mixed with 200 μl of 10% TFA butanone, and the organic phase was concentrated by speed vacuum. Average masses of the sample were determined using a PerSeptive Biosystems Voyager Linear MALDI/TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with a nitrogen laser (337 nm) and operated in the linear mode. The sample was crystallized with α-cyano-4-hydroxycinnamic acid (10 mg/ml in 50% acetonitrile/0.1% TFA/H₂O).

Docking of SB into the Active Site of the P450 2B1 Model

The P450 2B1 structure was obtained previously using a consensus strategy and verified with the Profiles-3D program, as described earlier (16). The SB structure was constructed using the InsightII (Biosym/MSI, San Diego, CA) and verified with the Cambridge Structural Data Base (CSD) against crystal structures of similar compounds. The structures were displayed on a Silicon Graphics workstation. Energy minimization and molecular dynamics calculations were carried out with the Discover simulation program (Biosym/MSI, San Diego, CA) with the consistent valence force field. The parameters for heme and ferryl oxygen were those described by Paulsen and Ornstein (22, 23).

Docking Conditions for SB Epoxidation—SB was placed in the active site of the P450 2B1 model, with the internal carbon of the olefinic SB-sidechain placed 4.5–4.9 Å from the heme iron aligned with Fe and S of Cys-436, resulting in the double bond paralleling the heme. This distance allows for van der Waals contacts between ferryl oxygen and the internal carbon, and thus apparently leads to SB oxidation at this carbon. The substrate can be oriented with the “si” or “re” face toward the heme. Since the SB molecule can assume numerous conformations, it was docked into the active site of the P450 2B1 model using molecular dynamics. For these simulations, both the C and H atoms of the SB double bond were fixed, while the rest of the molecule was allowed to move. Initially, the docked substrate was minimized with the Discover program, using the steepest descent algorithm and harmonic potential, with a non-bond cutoff of 8 Å, to a maximum gradient of 1 kcal/mol Å. The dielectric constant used was 1.0; no morse or cross-terms were included (16, 22). Subsequently, SB was subjected to one cycle of molecular dynamics using the leap-frog algorithm. The system was equilibrated for 0.1 ps, and the simulations were continued for 1 ps at 300 K using 1-fs time steps. This was followed by minimization using the steepest descent gradient, as described above. Finally, minimization was performed on side chains of P450 2B1 residues that contact the substrate (distance less than 5 Å), using the steepest descent method until the gradient was less than 1 kcal/mol Å. The non-bond interaction energy between the docked SB and the protein was calculated using the Docking module of InsightII package. Both electrostatic and van der Waals interactions were evaluated using the cutoff of 10 Å. The potential energy of SB was also calculated.

Docking Conditions for SB Heme Adduct Formation—The SB product, with the OH group at the internal carbon of the SB double bond, was placed above heme ring A (the major adduct formation site) at a distance that would allow for the C₇N₇ bond formation (that is, the terminal olefinic C of the SB product and N₇ of the heme). The chirality of the SB product at the “internal” C is S, since it was formed as a result of “re” face oxidation. Minimization and molecular dynamics of SB docked in an orientation consistent with the heme addition reaction was performed as described in the case of SB epoxidation. However, in the case of the SB-heme adduct, only the terminal olefinic C was fixed. Final minimization was carried out on the side chains of the residues contacting the substrate, as described earlier. The energy of interactions was also evaluated.

RESULTS AND DISCUSSION

SB Epoxidation by P450 2B1 and Its Mutants—The SB epoxide formed during the incubation was monitored by HPLC with UV detection at 210 nm. A peak with a retention time of 7.2 min was observed at 1 ml/min with 415 nm detection. The fractions were collected every 2 min and subjected to scintillation counting.

SB Heme Adduct Formation—The energy of interactions was also evaluated. The energy of interactions was also evaluated.

Energy of interactions was also evaluated.
SB Inactivation of P450 2B1 and Its Structural Mutants

similar to that of the authentic SB-epoxide eluting at 5.5 min was detected in the NADPH-supplemented incubations but not in the control (NADPH-devoid) incubations (Fig. 2). The identity of the SB-epoxide was further confirmed by its relative \[^{14}\text{C}]\text{SB}-derived radioactivity, when \[^{14}\text{C}]\text{SB} was included in both the NADPH-supplemented and control incubations (Fig. 2). SB-epoxide formation by the wild type \textit{E. coli} expressed enzyme was comparable to that of the purified functionally reconstituted rat liver enzyme (i.e. 50 and 59 nmol/nmol of P450/15 min, respectively). The mutant 2B1 T302S, F206L, I114V, V363L, V367A, and G478S enzymes catalyzed SB-epoxidation to a roughly comparable extent to the wild type 2B1, while V363A exhibited relatively lower activity (approximately 34%) (Table I). This activity profile was quite different from that of the corresponding PROD activity, wherein I114V retained 100% of the activity, but T302S retained only about 50%, F206L and V363A, 30%, and V363L, V367A, and G478S less than 2% of the activity (Table I). These findings thus indicate that specific mutations in the various SRS regions differentially affect P450 2B1-dependent metabolism of SB and pentoxyresorufin, thereby implicating individual differences in corresponding substrate-active site fits.

Detection of SB-Heme Adduct by Visible Electronic Absorption Spectroscopy and Mass Spectrometry—After P450 2B1 was inactivated by SB in a functionally reconstituted system, an electronic absorption difference spectrum for the NADPH-supp-

![Diagram](Image)

**FIG. 1.** The proposed scheme for the formation of the reactive SB-intermediate that alkylates the P450 2B1 heme or protein during its SB-mediated inactivation.

![HPLC Graph](Image)

**FIG. 2.** HPLC determination of SB-epoxide generated in P450 2B1 reconstituted system with 210 nm detection (**A**) and scintillation counting (**B**). **C** depicts the corresponding HPLC elution profile of the –NADPH control; **D** is that of the SB-epoxide standard. For experimental details, see "Materials and Methods."
implemented versus the NADPH-devoid incubation was obtained with an absorbance maximum at 445 nm and a trough at 418 nm (Fig. 3). A 445 nm absorbance was also obtained when the SB-inactivated P450 2B1 incubation mixture was scanned against aliquots of this same mixture taken at time 0 and placed in ice (not incubated at 37 °C). This spectral absorption was dependent on the olefinic moiety of the drug, because amobarbital, the saturated analog of SB, failed to destroy the enzyme as well as yield a corresponding spectrum, when it replaced SB in the incubation mixture. It is conceivable that this spectrum reflects the presence of SB-N-heme (Fe³⁺) adduct in this in vitro system, because it is similar to that of the purified iron-complexed N-ethylporphyrins, that exhibit absorbance maxima at 442 nm (25). Furthermore, consistent with this possibility, the absolute spectra of phenylacetylene- or 3-alkylsydnone-inactivated P450 2B1 preparations that yield N-modified heme adducts also exhibit a time-dependent increase in a 445 nm shoulder (26, 27). It is interesting that no corresponding spectra are detected after inactivation of P450 2B1 by allylisopropylacetamide, or other P450s by 3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydroxypryridene, in spite of the fact that much larger quantities of N-alkylated porphyrins are formed and isolated from such inactivation systems.² To our knowledge, this is the first spectral documentation of a P450 heme-drug adduct in situ. The ability to form SB-heme adducts as reflected by the magnitude of the corresponding 445 nm absorbance, varied significantly among different P450 2B1 mutants, indicating that either they were not equally susceptible to SB-heme alkylation or that their SB-heme adduct did not persist sufficiently long after formation to be detected (Fig. 3, Table I).

The SB-heme adduct was also detected in the TFA/butanone extracts of the SB-inactivated P450 2B1 incubation mixture (Fig. 4). MALDI/TOF mass spectrometric analyses of the extracts yielded a mass (MH⁺) of 818.0 Da, in good agreement with the mass of 816.9 Da expected for the hydroxy-SB-protoporphyrin IX adduct, formed after Fe³⁺ removal from the SB-modified P450 2B1 heme. No other SB-modified heme-derived species were detected in the TFA/butanone extracts (Fig. 4).

² L. M. Bornheim, K. He, and M. A. Correia, unpublished observations.

**TABLE I**

| 2B1 enzymes | PROD activity | SB epoxidation | Heme content | A/B | A/C | B + C | A/(B + C) |
|--------------|--------------|----------------|--------------|-----|-----|-------|-----------|
| Wild type    | 9.69         | 50             | 1.49         | 0.31| 3.1 | 34    | 1.80      |
| I114V (SRS-1)| 10.4         | 40             | 1.62         | 0.31| 3.1 | 25    | 1.93      |
| T302S (SRS-4)| 4.85         | 88             | 0.37         | 0.21| 3.7 | 238   | 0.58      |
| V363A (SRS-5)| 2.42         | 17             | 0.82         | 0.36| 4.2 | 21    | 1.18      |
| V363L (SRS-5)| 0.17         | 32             | 0.39         | 0.12| 1.8 | 82    | 0.51      |
| V367A (SRS-5)| 0.17         | 41             | 0.82         | 0.05| 5.4 | 50    | 0.87      |
| G478S (SRS-6)| 0.00         | 38             | 0.81         | 0.15| 1.7 | 47    | 0.98      |

**FIG. 3.** The electronic absorption difference spectrum of SB-heme adduct formed in P450 2B1 reconstituted system in vitro (left panel) and the relative spectrally determined amounts of the SB-heme adducts generated during SB-mediated inactivation of P450 2B1 and its mutants (right panel). For experimental details, see “Materials and Methods.”
The relative intensity of this SB-protoporphyrin adduct signal increased over the 15-min incubation period, in parallel with the decrease in PROD activity of the inactivated enzyme (Fig. 5).

Once formed, however, unlike the transient heme adducts generated through N-alkylation of chloroperoxidase by terminal alkenes and alkynes (30), the SB-heme adduct was not reversible. Under similar conditions and within the temporal limits of P450 stability, it could not be reverted back to the parent unmodified heme that would restore the native chromophore and functional activity of the enzyme (not shown).

Preliminary 1H NMR analyses of the SB-heme adducts obtained from SB-treated rats whose liver P450 2B1 had been induced by phenobarbital pretreatment revealed that the majority of the adducts contained SB on the Nα pyrrole ring of the heme (not shown). This result is consistent with the known topology of the P450 2B1 active site, wherein the Nβ and Nγ pyrrole rings are believed to be largely masked by the protein (28, 29).

**HPLC Determination of the SB-Protoporphyrin Adducts Formed in the Functionally Reconstituted P450 2B1 System—**

HPLC analyses of the TFA/butanone extracts of the SB-inactivated P450 2B1 reconstituted system. Top panel, −NADPH; bottom panel, + NADPH. For experimental details, see "Materials and Methods."
vated and control incubation mixtures showed a peak with 415 nm absorbance and a retention time of 27–29 min that contained \[^{14}\text{C}\]SB-derived radioactivity (Fig. 6). MALDI/TOF mass spectrometric analyses of this peak confirmed it to be the hydroxy-SB-protoporphyrin IX adduct with a mass (MH\(^+\)) of 817.7 Da (not shown). The electronic absorption spectrum of its zinc complex revealed the characteristic absorbances at 430, 541, 582, and 628 nm for an N-alkylated heme species. On the basis of its specific \[^{14}\text{C}\]SB radioactivity, the SB-heme adduct could be quantified in the concentration range of 0.1–2 nmol with a recovery of 94.3 ± 1.8% (n = 4).

**SB-mediated Heme and Protein Modification of P450 2B1 and Its Mutants**—The above approach was used to quantitate the relative extent of SB-heme adduct formation and protein modification of the wild type 2B1 and its structural mutants. The wild type P450 2B1 formed 1.49 nmol of SB-heme adduct per nmol of P450 inactivated in 15 min. The SB-mediated heme N-alkylation of T302S and V363L mutant enzymes was dramatically decreased, whereas that of I114V mutant was slightly increased, relative to the corresponding wild type value (Table I). On the other hand, the other structural mutants (F206L, V363A, V367A, and G478S) still retained greater than 50% of their wild type susceptibility to SB-heme N-alkylation as revealed by their relative yields of SB-heme adducts. It is noteworthy that these values, albeit lower than that of the wild type, are equivalent to the values observed with the purified rat liver 2B1. Thus, it is unclear whether these lower values reflect poor recycling of available heme and/or poor catalytic efficiency of the enzyme, since the formation of the additional SB-heme adducts from the available heme is dependent both on structural reassembly and fresh inactivation cycles.

Furthermore, the differences in SB-heme adduct formation appear to be dictated by the structural differences at the active site rather than by the adventitious heme availability. Accordingly, the T302S membranes contained just as much heme as the wild type, if not more, whereas, although less heme was available in the V363L preparation, its heme content was comparable to that of the G478S preparation, that yielded substantially higher levels of the SB-heme adduct (Table I).

When SB-induced protein modification of the wild type 2B1
and its structural mutants was examined, we found that the values for V363L, G478S, and T302S mutants were, respectively, about 60, 50, and 30% lower than that of the wild type P450 2B1 (Table I). Since these mutations reside on SRS-5, SRS-6, and SRS-4, respectively, it appears that the structural intactness of each of these domains is an important determinant of SB-induced protein modification. The critical importance of the SRS-5 domain is further underscored by the finding that the SRS-5 V367A mutant was found to be relatively less susceptible to protein modification during SB-mediated inactivation. Such poor SB modification of the V367A protein does not appear to be due to impairment of its functional capacity, since it was quite capable of concurrently supporting SB epoxidation and of incurring heme N-alkylation. Thus, the low susceptibility of this particular mutant to protein modification is mechanistically different from its resistance to chloramphenicol-mediated inactivation, wherein the observed negligible covalent chloramphenicol-protein binding was attributed to its low chloramphenicol metabolizing capacity (15). Indeed, most likely, the poor SB-induced protein modification of V367A in the SRS-5 domain is due to structural perturbations of its active site microenvironment. It is interesting in this regard, that just 4 residues upstream from the Val-367 site on SRS-5, the Val-363 mutation to Leu but not Ala, also impairs SB-induced protein modification, indicating that the extension of the Val residue by a CH2 unit in the Leu residue is sufficient to interfere in the SB-induced protein modification.

It is noteworthy that the stoichiometry of the inactivation (moles of [14C]SB bound to heme and protein per mol of P450 inactivated) was apparently greater than 1, for the membrane-bound wild type and some mutant enzymes. Because the heme content of the solubilized membranes from wild type P450 2B1 and its structural mutants 1114V, F206L, T302S, V363A, and V367A was greater than 3 nmol per nmol of P450, whereas V363L and G478S exhibited a corresponding value of about 2 nmol of heme per nmol of P450 (Table I), it appears that this adventitious heme could replace the alkylated heme to restore the enzyme activity through sequential futile inactivation/reconstitution cycles, as long as sufficient SB and NADPH are present (31). On the other hand, in purified functionally reconstituted P450 2B1 systems, wherein no adventitious heme is present other than that of the added catalase, the moles of [14C]SB bound to heme and protein is ∼0.83 and 0.20, respectively, per mol of P450 inactivated, with the stoichiometry of the inactivation event ∼1, and a partition ratio for SB-epoxidation to P450 inactivation of the order of ∼59:1.

The partition ratio for a given mechanism-based inactivator of an enzyme is a measure of its inactivating efficiency, i.e. the relative number of productive turnover cycles per inactivating event (32). The lower this ratio, the more potent the inactivator. Usually, these ratios are used to compare the relative inactivating potentials of various suicide inactivators for a given enzyme. As in the present case, they can also be exploited to gain some insight into the topological influence that individual structural mutations within the active site of the target enzyme P450 2B1 might exert on the partitioning of SB into productive (SB-epoxide) and destructive (heme N-alkylation and protein modification). On the whole, the values for individual partition ratios calculated for SB-epoxidation relative to either SB-induced heme or protein modification observed with the wild type 2B1 or each of its site-directed structural mutants showed similar quantitative trends, with some notable exceptions (Table I). That is, such analyses revealed that the T302S mutant largely favored productive SB metabolism over the destructive pathway, irrespective of whether the ratio is expressed on the basis of SB-epoxide formed to heme- or protein-modified. The reverse appears to be true of the V363A mutant, which largely favored destructive events over productive metabolism. The salient exception is the SB partitioning catalyzed by the V367A mutant which apparently is quite competent in SB-epoxidation and heme N-alkylation but not protein modification. This to a much lesser extent is also true of the G478S mutant, indicating the importance of these SRS-5 and SRS-6 regions in dictating the specific course of the inactivation event.

A very comparable profile emerged when the partition ratios were expressed on the basis of epoxide formed to the cumulative destruction events (heme and protein modification) (Table I). However, the ratio of the V367A mutant so expressed provided no clue to its distinctive recalcitrance to protein modification (Table I).

**SB Docking into the Active Site of the P450 2B1 Model**—To assist us in a reasonable interpretation of the above findings, we docked SB in a refined molecular model of P450 2B1 constructed using consensus strategy and based on the crystallographic structures of P450s 101, 102, and 108 (16). That model has been shown to be consistent with the results from site-directed mutagenesis of cytochromes P450 2B and provided...
plausible explanations for alterations in regio- and stereospecificity of steroid hydroxylation in various mutants of P450 2B1 (16). Molecular modeling of SB in the active site of this P450 2B1 model revealed that of the several nucleophilic amino acid residues in the SB-modified helix I peptide fragment (residues 277–323), possibly four could be singled out as potential candidates for SB-mediated alkylation, on the basis of their relative distances to the SB bound in the active site, their side-chain orientation within the active site, and shielding effect of other residues. Of these, Thr-302 was the best candidate, with Ser-294, Thr-305, and Thr-306 plausible, but less likely. The likelihood of Thr-302 as the target residue is further strengthened by our finding that HPLC-peptide mapping of pepsin digests of [14C]SB-inactivated 2B1 (which would reduce the size of the [14C]SB-modified peptide), yielded a 14C-labeled digest of [14C]SB-modified Thr-302, on the basis of relative distances to the SB bound in the active site, their side-chain orientation within the active site, and shielding effect of other residues. Of these, Thr-302 was the best candidate, with Ser-294, Thr-305, and Thr-306 plausible, but less likely. The likelihood of Thr-302 as the target residue is further strengthened by our finding that HPLC-peptide mapping of pepsin digests of [14C]SB-inactivated 2B1 (which would reduce the size of the [14C]SB-modified peptide), yielded a 14C-labeled peptide whose MALDI-MS analyses gave a mass of 835.9, consistent with that of a SB-alkylated peptide. This finding thus confines the site of modification to the Thr-302-containing hexapeptide domain of the previously identified, 46-residue-long, [14C]SB-modified Lys-C peptide 277–323.

Simulations with either the S or R isomer of SB gave similar results, indicating that the SB stereochemistry did not influence its binding, a result expected from the high flexibility of the SB side chain. Furthermore, both si and re orientations were possible, but the si orientation seemed to be energetically more favorable, considering the docking energy and the potential energy of the substrate. Docked in this orientation, SB can directly interact with Thr-302, Val-363, Val-367, and Gly-478, whereas residues Ile-114 and Phe-206 are in the vicinity of the docked substrate, but not sufficiently close for direct interaction (Fig. 7). The first four residues are located in regions that are highly conserved in the four known crystal structures, while residues 114 and 206 are in regions less structurally conserved. However, in P450 2B2 enzymes, all six positions were shown to control steroid hydroxylation (15, 16). In the case of SB, modeling results are consistent with the data indicating that mutation of residues 302, 363, 367, and 478 affected epoxidation and/or partitioning of the reactive intermediate(s) (Table I). Interestingly, in contrast to the other mutants, the T302S mutant exhibited a marked increase in SB epoxidation, a result that may be rationalized by its ability to enable SB to assume another binding orientation with the re face toward the heme. This orientation would not be possible with Thr at position 302, since its methyl group would create van der Waals overlaps with SB.

Structural characterization of the SB-modified heme adducts isolated from SB-treated rats indicated that the major component is the Nα isomer. Docking of the oxidized SB cation radical above heme ring A to enable its terminal olefinic carbon to bond with pyrrolic Nα revealed that this SB product interacted closely with Thr-302 and Val-363 (Fig. 8). The decrease in heme adduct formation with the T302S mutant could be related to the increased mobility of the SB product in the active site pocket. It is likely that the side chain methyl group of Thr helps to stabilize the SB intermediate in an orientation that allows heme-adduct formation. A similar decrease with the V363L mutant, on the other hand, appears to be due to the van der Waals overlaps with the oxidized SB product, a result of the CH₂ increased length of the Leu residue side chain.

**Conclusions**—The above findings reveal that specific active site residues located in putative SRS domains may play important roles in dictating the trajectory of SB metabolism during its mechanism-based inactivation of P450 2B1. Accordingly, the SRS-1 domain does not appear to play a significant role given that critical mutations in this region failed to appreciably affect any of the SB metabolic parameters examined. Active site residue Thr-302 on SRS-4 is a critical determinant of the SB partitioning into both productive SB-epoxide formation and enzyme inactivation. On the other hand, Val-367 on SRS-5 apparently is a critical determinant of 2B1 protein modification, since its mutation to Ala drastically reduces this component, without altering either SB epoxidation or heme N-alkylation. Finally, Val-363, also on SRS-5, appears to critically control both epoxidation and heme N-alkylation. Replacement of this residue with the one CH₂ unit shorter, Ala, markedly reduces SB epoxidation but not heme N-alkylation or protein modification, whereas its replacement with the one CH₃ unit longer Leu appears to suppress its catalytic activity. This is implicated from the finding that the mutant shows decreases in all the three parameters examined. Thus, the lower heme N-alkylation of the V363L mutant can be attributed to its lower SB-metabolizing capacity as well as van der Waals overlaps.
imposed by the larger Leu residue. The lower heme N-alkylation of the T302S mutant on the other hand appears to be due not only to a more efficient conversion of the SB intermediate to the epoxide from both the re and si orientations, but also to the increased mobility of the oxidized SB product, features enabled by the Ser substitution that eliminates the steric constraints normally posed by the $-CH_3$ group of Thr-302.

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Secobarbital-mediated Inactivation of Cytochrome P450 2B1 and Its Active Site Mutants: PARTITIONING BETWEEN HEME AND PROTEIN ALKYLATION AND EPOXIDATION

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