Minireview

MECHANISM-BASED INACTIVATION AND REVERSIBILITY: IS THERE A NEW TREND IN THE INACTIVATION OF CYTOCHROME P450 ENZYMES?

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ABSTRACT:
Recent studies with cytochrome P450 (P450) enzymes from the 2E and 2B subfamilies have shed light on what may be a new trend in the mechanism-based inactivation of P450s: reversibility. The reversible inactivation of P450-type enzymes was first reported in the mid-1990s by Dexter and Hager [Dexter AF and Hager LP (1995) J Am Chem Soc 117:817–818], who studied the transient heme N-alkylation of chloroperoxidase by allylbenezene and 1-hexyne. While characterizing small tert-butyl acetylenes as mechanism-based inactivators of P450s 2E1 and 2B4, Hollenberg and coworkers observed the reversible inactivation of an acetylene-inactivated T303A mutant of P450 2E1. The mechanism of reversibility was a combined product of the structure of the inactivator and the positioning of conserved amino acid residues, threonine 303 (alane in the mutant) and glutamate 302, in the enzyme active site. Reversibility was also observed with both wild-type P450 2B4 and the T302A mutant of 2B4, although this inactivation and reversibility did not seem to depend on the T302 residue. Subsequent studies have attempted to elucidate the chemical/structural requirements of the inactivator in determining reversibility and have shown that both the size and the chemical nature of functional groups play an important role. At this time, reversibility has only been observed with P450 2E and 2B enzymes during their mechanism-based inactivation by terminal alkynes. Future studies with P450s from other subfamilies and structurally distinct inactivators will greatly aid our understanding of the molecular and chemical determinants of reversibility.

Mechanism-Based Inactivation and Reversibility (Overview)

A wide variety of xenobiotic compounds have been shown to undergo metabolic activation by the cytochrome P450 enzymes to form biologically reactive intermediates that may, in turn, target the P450 for inactivation. The specific binding of these reactive intermediates to the active sites of the P450s has been exploited in the design of irreversible inhibitors, otherwise defined as suicide or mechanism-based inactivators (Massey et al., 1970; Rando, 1984). Mechanism-based inactivators can be broadly classified as substrates for the P450 that, in the process of metabolism by the enzyme, are converted to a reactive intermediate, which then irreversibly inactivates the enzyme without leaving the active site (Silverman, 1995). There are three different mechanisms by which the reactive intermediate is able to inactivate the P450: covalent adduction to an amino acid residue within the enzyme active site, arylation or alkylation of the prosthetic heme moiety, and destruction of the heme group, leading to heme-derived products that form crosslinks with the P450 apoprotein (Osawa and Pohl, 1989).

The kinetic scheme for mechanism-based inactivation (Silverman, 1995) is shown in Scheme 1, where E is defined as the enzyme and I represents the inactivator. Briefly, the inactivator reversibly binds to the enzyme active site and is catalytically converted to a reactive intermediate, I*. The reactive intermediate can either be released as product (P) or it can become covalently adducted within the active site, leading to an irreversible inactivation of the enzyme (EI†). A number of criteria are routinely used to determine whether a substrate for a particular P450 is a mechanism-based inactivator, including time- and concentration-dependent enzyme inactivation, an absolute requirement for NADPH, substrate protection against P450 inactivation, irreversibility of inactivation by dialysis or gel filtration, and a lack of effect on the rate of inactivation in the presence of exogenous nucleophiles (Kent et al., 2001).

In 1995, Dexter and Hager reported for the first time the transient heme N-alkylation of the enzyme chloroperoxidase (CPO) by terminal alkynes and alkynes (Dexter and Hager, 1995). These data suggested that chloroperoxidase was inactivated in a P450-type reaction involving the mechanism-based formation of N-alkylporphyrins during the oxidation of allylbenezene and 1-hexyne. These structurally distinct compounds inactivated chloroperoxidase in a time- and concentration-dependent manner with losses in the enzymatic activity corresponding to losses in the native heme and the formation of N-alkyl heme adducts. The inactivated CPO formed in these reactions then underwent a spontaneous loss of the heme adducts (as observed by electrospray mass spectrometry analysis) with a restoration of enzymatic

ABBREVIATIONS: P450, cytochrome P450; tBA, tert-butyl acetylene; tBMP, tert-butyl 1-methyl-2-propynyl ether; CPO, chloroperoxidase; 4-MP, 4-methyl-1-pentyne; tBPA, 4-tert-butylphenylacetylene; 3-PP, 3-phenyl-1-propyne.
activity and native heme (Dexter and Hager, 1995; Debrunner et al., 1996). At the time Dexter and Hager (1995) reported these results, this type of reversible inactivation mechanism had not been previously reported for CPO or the cytochrome P450 enzymes.

This review will attempt to summarize recent data obtained on the reversible inactivation of P450s from the 2E and 2B subfamilies of cytochrome P450 enzymes by small tert-butyl acetylenic compounds. Interestingly, the reversibility of P450 inactivation can be influenced by both the architecture of the enzyme active site and changes in the structure of the inactivator. It may be that reversibility in P450 systems is more common and, in fact, more complex, than previously thought. Future experiments with P450s from other families and subfamilies that specifically probe compounds for a reversible inactivation mechanism will greatly aid in our understanding of this interesting phenomenon. The importance of reversibility mechanisms in vivo must also be assessed and may alter our current interpretation of the mechanism-based inactivation of human cytochrome P450 enzymes by xenobiotics and clinically relevant drugs.

Acetylenic Mechanism-Based Inactivators

A variety of different compounds containing an acetylenic functional group have been shown to inactivate P450 enzymes in a mechanism-based manner. Ortiz de Montellano and coworkers have described two mechanisms for the inactivation of cytochrome P450 enzymes by acetylenes (Ortiz de Montellano, 1985, 1991; Ortiz de Montellano and Komives, 1985; Ortiz de Montellano and Reich, 1986). Insertion of the oxygen atom from the P450-derived activated oxygen species at the internal carbon of the acetylene results in the formation of a reactive intermediate that leads to heme adduction and destruction of the heme chromophore. Transfer of the oxygen atom to the terminal acetylenic carbon results in a reactive intermediate that undergoes rearrangement to form a ketene species. This reactive ketene can either be hydrolyzed to produce a carboxylic acid product or it can acylate nucleophilic amino acid residues within the active site of P450s, resulting in enzyme inactivation. Examples of both types of reactions have been reported and are shown in Fig. 1 for comparison.

The construction of acetylene-derived mechanism-based inactivators for the cytochromes P450 has used a variety of different carrier structures, including fatty acids (Ortiz de Montellano and Reich, 1984; Shak et al., 1985; CaJacob and Ortiz de Montellano, 1986; Muerhoff et al., 1984; Ortiz de Montellano and Komives, 1985; Komives and Ortiz de Montellano, 1987; Hammons et al., 1989), aromatic hydrocarbons (Gan et al., 1984; Ortiz de Montellano and Komives, 1985; Komives and Ortiz de Montellano, 1987; Hammons et al., 1989), and steroids (Covey et al., 1981; Nagahisa et al., 1983; Halpert et al., 1989). Previously, the inactivation of P450 2B1 by substituted phenylacetylenes was characterized and shown to primarily result in heme alkylation (Komives and Ortiz de Montellano, 1987). Another acetylene, 2-ethynylnaphthalene, has been shown to inactivate purified rat P450s 1A1 and 1A2 in a mechanism-based manner through covalent adduction of the apoproteins (Hammons et al., 1989). Hollenberg and coworkers demonstrated the mechanism-based inactivation of P450s 2E1 and 2E1 T303A by two structurally similar compounds (Fig. 2) that contained a tert-butyl moiety for P450 2E1 specificity and an ethynyl functional group for P450-dependent metabolism to a reactive intermediate capable of covalently modifying the active site through heme or protein adduction (Blobaum et al., 2002). tert-Butyl acetylene (BA) and tert-butyl 1-methyl-2-propynyl ether (tBMP) inactivated the P450s 2E1 through three distinct mechanisms: 1) covalent alkylation

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**SCHEME 1.** Equation for the mechanism-based inactivation of an enzyme. Adapted from the original mechanism proposed by Silverman (1995). E is defined as the enzyme and I represents the inactivator. The inactivator reversibly binds to the enzyme active site and is catalytically converted to a reactive intermediate, I*. The reactive intermediate can either be released as product (P) or can become covalently adducted within the active site, leading to an irreversible inactivation of the enzyme (EI†).

**FIG. 1.** Mechanisms for heme and protein adduction of acetylenic compounds. Insertion of the oxygen atom from the P450-derived activated oxygen species at the internal carbon of the acetylene results in the formation of a reactive intermediate that leads to heme adduction (pathway b). Transfer of the oxygen atom to the terminal acetylenic carbon results in a reactive intermediate that undergoes rearrangement to form a ketene species. This reactive ketene can either be hydrolyzed to produce a carboxylic acid product or it can adduct to amino acid residues within the active site of P450s resulting in enzyme inactivation (pathway a). Adapted from Ortiz de Montellano and colleagues (Ortiz de Montellano, 1985, 1991; Ortiz de Montellano and Komives, 1985; Ortiz de Montellano and Reich, 1986).
of the heme prosthetic moiety (tBA and tBMP inactivation of P450 2E1 and 2E1 T303A), 2) a combination of heme alkylation and protein adduction (tBA inactivation of P450 2E1), and 3) a novel and not previously described reversible alkylation of the P450 heme (tBA inactivation of the T303A mutant). Characterization of this reversible inactivation mechanism demonstrated that losses in the activity and native heme of a tBA-inactivated T303A sample could be restored by dialysis or spin column gel filtration (Blobaum et al., 2004a). Acetylene heme adducts with m/z of 661 Da were shown to be reversible with time, as described for the reversible inactivation of chloroperoxidase by allylbenzene (Dexter and Hager, 1995). Interestingly, a source of exogenous protons was required to observe stable heme adducts in the tBA-inactivated T303A mutant, whereas the wild-type P450 2E1 was able to form these tBA adducts under the same conditions regardless of prior preacidification (Blobaum et al., 2004a). These data suggested an important role for the highly conserved threonine 303 residue as a possible participant in a proton relay network to the active site of P450 2E1. In addition, studies with alternate oxidants capable of supporting enzyme inactivation in the
absence of NADPH and reductase suggested the formation and utilization of a hydroperoxo-Iron species for substrate oxygenation by the T303A mutant and an iron-oxo species for use by the wild-type 2E1 enzyme, confirming the disruption of proton delivery to the active site of the threonine mutant (Blobaum et al., 2004b). Scheme 2 depicts a speculative mechanism for the reversible inactivation of P450 2E1 T303A by a small acetylene such as tBA. In this chemical scheme, an inactivating intermediate is formed from the insertion of oxygen into an acetylenic inhibitor (tBA) by a hydroperoxy-Iron species in the T303A mutant (Blobaum et al., 2004a). This intermediate is responsible for the reversible loss in the enzymatic activity of 2E1 T303A and is shown in the scheme to have two possible fates: 1) the formation of the intermediate is reversible over time and will re-form an activated enzyme with intact heme and an acetylene-derived carboxylic acid reversal product; or 2) the inactivating intermediate is stabilized in the presence of exogenous protons and will lead to irreversible N-alkylation of the P450 heme. It is this second pathway that is identical to the sequence of steps that are involved in the irreversible inactivation of the wild-type P450 2E1 enzyme by tBA.

Unique Spectral Intermediates Formed during Reversible Inactivation

Clorgyline was previously shown to inactivate P450 2B1 through the formation of a metabolic intermediate (MI) complex that could be reversed to regenerate the active enzyme (Sharma et al., 1996). Incubation with clorgyline and NADPH resulted in an absorbance peak with a maximum at 455 nm in the difference spectrum that is characteristic of a MI complex. Although MI complexes have been well documented in the literature and are known to have an absorbance maximum at 455 nm, there has also been evidence for the formation of other types of spectral intermediate complexes that are characterized by absorbance maxima at longer wavelengths (Battioni et al., 1983; Mansuy and Fontecave, 1983). To investigate the possible formation of a metabolic intermediate complex or some other type of spectral intermediate by the tBA-inactivated T303A mutant of P450 2E1, extensive spectral analyses were performed (Blobaum et al., 2004b). Interestingly, a peak having an absorption maximum at 485 nm was observed with the 2E1 T303A samples; however, a similar peak was not detected in the spectrum of the wild-type enzyme. The formation of the 485-nm peak in the T303A enzyme was monitored with time and showed a rapid accumulation during the first 10 min with a plateau after 20 min. Importantly, the formation of the peak at 485 nm required oxygen, and after reversion to the active enzyme after overnight dialysis, the peak could be regenerated by the addition of fresh tBA and NADPH (Blobaum et al., 2004b). Given that typical MI complexes absorb at 455 nm, this spectral intermediate having a maximum absorption at 485 nm was believed to be a newly discovered tBA-Fe intermediate. Because protons are required to support the formation of the N-alkylated tBA heme adds (Blobaum et al., 2004a), this acetylene-Iron spectral intermediate may be a chelated structure in which the oxygenated acetylene forms a bridge between the heme iron atom and one of the pyrrole nitrogens of the heme moiety (Fig. 3). Scheme 2 proposes the formation of such an intermediate during the reversible inactivation of P450 2E1 T303A by tBA. In this scheme, the inactivating intermediate corresponds to the spectral intermediate observed at 485 nm, and can either lead to reversion to the active enzyme or can slowly decompose over time to regenerate the native enzyme with full catalytic activity.

The Role of P450 Active Site Architecture in Reversibility

Active site models of P450 2E1 have shown a highly conserved threonine (T303) positioned directly over the plane of the heme moiety and within hydrogen bonding distance to the activated oxygen species (Tan et al., 1997). Over the years, many groups have proposed that this threonine residue (T303 in P450 2E1, T302 in P450 2B4, and T252 in P450cam) is involved in a proton shuttle network that shuttles protons to the activated oxygen species in the enzyme active site (Imai et al., 1989; Raag et al., 1991; Vaz et al., 1996, 1998; Jin et al., 2003). Much of our understanding of the important role played by this conserved threonine residue in proton relay and dioxygen activation has come from studies with the camphor monooxygenase, P450cam. Mutagenesis of T252 to aliphatic residues in P450cam led to a loss in enzymatic activity and the production of peroxide in what is generally considered to be an uncoupling reaction (Imai et al., 1989; Martinis et al., 1989). The X-ray crystal structure of the T252A mutant in the ferric state indicated a role for this conserved residue in a proton shuttle pathway that connects the surface of the protein with the enzyme active site (Raag et al., 1991). The elucidation of the ferrous dioxygen complex of P450cam provided evidence for a continuous hydrogen-bonded link between the conserved T252, water molecules, and the dioxygen ligand (Schlichting et al., 2000). Recently, the T252A ferrous dioxygen structure was solved by Nagano and Poulos (2005) and was unexpectedly shown to contain two “catalytic” waters in the active site, similar to waters that are observed in the ferrous dioxygen complex of wild-type P450cam. In addition, the replacement of the free hydroxyl group (OH) on T252 with a methoxy group (OCH3) does not significantly alter the catalytic activity of the enzyme (Kimata et al., 1995). It has thus been suggested that this conserved threonine residue may not serve as a proton donor in dioxygen activation but, rather, may promote the addition of the second proton in the P450 catalytic cycle to the distal oxygen by accepting a hydrogen bond from the hydroperoxy-Iron species. Finally, and of great interest, these results demonstrate that even in the absence of the conserved threonine, water molecules or other compensatory networks for proton delivery may form in the enzyme active site. As the crystal structures of the threonine mutants of mammalian P450s in either the ferric or ferrous dioxygen states are unavailable at this time, it is not known whether these same observations will hold true.

Threonine 303 in the mammalian cytochrome P450 2E1 was shown to be important in the mechanism-based inactivation of P450s 2E1 and 2E1 T303A by the small tert-butyl acetylene, tBA (Blobaum et al., 2002, 2004a). In the 2E1 mutant lacking the conserved threonine,
and is thought to be involved in proton delivery to the active sites of these P450s, the role of this conserved residue and proton relay networks in reversibility was examined in P450 2B4 and its T302A mutant. Research showed that the same acetylenic inactivators (tBA and tBMP) were able to inactivate the two P450s in a mechanism-based manner, through the formation of acetylene adducts to the P450 heme (von Weymarn et al., 2004; Blobaum et al., 2005). Interestingly, these inactivations were found to be partially reversible (20–30%) with dialysis and spin column gel filtration. Protons were required to form stable tBA or tBMP heme adducts in both wild-type and mutant 2B4 P450s, demonstrating a significant deviation from the 2E1 studies mentioned above. Active site models of P450 2B4 and the T302A mutant based on the 2B4 crystal structure (Scott et al., 2004) showed that the T302A mutation does not significantly alter the architecture of the enzyme active site or the proton delivery networks therein (Blobaum et al., 2005). As with P450 2E1, two possible networks for proton delivery exist in the 2B4 P450s. However, the glutamate (E301) and threonine (T302) network remains intact in the T302A mutant of 2B4, suggesting that there is still efficient delivery of protons in this enzyme. Thus, it was deduced from mass spectral data and computational modeling that the conserved threonine residue in P450 2B4 is not involved in the delivery of protons to the acetylene reactive intermediate of the heme or in the observed partial reversibility with the 2B4 enzymes. It can be concluded from these studies that active site architecture and proton relay may play a significant role in the determinants of reversibility in these P450s. Since these glutamate and threonine residues are highly conserved among all P450s, targeted mutations in other mammalian P450s may prove useful in determining their role in proton delivery and reversibility.

### Chemical Determinants of Reversibility

The chemical nature of the inactivator also seems to play a role in determining reversibility with P450 enzymes. When P450s 2E1 and 2E1 T303A were inactivated by tBA and tBMP, only the small tBA compound was able to demonstrate reversibility with the T303A mutant (Blobaum et al., 2002, 2004a). The larger tBMP inactivated the 2E1 T303A enzyme in an irreversible manner, and it was thought that the internal oxygen moiety of tBMP and its elongated structure may have provided more surface contacts for stabilizing hydrogen bonding in the enzyme active site. Terminal acetylenes and not their olefin counterparts are capable of inactivation and reversibility with P450s 2E and 2B (unpublished observation). For example, in testing the double-bonded tert-butyl olefin for its ability to reversibly inactivate the 2E1 T303A mutant in a mechanism-based manner, inactivation of the enzyme was not observed. This suggested a requirement of an acetylene group for metabolic activation to a reactive intermediate capable of inactivating the enzyme. The relative size and chemical nature of the functional groups attached to terminal acetylenic compounds also influence the determinants of reversibility with P450 2E1 and the 2E1 T303A mutant. Several additional acetylenes (Fig. 5) were tested for their ability to inactivate these P450s in a mechanism-based manner and were monitored for their ability to reverse these inactivations with dialysis and spin column gel filtration (Table 1; unpublished observations). Larger, aromatic acetylenes (tBPA) were only competitive inhibitors, whereas elongated acetylenes without internal oxygen atoms (3-PP) were irreversible mechanism-based inactivators of both 2E1 P450s. Small terminal acetylenes (4-MP), on the other hand, were able to reversibly inactivate the T303A mutant of 2E1 in a mechanism-based manner. Spectral analyses showed the formation of a P450 spectral intermediate at 483 nm with 4-MP and a requirement for protons to form stable acetylene heme adducts (Fig. 6). Interestingly, the structural requirement for reversibility does not
The topic of reversibility in the context of mechanism-based inactivation of P450 enzymes has never really been considered an object of much discussion. It was thought that compounds that fulfilled the criteria of a mechanism-based inactivator should show an irreversible inactivation mechanism, indicative of a covalent modification of the inactivator itself. Whether it is an irreversible or a reversible mechanism-based inactivator is crucial for our understanding of P450 inactivation in biological systems.

Conclusions and Future Directions

The topic of reversibility in the context of mechanism-based inactivation of P450 enzymes has never really been considered an object of much discussion. It was thought that compounds that fulfilled the criteria of a mechanism-based inactivator should show an irreversible inactivation mechanism, indicative of a covalent modification of the enzyme active site resulting in a permanent loss of enzyme activity. The observation that mechanism-based inactivators can fulfill all the criteria set forth by Silverman (1995) and yet can inactivate in a reversible manner is a new trend in P450 literature. The reversible inactivations of chloroperoxidase and P450 enzymes by acetylenic compounds are the first such reports of reversible mechanism-based inactivators. As discussed in this review, it is apparent that there are multiple determinants of reversibility. Enzyme active site architecture and the location and distance of critical amino acids residues from the inactivator itself seem to influence the mechanism of reversibility. In addition, the size and chemical nature of the compound will determine whether it is an irreversible or a reversible mechanism-based inactivator. Is the reversible inactivation of P450s a new trend that we must consider in terms of our viewpoints on P450 inhibition literature and P450-inactivator relationships? Experiments that answer these critical questions and determine the enzymatic and chemical requirements for reversibility are crucial for our understanding of P450 inactivation in biological systems.

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References


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Dr. Blobaum is currently a postdoctoral fellow in the laboratory of Dr. Lawrence J. Marnett in the biochemistry department at Vanderbilt University, and her work focuses on the determinants of the selectivity for cytoxygenase enzyme inhibition by structurally novel COX-2 inhibitors.