Site-directed Mutagenesis of Putative Substrate-binding Residues Reveals a Mechanism Controlling the Different Stereospecificities of Two Tropinone Reductases

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Two tropinone reductases (TRs) constitute a key branch point in the biosynthetic pathway of tropane alkaloids, which are mainly produced in several solanaceous plants. The two TRs share 64% identical amino acid residues and reduce the 3-carbonyl group of a common substrate, tropinone, but they produce distinct alcohol products with different stereospecific configurations. Previous x-ray crystallographic analysis has revealed their highly conserved overall folding, and the modeling of tropinone within the putative substrate-binding sites has suggested that the different stereospecificities may be determined solely by the different binding orientations of tropinone to the enzymes. In this study, we have constructed various mutant TRs, in which putative substrate-binding residues from one TR were substituted with those found in the corresponding positions of the other TR. Substitution of five amino acid residues resulted in an almost complete reversal of stereospecificity, indicating that the different stereospecificities are indeed determined by the binding orientation of tropinone. Detailed kinetic analysis of the mutant enzymes has shown that TR stereospecificity is determined by varying the contributions from electrostatic and hydrophobic interactions and that the present TR structures represent highly evolved forms, in which strict stereospecificities and rapid turnover are accomplished together.

Two tropinone reductases (TRs) catalyze key branch point steps in the biosynthesis of tropane alkaloids, which include several medicinally important compounds such as hyoscyamine (its racemic form, atropine) and scopoline (1). The two enzymes share a common substrate, tropinone, and transfer the pro-S hydrogen atom of the cofactor NADPH to the 3-carbonyl carbon atom of the substrate. However, the resulting alcohol products have opposite configurations at the hydroxyl group, i.e. one enzyme, TR-I (EC 1.1.1.206), produces tropine with a 3α-hydroxy group, whereas the other, TR-II (EC 1.1.1.236), produces pseudotropine (ψ-tropine) with a β-hydroxy group (Fig. 1). Tropine and ψ-tropine are not interconverted in vivo (2) and are further metabolized to various alkaloid products.

cDNAs encoding TRs have so far been isolated from two plant species, Datura stramonium and Hyoscyamus niger, both of which belong to the family Solanaceae (3, 4). All four TRs have been expressed in Escherichia coli as native proteins, and gel filtration experiments with these recombinant TRs have revealed that the enzymes of D. stramonium exist as a homodimer, whereas the TRs of H. niger most likely exist as a homotetramer (5). In both species, TR-I and TR-II share 64% identical amino acids from the 260–274 residues comprising each subunit, suggesting that the two enzymes diverged relatively recently from the same ancestral protein. The amino acid sequences also indicated that the TRs belong to the short chain dehydrogenase/reductase family (6). The most intriguing question about the TRs asks how these enzymes have evolved to catalyze opposite stereospecific reductions of the same substrate, and what molecular mechanism enables these homologous enzymes to control the stereospecificity in such a strict manner? A clue to answering these questions was first obtained from the analysis of a series of chimeric TR enzymes, which suggested that the different stereospecificity was conferred by a structural difference in their substrate-binding sites (5). Recent x-ray crystallographic analysis of the TRs from D. stramonium has revealed that the two enzymes share almost identical overall protein folding and that both the cofactor-binding site and the catalytically important Tyr residue are structurally very well conserved within their three-dimensional structures (7). Well conserved positions for both the hydride donor (NADPH) and the proton donor (catalytic Tyr residue) have suggested that the configurations of the reaction products (3α- or 3β-hydroxyl group) are determined solely by the binding orientations of tropinone to the enzymes (7). Furthermore, the predicted substrate-binding site of each enzyme appears to be consistent with the predicted binding orientation of tropinone that produces the hydroxyl group with the relevant configuration (Fig. 2, A and B) (7). The substrate-binding sites of the TRs have different electrostatic charge distributions that may fix opposite orientations for tropinone; in TR-I, the positive charge of His112 may repulse the negative charge of the tropinone nitrogen atom, whereas in TR-II, the favorable interaction between the negative charge of Glu156 and the positive charge of the tropinone nitrogen atom may fix the opposite binding orientation. In addition, the putative tropinone-binding sites of both TRs were composed mostly of hydrophobic amino acids, some of which were replaced with different amino acids in TR-I and TR-II. Such changes may differentially shape the substrate-binding sites and thereby aid in fixing the binding orientation of tropinone. Although bound tropinone is predicted to be surrounded by 11 amino acid residues, comparison of the four TR sequences (two TRs from the two plant species) has

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§ The abbreviations used are: TR, tropinone reductase; ψ-tropine, pseudotropine.

K. Nakajima and T. Hashimoto, unpublished observations.
Site-directed Mutagenesis of Tropinone Reductases

RESULTS

Construction of Mutant TR Enzymes—A previous x-ray crystallographic study predicted 11 amino acid residues from each TR that would possibly come in contact with tropinone bound to the enzyme. Comparison of the amino acid sequences of TR-I and TR-II from the two plant species revealed that only five of these 11 residues could be correlated with stereospecificity, i.e. they are numbered in tropine.

A potential method to confirm these hypothetical tropinone binding modes would be the crystallographic analysis of the protein-cofactor-tropinone ternary complexes. However, such complex crystals cannot be obtained in all cases, and even if successful, the structure alone is not sufficient to understand the role of each amino acid residue. Site-directed mutagenesis is another method that could be used to confirm the hypothetical tropinone binding mode and thereby compensate for the present absence of crystallographic studies of the ternary complexes. Here we report the construction and characterization of a series of mutant TR enzymes. Substitution of the five amino acids involved in the putative TR substrate-binding sites was determined from [S] versus [S]0 plots using at least five different [S] values, where [S] is the concentration of the substrate and v is the activity at each substrate concentration.

Enzyme Assay and Kinetic Analysis—TR activity was measured by either product quantification with gas-liquid chromatography or photometric measurement of NADPH consumption (9). For kinetic analysis, the photometric method was used because of its accuracy in measuring the steady state velocity of the reaction. $K_m$ and $k_{cat}$ values were determined from [S] versus [S]0 plots using at least five different [S] values, where [S] is the concentration of the substrate and v is the activity at each substrate concentration.

Site-directed Mutagenesis of Tropinone Reductases

Site-directed Mutagenesis—Mutations were introduced into the TR-coding regions of the expression vectors, pETTR1 and pETTR2, which respectively express the native TR-I and TR-II enzymes of D. stramonium under the control of the T7 promoter (7). Mutagenesis was performed by the “splicing by overlap extension” method (8), using two complementary primers that contain the desired mutation(s) and two other primers that anneal to either upstream or downstream regions of the vector (T7 promoter or T7 terminator primers). Amplified DNA from the second round of polymerase chain reactions was digested at the XbaI and EcoRI sites derived from the multicloning site of the pET21d vector. The digested DNA was purified by a Chroma Spin 400 column (CLONTECH) and ligated into new pET21d vector at the XbaI and EcoRI sites, so as to reconstruct the vector sequences. Because the mutagenesis was performed using many cycles of polymerase chain reactions with Taq polymerase, all the mutated plasmids were sequenced, and only plasmids without unintended missense mutations were used to express proteins.

Protein Expression and Purification—Mutant and wild-type TR enzymes were expressed in E. coli strain BL21 (DE3) and extracted as described previously (7). Buffer-soluble proteins were fractionated by precipitation with ammonium sulfate. Proteins precipitating at 45–75% saturation of ammonium sulfate were dissolved in buffer A (10 mM potassium phosphate, pH 7.0, 1 mM dithiothreitol, 0.1% (v/v) glycerol) containing 35% saturation ammonium sulfate and loaded onto a butyl-Sepharose fast flow column (1.5 × 2.7 cm, Amersham Pharmacia Biotech) and eluted with a stepwise gradient of KCl (0–1.0 M) in buffer A. Affinity chromatography fractions were desalted by PD-10, aliquoted, and stored at −80 °C until further use.

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Because the mutant enzymes had differing affinities for tropinone, as seen in Table I, enzyme reactions were performed at four different tropinone concentrations (0.05, 0.5, and 5 mM, plus the \(K_a\) of each enzyme). Fig. 4 shows the TR-I/TR-II activity ratios measured as described above. Among the single mutants of the three A residues, only the mutation at A3 affected stereospecificity. In TR-II this position is occupied by Glu, an acidic residue predicted to be the most important determinant of tropinone binding orientation. When mutations at residues A1 and A2, which alone had no effect on stereospecificity, were added to the above A3 mutants, stereospecificity was shifted in a cumulative fashion. At a tropinone concentration of 5 mM, the stereospecificity of the triple mutants of both TR-I and TR-II was shifted by more than 90%. At lower tropinone concentrations, however, the stereospecificity shift was diminished, especially for the TR-II mutant, which possessed two productive tropinone binding orientations, with the one producing \(\psi\)-tropine (TR-II orientation) still being of higher affinity.

In both TR-I and TR-II, substitution of the B residues alone had no effect on stereospecificity. When these group B mutations and the group A triple mutations were combined in a single enzyme to give quintuple mutants, the stereospecificity shifts were further enhanced, and the dependence on tropinone concentration seen in the triple mutant disappeared.

**Role of His\(^{112}\) in TR-I Catalysis**—The results obtained above disagreed with our previous prediction concerning the role of His\(^{112}\) in TR-I. The positive charge that would arise from His\(^{112}\) seems to have no direct contribution to stereospecificity, because the H112Y mutant retained wild-type TR-I stereospecificity. To understand the role of His\(^{112}\) in TR-I catalysis, we constructed an additional mutant, H112F, in which His\(^{112}\) was replaced with Phe, an amino acid with similar size but higher hydrophobicity. This mutant retained TR-I stereospecificity, as expected from the H112Y mutant (data not shown), and its affinity for tropinone was also similar to that of the H112Y mutant (Table I). However, the H112F mutant showed a reduced turnover rate, with \(k_{cat}\) values at pH 7.0 being 20 and 31% of the wild-type and the H112Y mutant, respectively (Table I). These observations suggest that although the positive charge of His\(^{112}\) was dispensable for stereospecificity, some degree of polarity at this position is necessary for efficient TR-I catalysis (and possibly for TR-II as well). This was confirmed by analyzing the kinetic parameters of the mutants at various pH values. Both H112Y and H112F mutants lost the pH dependence of \(k_{cat}\) values that was observed in the wild-type TR-I, especially the sharp increase in \(k_{cat}\) at acidic pH (Fig. 5A). Conversely, the Y100H mutation conferred a pH dependence to the \(k_{cat}\) values of TR-II (Fig. 5B). Given that the \(pK_a\) of the His side chain is about 6.4 (12), the observed pH dependence of the \(k_{cat}\) values likely reflects the change in hydrophobicity of His\(^{112}\), Tyr\(^{200}\) of TR-II (\(pK_a\) 9.7) is thought to be protonated within the pH range tested, but the e-hydroxyl group may still
The different substrate recognition mechanisms of TR-I and TR-II raise the question as to why TR-I does not use electrostatic interaction with Glu or Asp in place of the nitrogen atom of tropinone, with the other amino acids helping to increase binding affinity and fine-tuning binding orientation. Without these favorable electrostatic interactions, the hydrophobic amino acids of the TR-I substrate-binding site provide a more complementary fit to tropinone than does TR-II. Consistent with this prediction are the results obtained from the group B mutations. The I223L/F226L mutations impaired the affinity of TR-I for tropinone, with the $K_m$ value of the mutant being 5.11 mM (a 44-fold increase from the wild-type), whereas the $K_m$ of the L210I/L213F TR-II mutant remained low at 0.0048 mM (Table I), indicating these two hydrophobic residues to be relatively more important for substrate binding in TR-I. Furthermore, among the 11 residue pairs that were predicted to form the TR substrate-binding sites, in TR-I, only one pair shows any interresidue variety (Val–Ile), whereas there are three such residue pairs in TR-II (Val–Ile, Val–Leu, and Leu–Met) (Fig. 2C). The diminished variety of TR-I residues may reflect the importance of these noncharged amino acids for the high affinity binding of tropinone to TR-I.

The different substrate recognition mechanisms of TR-I and TR-II raise the question as to why TR-I does not use electrostatic interaction to increase its affinity for tropinone. To address this question, we introduced either Glu or Asp in place of the nitrogen atom of tropinone. These introduced residues significantly increased the affinity of TR-I for tropinone, such that the $K_m$ value of the mutant being 5.11 mM (a 44-fold increase from the wild-type), whereas the $K_m$ of the L210I/L213F TR-II mutant remained low at 0.0048 mM (Table I), indicating these two hydrophobic residues to be relatively more important for substrate binding in TR-I. Furthermore, among the 11 residue pairs that were predicted to form the TR substrate-binding sites, in TR-I, only one pair shows any interresidue variety (Val–Ile), whereas there are three such residue pairs in TR-II (Val–Ile, Val–Leu, and Leu–Met) (Fig. 2C). The diminished variety of TR-I residues may reflect the importance of these noncharged amino acids for the high affinity binding of tropinone to TR-I.
Val^{203} was able to locate its side chain carboxyl group at a distance favorable to the tropinone nitrogen. These observations suggest that the TR backbone does not permit TR-I to use an electrostatic interaction in binding tropinone in a correct and productive orientation.

**Implication for the Evolution of TRs**—The impaired stereospecificity and reactivity (as measured by $k_{\text{cat}}/K_m$ values) observed in all the mutant enzymes indicate that the wild-type structures of both TRs represent highly evolved forms. For instance, His^{112} of TR-I, which appeared to have no direct contribution to stereospecificity, was, however, found to be important for both high affinity binding of tropinone and rapid turnover. The corresponding TR-II residue, Tyr^{100}, cannot replace His, because this mutation reduces the affinity for tropinone, and no increase in $k_{\text{cat}}$ can be expected at physiological pH (Fig. 5B). This could mean that the metabolism of tropinone may have evolved under strong selective pressure, i.e. the regulated production of two different stereoisomers might have conferred selective advantage to the plants producing them. In *H. niger*, tropine is esterified to an organic acid to give hyoscyamine and then scopolamine (Fig. 1) (1). \(\psi\)-Tropine can also be esterified to different organic acids, but most \(\psi\)-tropine is considered to be polyhydroxylated to give calystegines (Fig. 1) (13). Whereas hyoscyamine and scopolamine act on the nervous system of mammals, calystegines have a glycosidase-inhibiting activity and can also serve as nutritional mediators in the rhizosphere (14, 15). Different functions of the tropine- and \(\psi\)-tropine-derived alkaloids might have prompted the acquisition of separate TR enzymes to control the metabolic flow at this branching point.

Recent random cDNA sequencing projects have revealed that genes highly homologous to TRs are widely distributed in the plant kingdom. We have determined the full-length sequences of five such clones from *Arabidopsis* and found that they encode proteins with high homology to TRs along the entire polypeptide chain (47–54% identical residues) but have a different set of predicted substrate-binding residues.\(^2\) This indicates that the same protein backbone is also utilized for enzymes that function in different metabolic pathways. The original TR enzyme must have arisen from a common ancestor with those enzymes via mutations to the substrate-binding site. This original TR enzyme likely had random stereospecificity, because

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**Fig. 4. Reaction stereospecificities of wild-type and mutant TRs.** Ratios between tropine- and \(\psi\)-tropine-forming activities at three fixed concentrations of tropinone (0.05, 0.5, and 5 mM) are shown by shaded bars, whereas those at the $K_m$ of each enzyme (listed in Table 1) are shown by striped bars. Each bar and attached lines correspondingly represent mean value and standard error from two measurements.
our mutation experiments indicated that a strict stereospecificity can only be achieved after fine-tuning the substrate-binding structure, which requires mutations at several amino acid residues. After gene duplication, each of the progeny TRs may have evolved strict and opposite stereospecificities. An alternative evolutionary process can also be assumed. Because the stereospecificity of most of the TR mutants was dependent on tropinone concentration, the ancestral TR that potentially had a random stereospecificity might have produced only one stereoisomer, provided that only low concentrations of tropinone were present during the early stages of evolution. Therefore, it is possible that an ancient TR first evolved to have strict stereospecificity for one configuration, and the opposite stereospecificity was later acquired by switching its stereospecificity using a process similar to this mutagenesis experiment. Consistent with this latter evolutionary process is the recent discovery of calystegines (3β-nortropane alkaloids) in well known solanaceous plants such as potato, tomato, and sweet pepper, in which no 3α-tropane alkaloid has been reported (16, 17). Likewise, no plant species has so far been shown to possess only TR-I activity. These observations imply a wider occurrence of TR-II in this plant family, and hence the later divergence of TR-I from TR-II. Exploring the TR gene(s) in such plant species may provide insights into the emergence of the two configurations in the evolution of tropane alkaloid biosynthesis.

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