Two Tropinone Reductases with Distinct Stereospecificities from Cultured Roots of *Hyoscyamus niger* 1

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**ABSTRACT**

Tropinone is an alkaline intermediate at the branch point of biosynthetic pathways leading to various tropane alkaloids. Two stereospecifically distinct NADPH-dependent oxidoreductases, TR-I and TR-II, which, respectively, reduce tropinone to 3α-hydroxytropine (tropine) and 3β-hydroxytropine (ψ-tropine), were detected mainly in the root of tropane alkaloid-producing plants but not in nonproducing cultured root. Both reductases were purified to near homogeneity from cultured root of *Hyoscyamus niger* and characterized. The TR-I reaction was reversible, whereas the TR-II reaction was essentially irreversible, reduction of the ketone being highly favored over oxidation of the alcohol ψ-tropine. Marked differences were found between the two reductases in their affinities for tropinone substrate and in the effects of amino acid modification reagents. Some differences in substrate specificity were apparent. For example, N-propyl-4-piperidone was reduced by TR-II but not by TR-I. Conversely, 3-quinuclidinone and 8-thiabicyclo[3.2.1]octane-3-one were accepted as substrates by TR-I but hardly at all by TR-II. Both enzymes were shown to be class B oxidoreductases, which transfer the pro-S hydrogen of NADPH to their substrates. Possible roles of these tropinone reductases in alkaloid biosynthesis are discussed.

Tropinone is an alkaline intermediate at the branch point of biosynthetic pathways leading to various tropane alkaloids mainly found in Solanaceae (Fig. 1). The 3-keto group of tropinone can be reduced to hydroxyls having two different configurations: tropine has a hydroxy group on the opposite side of the tropane nitrogen atom (α-configuration), whereas ψ-tropine has the group on the same side (β-configuration). The 3α-hydroxy group of tropinone is then esterified with organic acids to give various tropane esters, such as hyoscyamine and scopoline (for a review, see ref. 20). In contrast, the metabolism of ψ-tropine is not well understood. Cocaine found in *Erythroxylum* is a well-known tropine ester with a ψ-tropine derivative.

An NADPH-dependent oxidoreductase (TR-I; Fig. 1) that converts tropinone to tropine was first discovered in cultured roots of *Datura stramonium* (15), but a similar enzyme with different stereospecificity that reduces tropinone to ψ-tropine (TR-II) was later found in cultured roots of *Hyoscyamus niger* (5). Subsequent studies by us (23) and others (4) indicated that the two stereospecifically distinct reductase activities can coexist in a given alkaloid-producing species. How the metabolic fates of tropinone are controlled by these reductases is an interesting question, particularly in view of our observation that the ψ-tropine-forming activity seemed to be predominant in cultured *H. niger* roots that accumulated mostly tropine-derived alkaloids (5). Here we report distribution of the two reductases in various plant species, their organ-specific expression, and detailed biochemical characterization of the enzymes highly purified from cultured roots of *H. niger*.

**MATERIALS AND METHODS**

**Plant Materials**

Cultured roots of *Hyoscyamus niger* L. have been maintained in our laboratory since 1984 as described elsewhere (13). Several plant species (*Physcholaima orientalis* M.B. Don, *Atropa acuminate* Royce ex Lindl, *Physalis alkekengi* L., and *Calystegia sepium* R. Br) were transformed with *Agrobacterium rhizogenes* (strain 15834) by Dr. P. Bachmann at our laboratory. A hairy root clone (D15/5) of *Datura stramonium* was obtained from Dr. R. Robins, Institute of Food Research, Norwich, UK. These roots were cultured in auxin-free B5 medium (7) supplemented with 3% (w/v) sucrose. All of the other cultured roots have been maintained in our laboratory for several years or were provided by Dr. P. Bachmann and cultured as described elsewhere (14). After the plants were harvested with a suction filter at the late growth phase, the roots were immediately frozen with liquid nitrogen and kept at −20°C until use.

Plants of *H. niger*, *D. stramonium*, and *Atropa belladonna* were grown in a growth chamber at 20°C and 80% RH with a 14-h light period and harvested for enzyme extraction when plants were flowering and had a few young fruits.

**Chemicals**

ψ-Tropine, hygrine, and TBON were, respectively, gifts from Professor G.G. Gross, University of Ulm, Ulm, West Germany; Professor S. Yamada, Jyosai University, Itado, Japan; and Dr. A.J. Parr, Institute of Food Research. 2-Carbomethoxy-3-tropine and ψ-pelletierine were generously provided by Professor E. Leete, University of Minnesota, Minneapolis, MN. Tropinone, tropine, and the other compounds used for the study of substrate specificity were...
potassium phosphate was producing distinguish reported before Enzyme Assays by DEAE-Sepharose purified synthesis, dehydrogenase, and phosphate.

Figure 1. Metabolism of tropinone. Tropinone is derived from ornithine and/or arginine by way of putrescine in several plant species, most notably in Solanaceae. The cyclic ketone is reduced stereospecifically either to 3α-hydroxytropane (tropine) by TR-I or to 3β-hydroxytropane (β-tropine) by TR-II. Tropine is esterified with various organic acids to give, for example, hyoscyamine and its epoxide derivative scopolamine, whereas the metabolism of β-tropine is not well understood. Carbons in the tropine skeleton of tropinone are numbered.

Enzyme Assays and Protein Determination

Because most crude enzyme extracts from tropane alkaloid-producing roots contained both TR activities, it was important to distinguish the two activities in the enzyme assays. This was accomplished by GLC separation of tropine and β-tropine. The reaction mixture of 1 mL contained, at a final concentration, 4 mM tropinone, 2 mM NADPH, 100 mM potassium phosphate buffer (pH 5.9), and enzyme. After incubation for 1 h at 37°C, the reaction was stopped by the addition of 100 µL of 25% NH4OH, and then 1 mL of the alkali solution was transferred to an Extrelut-1 column (Merck). Alkaloids were eluted from the column with 6 mL of chloroform, and the chloroform eluate was evaporated to dryness. The dry residue was then dissolved in 50 µL of a dioxane solution containing 0.1% (v/v) nicotine as an internal standard. Alkaloids were separated by GLC equipped with an apolar capillary column CB-1 (0.25 mm i.d. x 30 m; Shimadzu, Kyoto, Japan) at 120°C. The order of elution was tropinone, tropine, β-tropine, and nicotine.

After TR-I and TR-II were completely separated by column chromatography, the enzyme activities were measured by the consumption of NADPH at 340 nm (5, 15). The concentration of NADPH in the above reaction mixture was lowered to 0.2 mM to maintain low background absorbance. The reference reaction without enzyme was always included to offset possible nonenzymic decomposition of NADPH in the relatively acidic assay mixture at 37°C, except that the reaction without ketone substrate was used as the reference when substrate specificity was studied. The molar absorption coefficient of 6200 was used to calculate the amount of NADPH consumed.

Reverse oxidation reactions catalyzed by purified TRs were assayed similarly by the formation of NADP+, as monitored at 340 nm. The reaction mixtures contained 100 mM of various buffers, 0.5 mM of NADPH, and 10 mM of either tropine or β-tropine. The reaction without enzyme was used as the reference.

The protein concentration was determined by the method of Bradford (3) with BSA as the standard.

Buffers

The following buffers were used: (buffer A) 100 mM potassium phosphate buffer (pH 7.0) containing 3 mM DTT and 0.5 mM diisopropylfluorophosphatase; (buffer B) 10 mM potassium phosphate (pH 7.0) containing 1 mM DTT and 10% (v/v) glycerol; (buffer C) 10 mM potassium phosphate (pH 7.0) containing 1 mM DTT, 0.1 mM NADPH, and 10% (v/v) glycerol; (buffer D) 25 mM bis-tris (pH 6.3), adjusted by addition of HCl containing 1 mM DTT, 0.1 mM NADPH, and 10% (v/v) glycerol; and (buffer E) 10% (v/v) Polybuffer 74 (Pharmacia) (pH 3.9, adjusted by addition of HCl) containing 1 mM DTT, 0.1 mM NADPH, and 10% (v/v) glycerol.

Purification of TRs

For large-scale purification of enzymes, cultured roots of H. niger were transferred to 300-mL flasks containing 75 mL of auxin-free B5 medium supplemented with 3% (w/v) sucrose and then cultured at 90 rpm in the dark for 3 d. After harvest, the roots were frozen and stored as described above.

All purification procedures were carried out at 4°C unless noted otherwise. Frozen roots (525 g fresh weight) were homogenized thoroughly with a Polytron (Kinematica, Luzern, Switzerland) in 1.5 L of buffer A containing 40 g of insoluble PVP (Polyclar AT; GAF Chemical Corp., Osaka, Japan). The homogenate was passed through a cheesecloth-Miracloth (Calbiochem)-cheesecloth filter and centrifuged at 10,000g for 40 min. A small portion of the supernatant was purchased from Aldrich, Nacalai tesque (Kyoto, Japan), and Wako Pure Chemical Industries (Osaka, Japan).

(4S)-[4-2H] and (4R)-[4-2H]NADPH were prepared essentially by the method of Allemann et al. (1), starting with -glucose-1-1d as a 2H source and using hexokinase, glucose-6-phosphate dehydrogenase, and dihydrofolate reductase. After synthesis, each stereospecifically labeled cofactor was purified by DEAE-Sepharose chromatography, basically as reported before (1).

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passed through a PD-10 column (Pharmacia), and this desalted eluate was subsequently referred to as crude extract. The TRs in the supernatant precipitated at ammonium sulfate concentrations between 45 and 75% saturated. The pellet obtained after a second centrifugation was dissolved in 44 mL of buffer B containing 1 mM NADPH and dialyzed for 11 h against a total of 9 L of buffer B with two changes of the buffer. The dialyzed enzyme solution was loaded onto a DEAE-Sepharose Fast Flow column (Pharmacia; 2.5 × 18 cm) previously equilibrated with buffer C, and the column was washed with 240 mL of the same buffer. The enzymes were eluted with a linear gradient (540 mL) of 0 to 0.4 M KCl dissolved in buffer C, at a flow rate of 1 mL/min. TR-I and TR-II activities were eluted in different fractions. Active fractions for each enzyme were pooled and separately purified in subsequent steps.

Active TR-I fractions were concentrated to 15 mL by a YM10 membrane filter (Amicon). After solid NADPH was added to a final concentration of 1 mM, the concentrated enzyme solution was dialyzed against a total of 6 L of buffer B for 9 h with one exchange of the buffer and then loaded on a hydroxylapatite column (Bio-gel HTP, Bio-Rad; 1.6 × 12 cm) previously equilibrated with buffer C. The column was washed with 25 mL of the same buffer, and the enzyme was eluted with a linear gradient (140 mL) of 0 to 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT, 0.1 mM NADPH, and 10% (v/v) glycerol, at a flow rate of 0.25 mL/min. Active TR-I fractions were concentrated to 1.3 mL by a YM10 filter, after which the buffer was changed to buffer C containing ammonium sulfate at 25% of saturation using a PD-10 column.

TR-I was subsequently purified by two fast protein liquid chromatography columns (Pharmacia) at room temperature. The above enzyme solution was loaded on a Phenyl-Superose H 10/10 column previously equilibrated with buffer C containing ammonium sulfate at 25% of saturation, and the column was washed with 24 mL of the same buffer. TR-I was eluted with a linear gradient (96 mL) of ammonium sulfate at 25 to 0% of saturation in buffer C, at a flow rate of 1 mL/min. Fractions containing high TR-I activity were pooled and desalted using PD-10 columns, and the buffer was changed to buffer D and loaded on a Mono P HRS/20 column previously equilibrated with buffer D. After the flow of 12 mL of buffer D, TR-I was eluted with buffer E at a flow rate of 0.5 mL/min. To each fraction (1 mL) was added 50 μL of 1 M potassium phosphate (pH 7.0) for neutralization.

The DEAE-Sepharose fractions of TR-II were further purified by successive chromatography on hydroxylapatite, Phenyl-Superose and Mono P columns, basically in the same way as described for the purification of TR-I.

Crude enzyme extracts from various plant organs and from other root cultures were obtained by the procedures used for the *H. niger* enzyme, on much smaller scales.

**Alkaloid Analysis**

Samples (50 mg dry weight) were lyophilized, soaked in 4 mL of 0.1 N H2SO4, and homogenized in mortars. The homogenates were sonicated for 20 min and filtered through No. 2 filter paper (Advantec, Tokyo). After the filtrates were mixed with 0.4 mL of 25% NH4OH, a 1-mL portion of each mixture was applied to an Extrelut-1 column (Merck), and alkaloids were eluted with 6 mL of dichloromethane. For the analysis of hyoscyamine, scopolamine, and 6β-hydroxyhyoscyamine, the organic extract was dried at 35°C. The dry residues were dissolved in 20 μL of N,O-bis(trimethylsilyl)acetamide and 80 μL of a dioxane solution containing tricosane (625 μg/mL) and analyzed by GLC (Shimadzu model GC-7A) equipped with a capillary column CB-J-1 (0.25 mm i.d. × 30 m; Shimadzu) at a column temperature of 235°C. For the analysis of tropinone, tropine, and ψ-tropine, 100 μL of the above dioxane solution containing tricosane was added to the dichloromethane extract, which was then evaporated at 30°C until the volume of the solution was reduced to approximately 100 μL. The concentrated solution was directly analyzed by GLC. The column was held at 120°C for 8 min, and then the column temperature was increased to 250°C at a rate of 32°C/min.

For the analysis of alkaloids in the culture medium, 1 mL of the medium was mixed with 100 μL of 25% NH4OH and then directly loaded on the Extrelut-1 column. Subsequent procedures were the same as described above. The alkaloid amounts shown in this report were the sum of the alkaloids in the cells and the medium.

**Determination of Stereospecific Hydrogen Transfer**

Purified TR-I and TR-II were separately incubated in the reaction mixture (1 mL) containing 100 mM potassium phosphate buffer (pH 5.9), 0.16 μmol of (4R)[4-2H]NADPH or 0.18 μmol of (4S)[4-2H]NADPH, and 4 μmol of tropine, for 2 h at 37°C. The reaction products were extracted as described above and analyzed by GC-MS as described elsewhere (13). The electron impact ionization mode was set at 70 eV.

**Kinetic Analysis**

Enzyme activities were measured for more than five concentrations of the given substrates or cofactors, and the kinetic parameters (±sd) were calculated by Wilkinson's statistical analysis method (22).

**RESULTS**

**TR Activities in Various Plant Organs**

Tropine-forming TR-I activity and ψ-tropine-forming TR-II activity were measured in various plant organs and cultured tissues of three solanaceous species that synthesize either hyoscyamine or scopolamine as the main alkaloid (Fig. 2). Strong TR-I and TR-II activities were found in the root tissues of the three plants. TR-I activities were by far the strongest in developmentally young root tissues such as branch roots and cultured roots, whereas strong TR-II activities were observed not only in such roots but in the stem of *Datura* and *Atropa* as well. In all plant organs analyzed, TR-II activities were higher than TR-I activities in *Hyoscyamus* and *Atropa*, whereas the reverse relation was found in most organs of *Datura*. The high activity of the TRs in the root led us to investigate the enzyme activities in various cultured roots from several genera.
Hyoscyamus niger, presumably a genus Hyoscyamus, no species. As only these cultures detected more TR-II activity than TR-I, tetrope alkaloids were isolated. The ability to contain tigloyloxy esters of tropine and \( \psi \)-tropine, the genus Physochlaina is known to contain tigloyloxy esters of tropine and \( \psi \)-tropine, and calystegins, polyhydroxylated derivatives of \( \psi \)-tropine, were recently discovered in C. sepium (8). Among the root cultures tested, the root culture of H. niger that showed relatively high activities of both TRs and good root growth was chosen for further studies.

**Time Courses of Alkaloid Formation and TR Activities**

When cultured roots of H. niger were transferred to a fresh medium, the contents of three alkamine intermediates peaked at day 3 (Fig. 4A), whereas the scopolamine content increased at a later stage of root growth (Fig. 4B). In this culture, the contents of hyoscyamine and 6\( \beta \)-hydroxyhyoscyamine were low and remained relatively constant during root growth. The TR-I activity was highest at day 3, but no apparent peak of TR-II activity was observed during the culture period of 15 d (Fig. 4C). To obtain the highest enzyme activities for purification, the cultured roots were harvested on the third day after subculture.

**Enzyme Purification**

We found that TR activities, especially TR-I activity, were rapidly lost during purification using a simple buffer without stabilizing agents. A series of experiments (not shown), however, revealed that NADPH, NADP\(^+\), and glycerol were effective in preserving TR activities (especially TR-I) during storage at 4°C. Therefore, 0.1 mM NADPH and 10% glycerol were included in all buffers used for column chromatography. The crude enzyme preparation from cultured H. niger roots had TR-II activity about 2-fold higher than TR-I activity. The two activities, partially purified by ammonium sulfate precipitation, were separated by gel filtration on a Sephacryl S-200 column.

**Figure 2.** TR activities in various plant organs of three tropane alkaloid-producing solanaceous species. Note the different scales for enzyme activity (pKat/mg of protein) in each species.

**Figure 3.** Alkaloid contents and TR activities in various root cultures from 19 species. Note the different scales for alkaloid contents (% dry weight [DW]) in each group of metabolites.
precipitation, were reproducibly and almost completely separated from each other by column chromatography on DEAE-Sepharose (Fig. 5). Thereafter, TR-I and TR-II were separately purified by successive column chromatography on hydroxylapatite, Phenyl-Superose, and Mono P. SDS-PAGE analysis of the active fractions after Mono P chromatography showed single major protein bands (30 kD for TR-I and 29 kD for TR-II), which closely corresponded with the patterns of the respective TR activities (Fig. 6). Overall, TR-I was purified 1820-fold to 131 nKat/mg of protein, and TR-II was purified 2560-fold to 387 nKat/mg of protein. These final specific activities were significantly higher than those reported previously for TR-I (0.15 nKat/mg of protein; ref. 15) and for TR-II (18 nKat/mg of protein; ref. 5). Enzyme preparations after Phenyl-Superose chromatography were used for characterization of the enzymes reported below.

**Mol Wt and Effects of pH on Forward and Reverse Reactions**

The mol wt of \textit{H. niger} TRs, as determined by gel filtration on TSK-GEL G3000SW (7.5 mm × 60 cm; TOSOH, Tokyo), were 115,000 ± 2,000 for TR-I (mean of five measurements...
and 69,000 ± 1,000 (minor peak) for TR-II (means of four measurements ± sd). The subunit mol wt of 30,000 for TR-I and 29,000 for TR-II (Fig. 6) indicate that TR-I may be a homotramer and TR-II, a homotrimer or a homotetramer.

The TR activities that reduce tropinone to either tropine or ψ-tropine (forward reaction) and the reverse oxidative activities were measured over the pH range of 4.0 to 11.0. The purified TR-I preparation reduced tropinone only to tropine and oxidized tropine, but not ψ-tropine, to tropinone, whereas the purified TR-II preparation catalyzed the conversions between tropinone and ψ-tropine. The forward reactions catalyzed by the two TRs were efficient at a somewhat acidic pH; TR-I had a relatively narrow pH range, optimum at 6.1, whereas TR-II had a broad pH range, optimum from 5.3 to 6.5. The reverse reactions were catalyzed efficiently by TR-I within a narrow pH range, optimum at 7.6, and very inefficiently (about 1.3% of the forward activity, at most) by TR-II. The pH optima of TR-I from Datura stramonium have been reported to be 6.8 for the forward reaction and 9.5 for the reverse reaction (15). We (5) previously reported the pH optimum for the forward reaction of H. niger TR-II to be 5.8.

**Substrate and Cofactor Specificities**

Both TRs had a much higher affinity for NADPH than for NADH; the respective $K_m$ values for NADPH, NADP+, and NADH were 11.3 ± 1.7, 41.5 ± 3.7, and >10,000 μM for TR-I and 6.1 ± 1.0, 251 ± 68, and 7,600 ± 1,400 μM for TR-II. TR-I had $K_m$ values of 1010 ± 180 μM for tropinone and 2600 ± 130 μM for tropone, whereas TR-II had much lower $K_m$ values of 34.0 ± 8.6 μM for tropinone and 687 ± 47 μM for ψ-tropine.

Various compounds, mostly cyclic ketones, were tested at 4 mM to determine whether they can be reduced by TR-I and TR-II (Fig. 7). The two TRs showed relatively broad substrate specificities, in which some ketones were reduced by both TRs and other ketones preferentially by one TR. The two reductases did not reduce the cyclic ketones containing nitrogen next to a keto group, such as 2-piperidone, N-methyl-2-piperidone, 2-pyrrolidone, and N-methyl-2-pyrrolidone or the ketones that are larger than the tropane ring, such as ψ-pelletierine and 2-carboxymethoxy-3-tropinone, a possible biosynthetic precursor of cocaine (17). Even the addition of a 6-hydroxyl group to the tropane ring significantly lowered the reactivity of the substrate, 6-hydroxytropinone. Conversely, the C6-C7 bridge of tropinone was not essential for activity, and many monocyclic ketones was reduced to various degrees by the TRs. Hygrine, a postulated biosynthetic precursor of tropinone (17), was not a substrate for either TR.

Kinetic parameters ($K_m$ and relative $V_{max}$) were obtained for several substrates (Table 1). Comparison of the $K_m$ values for piperidones and cyclohexanones suggests that the C6-C7 bridge and the nitrogen atom of tropinone serve to decrease the affinity of tropinone for TR-I but to increase the affinity for TR-II. Similar experiments performed with essentially homogeneous TR-I also resulted in the same reactivities for N-methyl-4-piperidone and 4-methylcyclohexanone as described in Figure 7, thus excluding the possibility that other nonspecific reductases contaminated the TR-I preparation (data not shown). Replacing the methyl group of N-methyl-4-piperidone and 4-methylcyclohexanone with an ethyl or propyl group resulted in a higher affinity for TR-II, indicating hydrophobic interactions at the N-bridge in binding the substrate to the active site of TR-II. The active site of TR-I apparently was not large enough to accept N-propyl-4-piperidone. As a substrate for TR-I, the somewhat bulky 3-quinuclidinone was almost as efficient as tropinone but had a very low affinity for TR-II. The difference between the two TRs in their affinity for substrates was also evident for TBON, a sulfur analog of tropinone. For TR-I, the $K_m$ value of this sulfur compound was 30-fold lower than the value of tropinone, whereas TR-II had a $K_m$ value of TBON at least 100-fold higher than that of tropinone.

**Effects of Inhibitors**

The inhibiting effects of various compounds on the two TR activities were compared. The two TR activities were affected similarly by metals. At 1 mM, both activities were inhibited severely (more than 99%) by CuCl2 and HgCl2; moderately (26–43%) by CdCl2; and from slightly to negligibly (0–15%) by FeCl2, FeCl3, MgCl2, CaCl2, MnCl2, CoCl2, NiCl2, ZnCl2, and LiCl. Among the three sulfhydryl reagents

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**Figure 7.** Substrate specificities of TRs. Various ketones were tested at 4 mM as substrates for TR-I and TR-II, and their reactivities were expressed relative to those of tropinone. N.D., Not detected.
tested, p-chloromercuribenzoic acid inhibited both TRs most strongly (Table II). TR-II seemed to be more susceptible to this reagent, which caused a 76% inhibition of TR-II activity even at 0.01 mM. Diethyl pyrocarbonate, which preferentially modifies the imidazole group of histidine residues at neutral pH, abolished the TR-I activity at 0.1 mM, whereas much higher concentrations were required to significantly inhibit the TR-II activity. EDTA at 1 mM did not inhibit either TR activity, indicating that metal ions are not required for catalysis.

TBON effectively inhibited the reduction of tropinone by TR-I at concentrations of 0.1 mM or higher but had no effect on the tropinone reduction by TR-II even at 4 mM (Table II).

### Stereospecificity of Hydrogen Transfer

The reaction products formed after parallel incubations of (4R)-[4-2H]NADPH and (4S)-[4-2H]NADPH with tropinone and either TR were analyzed by GC-MS (Fig. 8). Both tropine and ψ-tropine, produced, respectively, by TR-I and TR-II in the presence of (4S)-[4-2H]NADPH, had the molecular ion of m/z 142, whereas the corresponding alcohols, produced in the presence of (4R)-[4-2H]NADPH, had the ion of m/z 141. The results show that both TRs transfer exclusively the pro-S hydrogen at C-4 of NADPH to tropinone. Accordingly, TR-I and TR-II are classified as B-specific oxidoreductases.

### DISCUSSION

Tropinone has been found in several tropine alkaloid-producing plants (see references cited in ref. 16) and shown to be metabolized to the tropine moiety of hyoscyamine and scopolamine when fed to intact *Datura innoxia* plants (16). Although the in vivo metabolism of tropinone to ψ-tropine or its derivatives has not been reported, it is reasonable to suppose that many esters with ψ-tropine isolated from plants (20) are also derived from tropinone. Accordingly, two reductase activities that differ in stereospecificity have been considered responsible for the reduction of tropinone to tropine and/or ψ-tropine. The tropine-forming activity was first found in a root culture of *D. stramonium* (15), and the ψ-tropine-forming activity was later found in a root culture of

### Table I. *K*<sub>m</sub> and Relative *V*<sub>max</sub> for Tropinone and Other Substrates

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*Not determined.*

### Table II. Effect of Inhibitors on TR Activities

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H. niger (5). However, an enzyme assay that measured the oxidation of NADPH in the presence of tropinone was used and, thus, these studies did not distinguish the alkaline product in the crude enzyme extracts and during enzyme purification. Recently, N-[methyl-14C]tropinone was used as a substrate in the enzyme assay, and the radioactive reaction product was separated on a silica gel column using HPLC to distinguish the two reductase activities (4). With that method, tropine-forming TR-I activity and β-tropine-forming TR-II activity were partially separated, but the two reductases were not further purified. For this study reported in this paper, we developed a simple assay method that distinguishes tropine and β-tropine by GLC and used this GLC method to measure the two TR activities simultaneously in crude enzyme extracts and during the initial stages of enzyme purification. Our studies and others (4) demonstrated that two distinct TRs catalyze highly stereospecific reduction of tropinone to either tropine or β-tropine, but not to both, and that the two TRs usually coexist in a given alkaloid-producing species, especially in the root.

Direct comparisons between highly purified TR-I and TR-II revealed both common and different properties in these reductases. Both TRs belong to the NADPH-dependent, class B oxidoreductases, of which the cinnamoyl-CoA reductase from Forsythia suspensa (9) is known to be another member from plants. The inability of EDTA to inhibit either of the TRs may indicate that neither of the TRs contains zinc, which is essential for catalysis in some oxidoreductases. Inhibition patterns by various metals were essentially the same for the two TRs. Both TRs reduced several common cyclic ketones, including tropinone. In contrast, there were several notable differences between TR-I and TR-II in the reversibility of their reactions, inhibition by the protein modification reagents, and their reactivity with several ketones, such as N-propyl-4-piperidone, 3-quinuclidinone, and TBON. Some of these properties have been reported for the relatively crude TR-I from D. stramonium (15) and for the partly purified TR-II from H. niger (5) and were basically confirmed in this report.

These observed similarities and differences between the two TRs inevitably lead to the question: how did these two enzymes evolve? The two TRs may have evolved divergently from a common ancestor or convergently to accept the common substrate tropinone. Studies of mutual cross-reactivity to antibodies raised against these TRs and information concerning the primary amino acid sequences and the organization of the genes will give us a better understanding of the evolutionary origin of TRs and, perhaps, of the evolution of diastereoisomers occasionally found in secondary products, in general. In this respect, it should be noted that a polyclonal antiserum raised against a pea isoflavone reductase that introduces a 35 chiral center recognized a stereospecifically distinct alfalfa isoflavone reductase that introduces an opposite 3R chiral center (18).

Our data concerning H. niger enzymes show that the specificity constant for TBON, expressed as $V_{max}/K_{m}$, is more than 10-fold higher in TR-I, but is much lower in TR-II, than that for tropinone. If TR-I and TR-II exist in the same cell and compete for tropinone, TBON administered to a root culture would be preferentially reduced by TR-I and competitively inhibit the formation of tropine, whereas TR-II would be little affected by the analog and would reduce accumulating tropinone to an increasing amount of β-tropine. This is exactly what was observed with a root culture of D. stramonium that had been fed with 4.2 mM TBON for 14 d. The treated Datura culture showed an increase in the β-tropine pool and a decrease in the pools of tropine, 3α-acetoxytropane, and hyoscyamine (19). A reduction product of TBON, presumably 8-thiabicyclo[3.2.1]octan-3α-ol, was also isolated from the culture. These results further indicate that both TRs are present in the same cells of the cultured roots.

TR-I activity is mostly restricted to branch roots and cultured roots. These tissues are developmentally young root tissues without secondary growth (12). Putrescine N-methyltransferase (14) and hyoscyamine 6β-hydroxylase (11), other enzymes involved in the biosynthesis of hyoscyamine and scopolamine, are also highly active in the young root tissues. Together with the classical grafting experiments between alkaloid-producing and non-producing plants (21), and alkaloid formation in organ cultures (13), these results suggest that hyoscyamine and scopolamine are mostly synthesized in the young root cells and translocated to the aerial parts of the plant. Recent immunohistochemical localization of hyoscyamine 6β-hydroxylase to the pericycle cells of the root (10)
further supports this conclusion. It will be studied whether TR-I is also expressed in the pericycle cells themselves when specific antibodies or gene probes become available.

The role of TR-II in alkaloid biosynthesis is not clear. Although TR-II activity is predominant over TR-I activity in *Hyoscyamus* and *Atropa*, it is the derivatives of tropine that accumulate in these genera. It may be that some $\psi$-tropine metabolites have escaped detection by conventional extraction and analysis methods of typical tropane alkaloids. Callystegins recently discovered in *Calystegia* and *Atropa* are polyhydroxylated $\psi$-tropines that because of their highly hydrophilic nature are not recovered in conventional alkaloid fractions (6, 8). It should be interesting to examine whether callystegins or similar hydrophilic $\psi$-tropine derivatives accumulate in the root cultures with high TR-II activity.

Whatever the potential metabolites of $\psi$-tropine may be, the fact that TR-II activity is high in branch roots and cultured roots may indicate a situation in which TR-I and TR-II compete for tropine in the alkaloid-synthesizing cells in the root. The feeding experiment with TBON as described above (19) is suggestive of such competition. Then, does the level of TR-II activity control the biosynthesis of tropine-derived alkaloids? TR-II has a much higher affinity for tropine than does TR-I, and the reduction of tropine is highly favored over the oxidation of $\psi$-tropine in the TR-II reaction. These in vitro data suggest that TR-II decreases the metabolite flow to tropine by efficiently removing tropine from the pathway, with an ultimate decrease in the contents of hyoscyamine and scopolamine. We could not, however, find any correlation between the ratio of TR-I activity to TR-II activity and the contents of hyoscyamine and scopolamine in various root cultures. Furthermore, cultured roots of *H. albus* and *H. bohemicus*, which have very low activities of both TRs, accumulate tropine, but the contents of tropine-derived alkaloids are, nevertheless, very high in these cultures. These observations suggest that the synthesis of tropine-derived alkaloids is usually not rate limited by TR-I activity and that potential competition between the two TRs in vivo does not notably influence the accumulation of hyoscyamine and its derivatives in normal root tissues. It should be noted that in most root cultures the activities of the TRs are generally much higher than those of other enzymes in the pathway: the specific activities of the TRs are in the range of 1 to 1200 pKat/mg of protein, whereas those of putrescine N-methyltransferase (ref. 14 and our unpublished results) and hyoscyamine 6β-hydroxylase (11) are in the range of 1 to 70 and 1 to 30 pKat/mg of protein, respectively. It may thus follow that reinforcement of TR-I activity or reduction of TR-II activity by means of genetic engineering would not have major impact on the productivity of hyoscyamine and scopolamine in transgenic medicinal plants.

**NOTE ADDED IN PROOF**

Partial purification of TRs from a *Datura* root culture was reported recently: A. Portstetten, B. Dräger, A. Nahrstedt (1992) Two tropine reducing enzymes from *Datura stramonium* transformed root cultures. Phytochemistry 31: 1135–1138.

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