The Second Naphthol Reductase of Fungal Melanin Biosynthesis in Magnaporthe grisea

TETRAHYDROXYNAPHTHALENE REDUCTASE*

James E. Thompson‡§, Stephen Fahnstock¶, Leonard Farrall, Der-Ing Liao§, Barbara Valent, and Douglas B. Jordan**‡‡

From the ‡DuPont Agricultural Products, Stine-Haskell Research Center, Newark, Delaware 19714 and §DuPont Central Research and Development, †DuPont Agricultural Products, and **DuPont Pharmaceuticals Company, Experimental Station, Wilmington, Delaware 19880-0400

Mutants of Magnaporthe grisea harboring a defective gene for 1,3,8-trihydroxynaphthalene reductase retain the capability to produce scytalone, thus suggesting the existence of a second naphthol reductase that can catalyze the reduction of 1,3,6,8-tetrahydroxynaphthalene to scytalone within the fungal melanin biosynthetic pathway. The second naphthol reductase gene was cloned from M. grisea by identification of cDNA fragments with weak homology to the cDNA of trihydroxynaphthalene reductase. The amino acid sequence for the second naphthol reductase is 46% identical to that of trihydroxynaphthalene reductase. The second naphthol reductase was produced in Escherichia coli and purified to homogeneity. Substrate competition experiments indicate that the second reductase prefers tetrahydroxynaphthalene over trihydroxynaphthalene by a factor of 310; trihydroxynaphthalene reductase prefers trihydroxynaphthalene over tetrahydroxynaphthalene by a factor of 4.2. On the basis of the 1300-fold difference in substrate specificities between the two reductases, the second reductase is designated tetrahydroxynaphthalene reductase. Tetrahydroxynaphthalene reductase has a 200-fold larger $K_i$ for the fungicide tricyclazole than that of trihydroxynaphthalene reductase, and this accounts for the latter enzyme being the primary physiological target of the fungicide. M. grisea mutants lacking activities for both trihydroxynaphthalene and tetrahydroxynaphthalene reductases do not produce scytalone, indicating that there are no other metabolic routes to scytalone.

Trihydroxynaphthalene (3HN) reductase is the biochemical target of three commercial agricultural fungicides (tricyclazole, pyroquilon, and phthalide of Fig. 1) that are applied to prevent blast disease in rice (1–8). 3HNR, a member of the enzyme super family known as the short chain dehydrogenases (9, 10), catalyzes the reduction of 1,3,8-trihydroxynaphthalene to vermelone, an intermediate step in fungal melanin biosynthesis (Fig. 1). In Magnaporthe grisea, the causal agent of rice blast, fungal melanin biosynthesis proceeds through a pentaketide route, joining acetate units to make 1,3,6,8-tetrahydroxynaphthalene (4HN) (7, 8). 4HN is transformed to 1,8-dihydroxynaphthalene through a succession of two reduction and two dehydration steps. 1,8-Dihydroxynaphthalene is considered to be the ultimate precursor of fungal melanin, a polymer that is employed by the pathogen during the initiation of disease (11, 12). Both dehydration reactions in the biosynthetic pathway are catalyzed by scytalone dehydratase (SD (13–15)), an enzyme that is also a target of a commercial fungicide (16–19), and others that are in development (20–23). X-ray structures of 3HNR, a homotetramer of 120 kDa, in the absence and presence of the inhibitor tricyclazole have been reported (5, 6, 24), and they provide the initial basis for a structure-based design program for inhibitors of the enzyme function.

Genetic studies on fungal melanin biosynthesis in M. grisea yielded three classes of melanin-deficient mutants based on pigmentation phenotypes (25). Instead of the black polymer of fungal melanin, Buf mutants lacking catalytic activity for 3HNR produce buff-colored pigment, and Ray mutants lacking catalytic activity for SD produce rosy-colored pigment. Accumulated pigments in the mutants result from the oxidation products of the respective precursors of the enzyme in the pathway, 3HN in the Buf mutants, and 4HN in the Ray mutants. Since the Buf mutant accumulates oxidation products of 3HN, it is obvious when considering the procession of the pathway (Fig. 1) that there should be another biochemical route to produce 3HN from 4HN without using 3HNR as the catalyst. Another indication of a second reductase is that the fungicides used for preventing rice blast (tricyclazole, pyroquilon, and phthalide) are known to cause the accumulation of 3HN byproducts in fungal cultures (1, 3, 4, 7, 8). A more quantitative assessment of the situation is obtained from measuring the accumulation in the media of scytalone, which is considerably more stable than 3HN or 4HN (Table I). The wild-type and the Buf mutant do not accumulate scytalone in their growth media, whereas the Ray mutant does. Crosses between the Buf and Ray mutants yield double mutants that accumulate scytalone in their growth medium to about half the extent as the Ray mutant. These results also suggest that there is either another reductase responsible for catalyzing the reduction of 4HN to scytalone or there is another route alto-
FIG. 1. The fungal melanin biosynthetic pathway.

gather for producing scytalone in \textit{M. grisea}. In this work we report that, indeed, there is another reductase in the pathway and that its presence in \textit{M. grisea} fully accounts for the accumulation of scytalone in the double mutant (\textit{buf} \textit{rsey}) described above. Additionally, we report that the new reductase is considerably more specific for 4HN over 3HN in comparison to 3HNR. Historically, the enzyme we denote as 3HNR (2, 5, 6, 24, 29, 30, 34) has been referred to by others (26–28) and in the enzyme nomenclature data base as tetrahydroxynaphthalene reductase, and this discrepancy is resolved.

**EXPERIMENTAL PROCEDURES**

**Materials and General Methods—**DDBO was synthesized as described (29). Scytalone was purified from cultures of \textit{Rsy} mutants of \textit{M. grisea} (25). The enzymatic synthesis of vermelone from scytalone was accomplished essentially as described (26). UV-visible spectrophotometric analyses were performed on HP 8452A or HP 8453 diode array spectrophotometers (Hewlett Packard). SDS-polyacrylamide gel electrophoresis analyses of proteins were conducted by using a PhastSystem (Amersham Pharmacia Biotech). Homogeneous 3HNR was purified as described (30). 3HNR subunit concentrations were estimated using a calculated \( \epsilon_{280} \) value of 20,760 M

\( \text{cm}^{-1} \). The molecular mass of 4HNR was determined using a Fisons VG Quattro II mass spectrometer with calibration against horse heart myoglobin as an external standard. \textit{M. grisea} and its mutants were grown in liquid media as described (25), and the scytalone concentrations in the media were determined by using scytalone dehydratase in an end point spectrophotometric determination (12).

**Preparation of 4HNR—**A ZipLox (Life Technologies, Inc.) library containing cDNA from \textit{M. grisea} strain 6043 (31) was probed with radiolabeled 3HNR cDNA, and a class of weakly hybridizing clones was isolated in addition to the homologous 3HNR clones.\(^2\) Sequencing of the weakly hybridizing cDNAs confirmed identification of a putative second reductase (HNR) gene. Genomic HNR clones were identified from a phage genomic excision library (31) containing DNA from strain 4091-I (Fig. 2). This vector (pFA156) was constructed by inserting a double-stranded synthetic oligonucleotide with the overlapping translational stop and restart signals into pET21c(+) (Novagen) in place of the multiple cloning site sequences between \textit{Bam}HI and \textit{XhoI} sites. The 4HNR coding sequence was inserted into pFA156 as an Ncol-Scl fragment from pFA127 to form the expression plasmid pFA168. The 4HNR coding sequence in pFA168 (and pCB1346) was found to carry a sequence alteration, presumably introduced during polymerase chain reaction cloning, resulting in a substitution of Leu (CTC) for the natural Phe (TTC), found in the genomic clone, at position 135. This mutation was corrected by site-directed mutagenesis using the QuikChange kit (Stratagene). The entire coding sequence was verified by DNA sequencing.

\textit{E. coli} BL21(DE3) cells transformed with the corrected pFA168 were grown to mid-log phase, induced with 1 mM 1-(isopropylthio)-\( \beta \)-galactopyranoside, harvested, resuspended in 25 mM Hepes-NaOH, pH 7.5, and lysed by sonication. Protein purification steps were at 0 to 4 °C. After centrifugation, the cleared lysate was brought to 25% of saturation with respect to (NH\(_4\))\(_2\)SO\(_4\) and centrifuged. The supernatant was subjected to hydrophobic interaction chromatography on a phenyl-Sepharose fast flow column (Amersham Pharmacia Biotech) equilibrated with 25 mM Hepes-NaOH, pH 7.5, containing 25% of saturation (NH\(_4\))\(_2\)SO\(_4\). Fractions containing 4HNR were dialyzed exhaustively against 25 mM Hepes-NaOH, pH 7.5. The protein was subjected to anion exchange chromatography (Mono-Q, Amersham Pharmacia Biotech) with a linear gradient (0 to 0.5 M NaCl in 50 mM Tris-HCl, pH 7.5) to obtain homogeneous 4HNR. 4HNR subunit concentrations were estimated using a calculated \( \epsilon_{280} \) value of 23,830 M

\( \text{cm}^{-1} \).

**Fungal Strains, Mutant Isolation—**Wild-type strain 4091-I-5-8, the \textit{Rsy} mutant CP485, and the \textit{Buf} mutant CP61 were described previously (25). Preparation of the remaining strains will be described in detail elsewhere.\(^2\) Briefly, the \textit{buf} \textit{rsey} double mutants 4442-4-3 and 4442-4-6 represent both pairs of double recombinant progeny from a nonparental ditype tetrad isolated from a genetic cross between \textit{Buf}-mutant CP2833 and a \textit{Rsy} strain derived from CP485. The Buf-mutants CP2831 and CP2833 were constructed by gene disruption techniques to ensure the total absence of 3HNR enzyme activity for these studies. Specifically, the internal 3HNR gene sequence encoding amino acids 39–253 was replaced by the \textit{BAR} gene, which confers bialaphos resistance to these mutants. A gene replacement vector for the 4HNR gene was produced by inserting the \textit{HPH} gene, conferring hygromycin resistance, into an internal \textit{NotI} site in the 4HNR coding sequence, and this vector was used to disrupt the 4HNR locus by homologous recombination. This 4HNR disruption vector was transformed into strain 4091-I-5-8 to produce the \textit{hnr} strain CP3097 and into the \( buf \) \textit{rsey} double mutant 4442-4-3 to produce triple mutants CP3100, CP3101, and CP3102. The \textit{buf} \textit{hnr} double mutant was obtained from tetrad analysis of a cross between the \textit{hnr} gene disruption strain CP3097 and \textit{buf} strain CP61.

**Determination of Steady-state Kinetic Parameters (Single Substrates)—**Reactions (1 ml) with varying concentrations of DDBO, scytalone, or vermelone contained 1 mM NADP\(^+\) in 50 mM MES, 25 mM Tris,
Tetrahydroxynaphthalene Reductase

25 mM ethanolamine, and 100 mM NaCl at pH 7.0 and 25 °C. Initial rates were measured spectrophotometrically after initiating reactions with enzyme. The following net extinction coefficients combining the products minus reactants (all in units of mM cm⁻¹) were used: DDDB, Δε₅₄₅ = 5.4; scytalone, Δε₅₅₅ = 9.38; vermelone, Δε₅₁₃ = 14.1. Reactions (1 ml) with PQ contained 200 μM NADP in 100 mM MOPS-NaOH and 1% MeSO at pH 7.0 and 25 °C. Reactions were initiated with PQ. Initial rates were monitored spectrophotometrically using Δε₅₁₃ and Δε₅₄₅ for 4HN and 3HN, respectively. Inhibitor concentrations exceeded enzyme concentrations in the ternary complex (enzyme-NADPH-inhibitor), respectively. Inhibitor concentrations exceeded enzyme concentrations in the incubations by at least 4-fold.

**RESULTS**

**Cloning and Mutation Analysis of 4HNR in M. grisea—**The putative second reductase gene was identified as an M. grisea gene with weak homology to the 3HNR gene. Mutants were produced by gene disruption techniques for phenotypic analysis. Hnr⁻ mutants have similar pigmentation properties as the wild-type strains. However, hnr⁻ buff⁻ double mutants were found to develop a cherry red pigment in their growth medium, a pigmentation phenotype that resembles that of the Ray⁻ mutants and reflects the accumulation of oxidation products of 4HN. Whereas double mutants (buff⁻ rsy⁻) lacking SD and 3HNR functions accumulate significant quantities of scytalone in their growth media, triple mutants (buff⁻ rsy⁻ hnr⁻) do not (Table I). The triple mutants develop red pigmentation similar to the buff⁻ hnr⁻ double mutants. It is concluded that the second reductase gene encodes for a 4HNR capable of catalyzing the reduction of 4HN to scytalone and that the activity of this 4HNR fully accounts for the conversion of 4HN to scytalone in the buff⁻ and buff⁻ rsy⁻ mutants.

**Expression of M. grisea 4HNR in E. coli—**The 4HNR gene product was produced in E. coli and purified to homogeneity. The predicted molecular mass of the product of the coding sequence is 28,637.5 Da. The molecular mass of the purified protein determined by electrospray ionization mass spectrometry is 28,436 Da. The protein was found to have the N-terminal sequence Pro-Ser-Ala-Asp lacking the N-terminal Met and Ala residues of the primary translation product, presumably as a consequence of the processing activity of methionine aminopeptidase and aminopeptidase P in E. coli (35). The predicted molecular mass of the aminopeptidase-processing protein is 28,435.2, in good agreement with the mass spectrometry results. A sequence alignment of 4HNR with 3HNR proteins indicates that the two proteins are 46% identical (Fig. 3). Based on the x-ray structure of the 3HNR-NADP³-trycyclazole complex (5), there are nine amino acid side chains within a 4-Å sphere around the inhibitor, which occupies the naphthol bind-

**TABLE I**

| Strain | Genotype | Scytalone in media |
|--------|----------|--------------------|
| 4091–5-8 | Wild-type | 3 ± 7 |
| CP345 | rsy⁻ | 460 ± 3 |
| CP3831 | buff⁻ | 2 ± 1 |
| 4442–4-3 | buff⁻ rsy⁻ | 180 ± 6 |
| 4442–4-6 | buff⁻ rsy⁻ | 170 ± 4 |
| 4446–5-3 | buff⁻ hnr⁻ | 4 ± 4 |
| CP3100 | buff⁻ rsy⁻ hnr⁻ | 4 ± 3 |
| CP3101 | buff⁻ hnr⁻ | 2 ± 2 |
| CP3102 | buff⁻ rsy⁻ hnr⁻ | 1 ± 2 |

Concentrations, Kₜ(eq) (vermelone), 3HN determinations were in 1-ml reactions containing 106 μM vermelone and 29, 58, or 116 μM NADP⁺. Reactions were initiated with 3HN (80 μg in 2 μl) and monitored until the absorbance at 348 nm was constant. A net extinction coefficient for products minus reactants (Δε₅₁₃ = 12.2 mM cm⁻¹) was used in calculating the equilibrium concentrations. Kₜ(eq) (vermelone), 3HN are determined after 7 days of growth in liquid media (n = 3). Genotypes ray⁻ buff⁻ and hnr⁻ lack catalytic activities for SD, 3HNR, and 4HNR, respectively.
FIG. 3. Comparison of the amino acid sequences of 3HNR (top) and 4HNR (bottom). The symbols between aligned amino acids indicate identity (1) and progressively smaller degrees of similarity between the side chains (colon, periods, or no symbol) as defined in Henikoff and Henikoff (42).

Substrate Specificities of 3HNR and 4HNR—Steady-state kinetic parameters for substrates scytalone and vermelone were determined for 4HNR and 3HNR under the same conditions at pH 7.0 and 25 °C (Table II). The individual determinations of substrate specificities for vermelone (Ke(cat)/Km (vermelone)) and scytalone (Ke(cat)/Km (scytalone)) suggest that the discrimination between the two enzymes is small and slightly favors the wrong selectivity according to our designations of the two enzymes based on the phenotypes of the M. grisea mutants. However, it is known that there are strong contributions from substrate inhibition in the determined values despite our efforts to diminish such effects. 3 and on this basis we must discount the specificity ratios. In the effort to determine true values for the relative substrate specificities, we employed a direct method for measuring substrate competition (34).

In this procedure the dehydrogenation rates for vermelone and scytalone are determined simultaneously in a single incubation for the individual enzymes, 3HNR and 4HNR. From the values reported in Table II using this method, 3HNR prefers vermelone over scytalone by a factor of 39; 4HNR has the opposite selectivity for scytalone over vermelone by a factor of 3.0. Equilibrium constants were determined spectrophotometrically at pH 7.0 and 25 °C: Kd(scytalone) = [scytalone][NADP+] /[4HNR][NADPH][H+] = 3.0 ± 0.5 × 107 and Kd(vermelone) = [vermelone][NADP+]/[3HNR][NADPH][H+] = 0.32 ± 0.04 × 107. They were used to calculate (Equation 6) the relative preferences for 3HNR and 4HNR; 3HNR prefers 3HN over 4HN by a factor of 3.0, and 4HNR prefers 4HN over 3HN by a factor of 39. Note that DDBO (Fig. 1), which more closely resembles scytalone than vermelone, is a much better substrate for 3HNR than 4HNR, whereas PQ (Fig. 1), which does not closely resemble either vermelone or scytalone, is accepted by 3HNR and 4HNR with similar substrate specificities.

Inhibitor Specificities of 3HNR and 4HNR—Inhibition constants were determined in the PQ reactions because NADPH

3 J. Thompson and D. Jordan, unpublished results.

| Kinetic parameter       | 4HNR      | 3HNR      |
|-------------------------|-----------|-----------|
| kcat (vermelone) (s⁻¹)  | 0.95 ± 0.05 | 2.0 ± 0.3  |
| Kd/Ke (vermelone) (M⁻¹) | 7.9 ± 0.7 × 10³ | 3.8 ± 0.4 × 10⁴ |
| Kd(scytalone) (s⁻¹)     | 0.26 ± 0.02 | 0.38 ± 0.02 |
| Kd(3HN) (s⁻¹)           | 1.1 ± 0.2 × 10⁴ | 6.3 ± 1.0 × 10⁸ |
| Kd(scytalone) (µM)      | 22 ± 5     | 6.0 ± 10²  |
| Km(3HN) (µM)            | 0.71 ± 0.1 | 0.60 ± 0.22 |
| Km(scytalone) (µM)      | 0.030 ± 0.004 | 39 ± 4     |
| Km(3HN) a               | 0.0032 ± 0.0004 | 4.2 ± 0.4  |
| Km(4HN) a               | 0.32 ± 0.02 | 14 ± 2²    |
| Kd(3HN) (DDBO) (s⁻¹)   | 9.1 ± 1.8 × 10³ | 2.9 ± 0.5 × 10⁵ |
| Kd(DDBO) (s⁻¹)          | 36 ± 7     | 5 ± 3⁸     |
| Kd(PQ) (s⁻¹)            | 12 ± 0.4   | 19 ± 0.9   |
| Kd(PQ) (s⁻¹)            | 6.3 ± 0.2 × 10⁶ | 4.1 ± 0.8 × 10⁶ |
| Kd(PQ) (µM)             | 5.4 ± 0.5  | 4.6 ± 0.7  |

Values obtained from competition experiments using vermelone and scytalone and transformed to reflect 3HN/4HN specificities using Equation 6.

Discussion

Substrate Preferences of 3HNR and 4HNR—The dilemma of an alternate route for the production of scytalone, as described in this study, originated from genetic studies and was addressed using a combination of molecular biology and biochemistry methods. Without doubt, 4HN is the precursor to scytalone. Also without doubt, there are two and only two naphthol reductases within the fungal melanin biosynthetic pathway. These conclusions are based on the results described in Table I where the triple mutants (lacking catalytic activities for 3HNR, 4HN, and SD) do not accumulate scytalone. Even though their active-site residues are similar, the two reductases catalyze the same naphthol reduction reactions with considerably different substrate specificities.

We chose to examine the reverse reactions of the reductases using scytalone and vermelone because these substrates are significantly more stable than their oxidized (naphthol) counterparts. Neither 5HN or 4HN behave nicely in their steady-state reactions with vermelone or scytalone because of severe product-substrate inhibition. Rate limitations for the 3HNR-catalyzed oxidation of scytalone have been attributed to product release (2). We have found that 3HN forms a tight dead end complex with 3HN-NADPH (5K = 60 nM), and this accounts for part of the problem in determining rate constants. It is interesting to speculate that 3HN, which has a pKₐ of 5 and exists as an anion at pH 7, has an attraction for the positively charged pyridine ring of NADPH as has been found with an anionic inhibitor of enoyl (acyl carrier protein) reductase complexed with NAD⁺ (36). As a result of the problems associated with determining steady-state kinetic parameters for scytalone and vermelone oxidation, the substrate specificities calculated from individually determined kcat/Km values suggest inaccurately that 3HNR prefers scytalone over vermelone and that 4HNR prefers scytalone over vermelone to a smaller extent than 3HNR (Table II). These results are not consistent with the genetics and highlight the difficulty of determining substrate specificities for 3HNR and 4HNR in this manner.
Viviani et al. (26, 27) and Vidal-Cros et al. (28) report cloning and characterization of a "tetrahydroxynaphthalene reductase" that varies from the 3HNR we have studied by three amino acids that lie outside the active site. We believe this difference owes to the two M. grisea strains used as the enzyme sources. Their gene was cloned by using the amino acid sequence of the protein purified from wild-type cultures of M. grisea (28). Our 3HNR gene was cloned by complementation of a Buf− mutant of M. grisea (30), so in vivo the gene product is the catalyst responsible for the conversion of 3HN to vermelone. The 99% sequence identity of their protein to ours makes it another 3HNR. Their description of the enzyme as a 4HNR was based on its substrate specificities for the naphthols, 3HN and 4HN. It is likely that their determination of kinetic parameters for the individual naphthol reductions also suffered from systematic errors owing to substrate and product inhibition.

In view of the problems involved in accurately determining $k_{cat}/K_m$ values for the substrates of 3HNR and 4HNR, it was necessary to employ a direct competition method (34) to determine the true preferences of 3HNR and 4HNR for vermelone. With this method, problems associated with nonproductive substrate binding and product inhibition are equalized because the two substrates are contained in the same incubation with the enzyme (37). The determinations from this method conclusively indicate that 3HNR prefers vermelone over scytalone by a factor of 39 and that 4HNR prefers scytalone over vermelone by a factor of 33 (Table II). Using Equation 6 with the equilibrium constants reported here shows that 3HNR prefers 3HN over 4HN by a factor of 1300. Computer modeling of 3HN and 4HN into the active site of 3HNR suggests that there is steric and electrostatic repulsion between the sulfur atom of Met283 (C-terminal residue) and the C6 hydroxyl group of 4HN but not with 3HN, which has a hydrogen at C6, thus accounting for the preference of 3HNR for 3HN.4 A homology model of 4HNR based on the x-ray structure of 3HNR does not show repulsion between the sulfur atom and the C6 hydroxyl group of 4HN because the C terminus of 4HNR is one residue shorter than that of 3HNR (Fig. 3); there is no methionine residue in the way, consistent with the better acceptance of 4HN by 3HNR than by 3HNR. The involvement of C-terminal residues in forming enzyme active sites has been documented in other systems (38–40). Why 4HNR prefers 4HN over 3HN by a factor of 310 remains a mystery. High quality crystals of 4HNR have been obtained,3 and perhaps a structural analysis of the protein will provide insights regarding its preference for 4HN. The enzyme nomenclature describes 3HNR as tetrahydroxynaphthalene reductase with a designation of EC 1.1.1.252. The results of this work require that our 3HNR be described as trihydroxynaphthalene reductase. Additionally, there should be a new entry for 4HNR as tetrahydroxynaphthalene reductase.6

**Table III**

| Inhibitor    | 4HNR | 3HNR |
|--------------|------|------|
| Tricyclazole | 480 ± 80 | 2.4 ± 0.4 |
| Pyroquilon   | 420 ± 20 | 14 ± 4 |
| Phthalide    | 51 ± 5  | 2.0 ± 0.4 |

Inhibitors and the Biology of Fungal Melanin—The fungicides (tricyclazole, pyroquilon, and phthalide) target the function of 3HNR in the prevention of disease. When wild-type M. grisea is incubated with the inhibitors, the fungus accumulates 3HN and its oxidized byproducts. 4HNR has much lower affinity for the inhibitors than 3HNR (by factors of 200, 30, and 25 for tricyclazole, pyroquilon, and phthalide, respectively; Table III), and this accounts for the accumulation of 3HN in the fungus instead of 4HN. At higher tricyclazole concentrations, the cherry red pigment characteristic of the hnr− buf− double mutant can appear in the wild-type,7 consistent with inhibition of 4HNR activity at elevated levels of the fungicide.

Whereas the 3HNR gene from M. grisea is required for pathogenicity of the rice blast fungus (25), the 4HNR gene is not.2 Certainly the activity of 4HNR is not absolutely required in cultures of M. grisea for the formation of fungal melanin as the hnr− mutant lacking the 4HNR gene has a wild-type phenotype. The catalytic activity of 3HNR must be responsible for reducing 4HN to scytalone in the mutants devoid of the 4HNR gene even though 3HNR has lower substrate specificity for 4HN in comparison with 3HN. In this work, we conclude that the catalytic activity of 4HNR is responsible for the production of scytalone in the double mutant buf− rsy− (lacking 3HNR and SD activities). Approximately half of the scytalone concentration is accumulated in the buf− rsy− double mutant in comparison to that of the rsy− single mutant (Table I), and this suggests that 4HNR has significant impact on flux through the melanin biosynthetic pathway.

Considered together, the genetic and biochemical data show that 4HNR might facilitate M. grisea infection, but that it is not required for it. Perhaps the activity of 4HNR in M. grisea is needed for the infection process under conditions that have not been examined. Otherwise, one might speculate that 4HNR and its gene are a conundrum of nature targeting geneticists and enzymologists. On the other hand, one might reflect that 4HNR is a potential element for providing resistance to commercial fungicides that target 3HNR. However, even though 4HNR has much lower affinities than 3HNR for the inhibitors (Table III), its relative specificity for 4HN over 3HN is 310, and its activity is not sufficient to support the reduction of 3HN to vermelone. If it were, the buf− mutant would have a wild-type phenotype. To date there are no reports of resistance to the 3HNR-targeted fungicides.

There are notable comparisons between the inhibitory complex formed by tricyclazole with 3HNR and the inhibitory complex formed by the antibacterial agent triclosan with its short chain dehydrogenase target enoyl (acyl carrier protein) reductase. The NAD+ form of the latter enzyme binds triclosan more strongly than the NADH or free enzyme forms (36). Ward et al. (36) point out that this is an advantage for the performance of the inhibitor in vivo because the NAD+ form of the enzyme is highly populated due to the 40-fold greater concentration of NAD+ over NADH in cells (36). 3HNR has the opposite disposition in that tricyclazole, and the other commercial inhibitors bind with the affinity progression 3HNR-NADPH > 3HNR-NADP+ > 3HNR (2). The inhibitors of 3HNR also select a highly populated form of the enzyme as the concentration of NADPH is higher in cells than that of NADP+ (41). On the basis of the equilibrium constants determined for this work, it is also clear that effective fungicides inhibit the thermodynamically more difficult of the two reduction reactions in the melanin biosynthetic pathway.

**Acknowledgments**—The technical assistance of Michael Picolletti in chemistry and Molecular Biology has been informed regarding the enzymes and their substrate specificities as reported here. 8 B. Valent, unpublished observation.
determining scytalone concentrations and Rand Schwartz in determining 3HNR inhibition constants are greatly appreciated.

REFERENCES

1. Tokoumbalides, M. C., and Sisler, H. D. (1978) *Pestic. Biochem. Physiol.* 8, 26–32
2. Thompson, J. E., Basarab, G. S., Andersson, A., Lindqvist, Y., and Jordan, D. B. (1997) *Biochemistry* 36, 1852–1860
3. Welschuk, C. P., Wolkow, P. M., and Sisler, H. D. (1981) *Pestic. Sci.* 12, 86–90
4. Ishida, M., and Nambu, K. (1975) *Noyaku Kagaku* 3, 10–26
5. Andersson, A., Jordan, D., Schneider, G., and Lindqvist, Y. (1996) *Structure (Lond.)* 4, 1161–1170
6. Liao, D.-I., Basarab, G. S., Gatenby, A. A., and Jordan, D. B. (2000) *Bioorg. Med. Chem. Lett.* 10, 491–494
7. Bell, A. A., and Wheeler, M. H. (1986) *Annu. Rev. Phytopathol.* 24, 411–451
8. Howard, R. J., and Valient, B. (1996) *Annu. Rev. Microbiol.* 50, 491–512
9. Joernvall, H., Persson, B, Krook, M., Atrian, S., Gonzalez-Duarte, R., Jeffrey, J., Bastmeyer, M. (1999) *Biochemistry* 38, 9931–9939
10. Wawrzak, Z., Sandalova, T., Steffens, J. J., Basarab, G. S., Lundqvist, T., and Schwartz, R. S. (1999) *Biochemistry* 38, 6003–6013
11. Joernvall, H., Hoog, J.-O., and Persson, B. (1999) *FEBS Lett.* 445, 261–264
12. Bechinger, C., Giebel, K.-F., Schnell, M., Deising, H. B., and Bastmeyer, M. (1999) *Science* 285, 1896–1899
13. Basarab, G. S., Steffens, J. J., Wawrzak, Z., Schwartz, R. S., Lindqvist, T., and Jordan, D. B. (1999) *Biochemistry* 38, 6012–6024
14. Jordan, D. B., Zheng, Y.-J., Locket, B. A., and Basarab, G. S. (2000) *Biochemistry* 39, 2276–2282
15. Jordan, D. B., Basarab, G. S., Steffens, J. J., Schwartz, R. S., and Doughty, J. G. (2000) *Biochemistry* 39, 6593–6602
16. Kurahashi, Y., Sakawa, S., Kimbara, T., Tanaka, K., and Kagabu, S. (1997) *Nippon Noyaku Gakkaishi* 22, 108–112
17. Tsuji, G., Takeda, T., Furusawa, I., Horino, O., and Kubo, Y. (1997) *Pestic. Biochem. Physiol.* 57, 211–219
18. Nakasako, M., Motoyama, T., Kurahashi, Y., and Yamaguchi, I. (1998) *Biochemistry* 37, 9931–9939
19. Wawrzak, Z., Sandalova, T., Steffens, J. J., Basarab, G. S., Lindqvist, T., Schwartz, R. S., and Jordan, D. B. (1999) *Proteins Struct. Funct. Genet.* 35, 425–439
20. Chen, J. M., Xu, S. L., Wawrzak, Z., Basarab, G. S., and Jordan, D. B. (1998) *Biochemistry* 37, 17735–17744
21. Jordan, D. B., Lessen, T., Wawrzak, Z., Bisaha, J. J., Gehret, T. C., Hansen, S. L., Schwartz, R. S., and Basarab, G. S. (1999) *Bioorg. Med. Chem. Lett.* 9, 1697–1612
22. Basarab, G. S., Jordan, D. B., Gehret, T. C., Schwartz, R. S., and Wawrzak, Z. (1999) *Bioorg. Med. Chem. Lett.* 9, 1613–1618
23. Jennings, I. D., Wawrzak, Z., Amoree, D., Schwartz, R. S., and Jordan, D. B. (1999) *Bioorg. Med. Chem. Lett.* 9, 2509–2514
24. Andersson, A., Jordan, D., Schneider, G., and Lindqvist, Y. (1997) *FEBS Lett.* 400, 173–176
25. Chumley, F. G., and Valent, B. (1990) *Mol. Plant-Microbe Interact.* 3, 135–143
26. Viviani, F., Gaudry, M., and Marquet, A. (1992) *N. J. Chem.* 16, 81–87
27. Viviani, F., Vors, J. P., Gaudry, M., and Marquet, A. (1993) *Bull. Soc. Chem. Fr.* 136, 395–404
28. Vidal-Cros, A., Viviani, F., Labesse, G., Boccara, M., and Gaudry, M. (1994) *Eur. J. Biochem.* 219, 985–992
29. Thompson, J. E., Basarab, G. S., Pierce, J., Hodge, C. N., and Jordan, D. B. (1998) *Anal. Biochem.* 256, 1–6
30. Andersson, A., Jordan, D., Schneider, G., Valent, B., and Lindqvist, Y. (1996) *Proteins Struct. Funct. Genet.* 24, 525–527
31. Sweigard, J. A., Carrell, A. M., Farrall, L., Chumley, F. G., and Valent, B. (1990) *Mol. Plant-Microbe Interact.* 11, 404–412
32. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
33. Schoner, B. E., Belagaje, R. M., and Schoner, R. G. (1990) *Methods Enzymol.* 185, 94–103
34. Thompson, J. E., and Jordan, D. B. (1998) *Anal. Biochem.* 256, 7–13
35. Yasue, H., Kikuchi, Y., Kojima, H., and Nagase, K. (1991) *Appl. Microbiol. Biotechnol.* 36, 211–215
36. Ward, W. H. J., Holdgate, G. A., ROWESEL, S., McLEAN, E. G., Clawson, A., Clayton, E., Nichols, W. W., COLLs, J. G., Minshull, C. A., JUDE, D. A., MISTRY, A., TIMMS, D., CAMBLE, R., HALEs, N. J., BRITTON, C. J., and TAYLOR, I. W. F. (1999) *Biochemistry* 38, 12514–12525
37. Fersht, A. (1994) *Enzyme Structure and Mechanism*, pp. 109–112, W. H. Freeman and Co.
38. Minor, W., STECZKO, J., BOLIN, J. T., OTWINOWSKI, Z., and Axelrod, B. (1993) *Biochemistry* 32, 6320–6323
39. Perry, K. M., Carreras, C. W., Chang, L. C., Santi, D. V., and Stroud, R. M. (1993) *Biochemistry* 32, 7116–7125
40. Guinard, G., Podobnik, M., Pungerec, J., Strukelj, B., Turk, V., and Turk, D. (1998) *Structure (Lond.)* 6, 51–61
41. Voet, D., and Voet, J. G. (1995) *Biochemistry*, 2nd Ed., p. 617, John Wiley & Sons, Inc., New York
42. Henikoff, S., and Henikoff, J. G. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 10915–10919
The Second Naphthol Reductase of Fungal Melanin Biosynthesis in *Magnaporthe grisea*: TETRAHYDROXYNAPHTHALENE REDUCTASE

James E. Thompson, Stephen Fahnestock, Leonard Farrall, Der-Ing Liao, Barbara Valent and Douglas B. Jordan

*J. Biol. Chem.* 2000, 275:34867-34872.
doi: 10.1074/jbc.M006659200 originally published online August 23, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M006659200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 39 references, 2 of which can be accessed free at
http://www.jbc.org/content/275/45/34867.full.html#ref-list-1