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Farnesol oxidation in insects: evidence that the biosynthesis of insect juvenile hormone is mediated by a specific alcohol oxidase

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Abstract

The oxidation of farnesol to farnesoic acid is a key step in insect juvenile hormone biosynthesis. We herein present preliminary characterization of the enzyme-catalyzed oxidation of farnesol to farnesal in larval corpora allata homogenates of the tobacco hornworm, *Manduca sexta*. This conversion, which is highly substrate specific, has a K_m apparent of 1 μM and a pH optimum between 6 and 7. Results from chemical modification experiments indicate that the enzyme possesses an active site tyrosine residue. Although farnesol oxidation in adult *M. sexta* corpora allata homogenates was previously identified as being catalyzed by a dehydrogenase, the corresponding conversion in larvae is not effected by the addition of nicotinamide cofactors. Instead, enzymatic activity is slightly enhanced by the addition of FAD, decreases when incubations are performed anaerobically, and is completely inhibited when either sodium dithionite or glucose oxidase is added. Although the effect of various additives suggests that the oxidation of farnesol to farnesal does not require a metal redox center, 1,10-phenanthroline (but not 4,7-phenanthroline) is a weak irreversible inhibitor of farnesol oxidation (IC_{50}=11 mM). The addition of exogenous metals (Fe^{2+}, Cu^{2+}, Ni^{2+}, and Co^{2+}) caused differential effects on farnesol metabolism, with Cu^{2+} being highly inhibitory. Taken together, this data suggests that the oxidation of farnesol to farnesal in larval corpora allata is mediated by a specific oxygen-dependent enzyme, perhaps a flavin and/or iron-dependent oxidase.

Keywords: Farnesol; Farnesal; Alcohol oxidase; Juvenile hormone

1. Introduction

Juvenile hormone (JH), a family of sesquiterpenoids whose structure is unique to invertebrates and some plants, is important for normal growth and development in insects (Kumaran, 1990). The most common juvenile hormone, JH III, is biosynthesized in the small, paired endocrine gland, the corpus allatum, from farnesyl pyrophosphate (FPP) (Schooley and Baker, 1985), a key intermediate of the mevalonate pathway (Porter and Spurgeon, 1981). The construction of JH in insects requires the oxidation of terpenol (formed upon hydrolysis of FPP or its homologs) to aldehyde and carboxylic acid. While this conversion was originally thought to be of limited importance to other metabolic pathways, studies related to the regulation of cholesterol biosynthesis have demonstrated that both farnesol and its oxidized metabolites play a critical role in regulating flux within the mevalonate pathway (Bostedor et al., 1997; Westfall et al., 1997; Correll et al., 1994). In addition, the oxidation of farnesol and its monoterpene analog, geraniol, is a required event for the construction of many plant natural products (Singh Sangwan et al., 1993; Ikeda et al., 1991; Banthorpe et al., 1976; Overton and Roberts, 1974). In vertebrates, plants, and fungi, the oxidation of farnesol to farnesal is typically mediated by nicotinamide-dependent dehydrogenases (Keung, 1991; Inoue et al., 1984; Chayet et al., 1973).

The first study of farnesol metabolism, as it relates to JH biosynthesis in insects, was performed over 15 years ago (Baker et al., 1983). Using corpora allata homogenates of the adult female sphinx moth, *Manduca sexta*, it was demonstrated that farnesol was sequentially converted to farnesal and farnesoic acid, and that acid formation was significantly enhanced by the addition of 2

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mM NAD. Although these results suggested that farnesol metabolism was mediated by a nicotinamide-dependent dehydrogenase, attempts to more clearly identify NAD involvement met with difficulties. Incubation of 1,5,9-[^{3}H]farnesol, followed by radio-HPLC analysis of the polar metabolites, indicated that neither [^{3}H]NADH nor [^{3}H]NADPH was present in corpora allata homogenates. However, the inability to detect radiolabeled cofactor did not disprove the formation of NAD(H), since other secondary redox processes may have occurred during incubation with the insect gland homogenate.

In this paper, we describe the partial characterization of farnesol metabolism in larval corpora cardiaca–corpora allata (CC–CA) homogenates of *M. sexta*. Despite the fact that farnesol oxidation in plants, vertebrates, and non-JH producing insect tissue is typically catalyzed by nicotinamide dependent dehydrogenases, the formation of farnesal in insect corpora allata is an oxygen-dependent process. Additive studies indicate that activity may require a metal cofactor and FAD, suggesting that JH biosynthesis involves the intermediacy of one or more alcohol oxidases.

2. Materials and methods

2.1. Chemical sources

Tween-80, nucleotide cofactors, yeast alcohol dehydrogenase (YADH), glucose oxidase, catalase, and geranylgeraniol were obtained from Sigma Chemical Co. (St. Louis, MO). All other materials were purchased from either Aldrich Chemical Co. (Milwaukee, WI) or Fisher Scientific (Pittsburgh, PA), except 1,2-[^{14}C]farnesol, which was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO) and was purified by silica gel column chromatography using a 5–10% ethyl acetate in hexane gradient.

2.2. Insects and tissue source

Manduca sexta larvae were reared on an artificial diet and maintained under constant photoperiod (18L:6D), using previously described procedures (Bell and Joachim, 1976). Animals were staged prior to molting at the 4th larval stadium by observing head capsule slippage and were made synchronous by starvation during the last larval molt. CC–CA complexes from newly emerged (0–12 h old), 5th stadium larvae (V/0), a developmental stage that is known to produce significant quantities of JH (Baker et al., 1987), were removed following previously established microdissection procedures (Bhaskaran and Jones, 1980). For all metal studies, corpora allata dissections were performed in the absence of *M. sexta* saline (4 mM NaCl, 40 mM KCl, 18 mM $MgCl_2$, and 3 mM $CaCl_2$).

2.3. Enzyme assay

The conversion of farnesol to farnesal within the corpus allatum was monitored as previously described (Sen and Garvin, 1995a). Briefly, CC–CA complexes were removed from V/0 *M. sexta* larvae, homogenized for 3 min on ice, in 100 mM Tris–HCl buffer, pH 7, and the resulting homogenate was centrifuged at 3,000*g* for 1 min. The supernatant was transferred to a microcentrifuge tube, the volume adjusted (typically to 0.25 gland pair equiv/25 μl), and Tween-80 was added to give a final concentration of 0.05% (w/v). After standing on ice for 5 min, the solution was centrifuged at 16,000*g* for 5 min and aliquots of supernatant (25–50 μl) were placed into 500 μl siliconized plastic microcentrifuge tubes (Fisher Scientific).

The conversion of farnesol to farnesal was assayed by adding radioactively labeled substrate (1,2-[^{14}C]farnesol, specific activity 27.5 mCi/mmol) to the prepared enzyme solution to give a final concentration of 10 μM. The solution was incubated at 26°C for 1–2 h, then quenched by the addition of acetonitrile containing farnesol, farnesal, and farnesoic acid standards. The reaction mixture was extracted with CH_2Cl_2 and the concentrated organic extract was redissolved in a minimum amount of CH_2Cl_2 and applied to a plastic-backed normal phase TLC plate (40×80 mm, Macherey-Nagel Polygram® Sil G/UV254, Bodman Industries, Aston, PA). Double elution with 10% ethyl acetate in hexane containing 5% triethylamine gave clean separation of starting material and product (R_f farnesol=0.5, R_f farnesal=0.85). The extent of farnesol oxidation (expressed as % conversion) was determined by cutting each TLC plate into five sections, then quantifying the amount of farnesol and farnesal present by liquid scintillation counting (Beckman LS 5801, using ScintiVerse BD, Fisher Scientific).

2.4. Additive studies

Substrate specificity, co-factor requirement, and inhibitor studies were determined by observing the effect (i.e., enhancement, no effect, or inhibition, as compared to appropriate controls) of various additives on farnesol metabolism. Enzyme (prepared as described in Section 2.3) was first preincubated with several concentrations of each additive (15–30 min for most experiments, except metal studies, which were preincubated 1–2 h), then assayed for activity. For all inhibitors, further studies were performed to determine the concentration of additive that yielded a 50% loss of enzyme activity.

2.5. K_m apparent determination

The K_m apparent for farnesol oxidation in *M. sexta* larval CC–CA homogenate was obtained by double reciprocal Lineweaver–Burk plot of the amount of farne-

sal formed at increasing concentrations of radioactively labeled farnesol (1–10 μM, using 10-[^{3}H]farnesol, 30 mCi/mmol) (Sen and Garvin, 1995b). The incubation time was adjusted to 30 min to provide conversions of no more than 15% at 10 μM substrate.

2.6. Enzyme localization

CC–CA homogenate was prepared in the absence of detergent (10 gland pair equiv/ml), and sequentially centrifuged at 3,000*g* (10 min), 16,000*g* (10 min), and 100,000*g* (1 h). The resulting 100,000*g* supernatant, and resupended 16,000*g* and 100,000*g* pellets were each diluted in buffer (0.5 gland pair equiv/50 μl) and Tween-80 was added to 0.05% (w/v). Aliquots (25 μl) were assayed for enzyme activity, as described in Section 2.3, above.

2.7. pH studies

A 5× enzyme solution was first prepared (Section 2.3), in 25 mM Tris–HCl, pH 7. Aliquots (10 μl) were diluted with 40 μl of appropriate 100 mM buffer (citrate–phosphate for pH 3–7 and Tris–HCl for pH 7–10) and assayed for enzymatic activity.

2.8. Filtration studies

Endogenous (non-covalently bound) cofactors were removed by sequential dilution and filtration of the homogenate or by gel filtration. Enzyme was prepared as usual (Section 2.3), in 75 μl of 100 mM Tris–HCl, pH 7 buffer. After removal of cellular debris, the solution was either subjected to gel filtration (Sephadex G-50, Sigma Chemical Co., see Section 2.9) or concentration through a 30,000 MW cutoff Microcon filter (Millipore, Bedford, MA). For the latter, the homogenate was diluted to 400 μl and concentrated to approximately 50 μl, a total of four times.

2.9. Metal studies

Stock solutions of several metal cations were prepared by dissolving the corresponding chloride salts (i.e., $FeCl_2$, $NiCl_2$, $CuCl_2$, and $CoCl_2$) in pH 2–3 H_2O. CC–CA homogenate was prepared as usual, using 100 mM MOPS, pH 7, in place of Tris–HCl. Enzyme aliquots (25 μl) were preincubated for 1 h in the presence of varying concentrations of metal (0.05–5 mM final concentration), then assayed for enzymatic activity.

For experiments involving the addition of metals to phenanthroline-treated enzyme, homogenate (250 μl, 10 CC–CA pair equiv) was first treated for 2 h with 1,10-phenantholine (20 mM), then passed through a 1×10 cm G-50 Sephadex column, using 100 mM MOPS as eluent. Fractions containing enzyme activity were pooled, and Tween-80 and dithiothreitol were added to give final concentrations of 0.05% (w/v) and 0–500 μM, respectively. Aliquots (50 μl) were preincubated with metal and subsequently assayed for enzymatic activity.

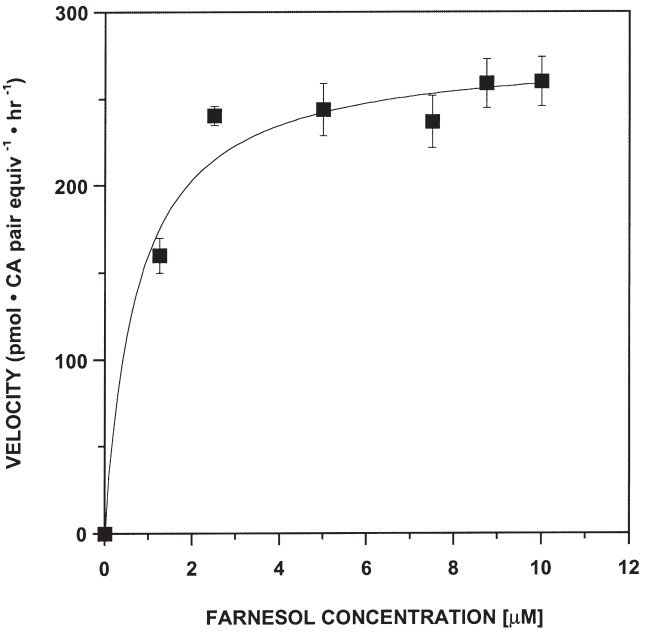

Fig. 1. Observed kinetics for the oxidation of farnesol to farnesal in larval *M. sexta* CC–CA homogenates (*n*=4 experiments performed in duplicate, data reported are from one experiment).

3. Results

3.1. General characteristics of farnesol oxidase in CC–CA homogenates

Farnesol oxidation displays conventional Michaelis–Menten kinetics (Fig. 1), yielding with a K_m apparent of 1 μM (R^2=0.995). Enzyme activity is localized in the 100,000*g* supernatant (Table 1) and is high over a broad pH range (Fig. 2), with optimum activity between 6 and 7.

3.2. Structure–activity relationship studies

A series of aliphatic and aromatic alcohols were tested for their ability to inhibit farnesol oxidation in CC–CA

Table 1
Enzyme location of farnesol dehydrogenase in larval *M. sexta* CC–CA homogenates (*n*=3 experiments in duplicate, data presented are from one experiment)

Cellular fraction	% Farnesal formation
16,000*g* pellet	1.9±0.2
100,000*g* pellet	1.2±0.1
100,000*g* supernatant	19.9±1.3

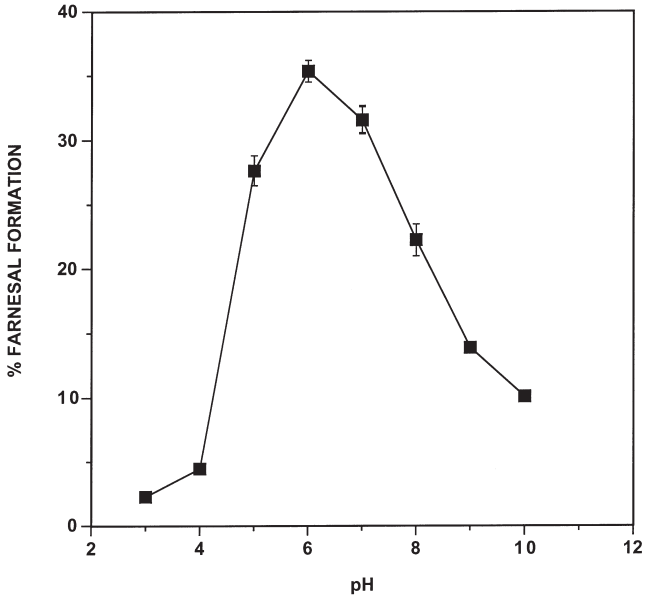

Fig. 2. Effect of pH on the oxidation of farnesol to farnesal in larval *M. sexta* CC–CA homogenates. Data was obtained using 100 mM citrate–phosphate (pH 3–7) and Tris–HCl (pH 7–10), as described in Section 2.7 (n=2 experiments in duplicate, data reported are from one experiment).

homogenates, including several terpenols. The results of these experiments are presented in Table 2. Only geraniol and geranylgeraniol were inhibitors of farnesol metabolism (IC_{50}=55 and 21 μM, respectively).

3.3. Co-factor studies

The effect of redox co-factors on farnesol metabolism in larval CC–CA homogenates was examined (Table 3). Neither the oxidized nor reduced forms of NAD(P), at concentrations up to 25 mM, had any effect on farnesal formation. Removal of endogenous nicotinamide co-factors, by filtration of the homogenate or by the addition of NADase and YADH, also had no effect on farnesol metabolism. While FMN caused no change in enzymatic activity, the presence of 5 mM FAD provided a modest increase in farnesal formation.

Table 2
Substrate specificity of farnesol oxidation in larval *M. sexta* CC–CA (n=2 experiments in duplicate)

Alcohol[a]	IC_{50} (μM)
Geraniol	55±3
Geranylgeraniol	21±4
Dimethylallyl alcohol	No inhibition
Ethanol	No inhibition
Octanol	No inhibition
Dodecanol	>500

[a] Tested at 0, 10, 50, 100, and 500 μM alcohol, added as a stock solution in DMSO.

Table 3
Cofactor requirement of farnesol dehydrogenase (n=3 experiments in duplicate)

Additive[a]	Concentration	% Activity remaining
NAD(P)[b]	25 mM	100
NAD(P)H	5 mM	97
NADase	0.02 U	100
YADH[c]	17 U	100
FAD	5 mM	126
FMN	5 mM	103

[a] Added as stock solutions in 100 mM Tris–HCl (pH 7), except NADase, which was prepared in 100 mM phosphate, pH 6.

[b] CC–CA homogenate was diluted and concentrated (4×) using a 30,000 MW cutoff filter prior to the addition of NAD.

[c] Incubation was performed in the presence of 5 mM ethanol as YADH substrate.

3.4. Inhibitor studies

Several additives were tested as potential inhibitors of farnesol metabolism (Table 4). Neither pyrazole, carbon monoxide, nor hydrazine inhibited the oxidation to farnesal in larval CC–CA homogenates. Likewise, the addition of ethyl carbazate had no effect on farnesol metabolism at up to 10 mM, and benzylhydrazine was only a modest inhibitor (IC_{50}=3.1 mM). Several amino acid tritrants were tested for inhibitory potency, including *N*-ethylmaleimide (NEM), iodoacetamide, diethyl pyrocarbonate, and tetranitromethane. NEM was inactive and both iodoacetamide and diethyl pyrocarbonate were only weakly inhibitory (IC_{50}=40 and 36 mM, respectively). In contrast, tetranitromethane was a potent inactivator of farnesol oxidation (IC_{50}=5 μM).

3.5. Oxygen dependency studies

We investigated the possible involvement of oxygen in the oxidative process (Table 5). Simple deoxygenation of all buffers and stock solutions used in the preparation of the CC–CA homogenates (by N_2 sparging) caused a drop in enzyme activity and the addition of sodium dithionite and of glucose oxidase resulted in significant inhibition of farnesol metabolism. At 15 mM sodium dithionite, the conversion of farnesol to farnesal was nearly completely inhibited.

3.6. Metal studies

CC–CA homogenates were incubated with a series of metal chelators, including EDTA, potassium tartrate, NaCN, 8-hydroxyquinoline, and 1,10-phenanthroline (Table 5). None of these compounds inhibited farnesol metabolism, with the exception of NaCN and 8-hydroxyquinoline, which were marginally inhibitory, and 1,10-phenanthroline, which exhibited dose-dependent,

Table 4
Effect of various additives on farnesol oxidation in larval *M. sexta* CC–CA (*n*=2 experiments in duplicate)

Additive[a]	Concentration	% Activity remaining	IC_{50} (mM)
Pyrazole	100 mM	100	–
CO	Saturated solution[b]	100[c]	–
Ethyl carbazate	10 mM	104	–
Benzylhydrazine	–	–	3.1±0.8
NH_2NH_2	500 μM	94	–
N-ethylmaleimide	50 mM	100	–
Iodoacetamide	–	–	40±10
Iodoacetamide[d]	50 mM	54	–
Tetranitromethane	–	–	0.005±0.001
Tetranitromethane[e]	500 μM	97	–
Diethyl pyrocarbonate	–	–	36±6

[a] Added as the following stock solutions: iodoacetamide, pyrazole, and NEM in 100 mM Tris–HCl, pH 7; ethyl carbazate and tetranitromethane in DMSO; benzylhydrazine and diethyl pyrocarbonate in ethanol.
[b] Prepared by diluting 10× homogenate with 200 mM phosphate buffer (pH 7.4), previously saturated with CO.
[c] As compared to control, which was prepared by diluting homogenate with N_2-saturated buffer.
[d] Additive was added after preincubation with 20 μM farnesol.
[e] Additive was added after preincubation with 400 μM farnesol.

Table 5
O_2 and metal dependence of farnesol dehydrogenase (*n*=2 experiments in duplicate)

Additive or procedure[a]	Concentration	% Activity remaining	IC_{50} (mM)
Degassing of buffers	–	41	–
$Na_2S_2O_4$	4 mM	66	5±1
Glucose oxidase	1.4 U	32	–
Glucose oxidase/catalase	1.4 U/5.6 U	21	–
NaCN[b]	50 mM	82	–
EDTA	50 mM	93	–
Potassium tartrate	20 mM	100	–
8-Hydroxyquinoline[c]	20 mM	82	–
1,10-Phenanthroline[c]	5 mM	86	11±4
$FeCl_2$[c]	5 mM	28	–
$CuCl_2$[c]	5 mM	15	–
$NiCl_2$[c]	5 mM	61	–
$CoCl_2$[c]	5 mM	86	–

[a] Additives prepared as follows: $Na_2S_2O_4$, glucose oxidase, catalase, NaCN, EDTA, and potassium tartrate in 100 mM Tris–HCl, pH 7; 8-hydroxyquinoline and 1,10-phenanthroline in ethanol; $FeCl_2$, $NiCl_2$, $CuCl_2$, and $CoCl_2$ in H_2O, pH 2–3.
[b] Assay performed in 500 mM sodium phosphate, pH 7.7.
[c] Assay performed in 100 mM MOPS, pH 7.

irreversible inactivation of farnesol oxidation, with an apparent IC_{50} of 11 mM. The corresponding 4,7-isomer of phenanthroline had little effect on farnesal metabolism at up to 20 mM concentration (data not shown).

Several metal salts, including $FeCl_2$, $CuCl_2$, $NiCl_2$, and $CoCl_2$, were added to larval CC–CA homogenates to investigate the possible role of a redox active metal center. All metals inhibited farnesol oxidation at high (5 mM) concentration (Table 5); however, only Cu^{2+} was strongly inhibitory at concentrations less than 1 mM (Fig. 3). We also examined the effect of "back-addition" of metals to phenanthroline-treated homogenates. After pre-incubation with 20 mM 1,10-phenanthroline, the homogenate was passed through a G-50 Sephadex column and active enzyme fractions (as determined by gel filtration of non-treated homogenate) were pooled and incubated with varying divalent metal cations. None of the metals tested, whether in the absence or presence of varying amounts of dithiothreitol (used as metal reductant) caused any activity enhancement.

4. Discussion

4.1. Farnesol metabolism in insect corpora allata homogenates is a selective enzymatic process

To evaluate farnesol oxidation in insects, as it relates to JH metabolism, CC–CA homogenates of larval *M. sexta* were incubated with 1,2-[^{14}C]farnesol and the

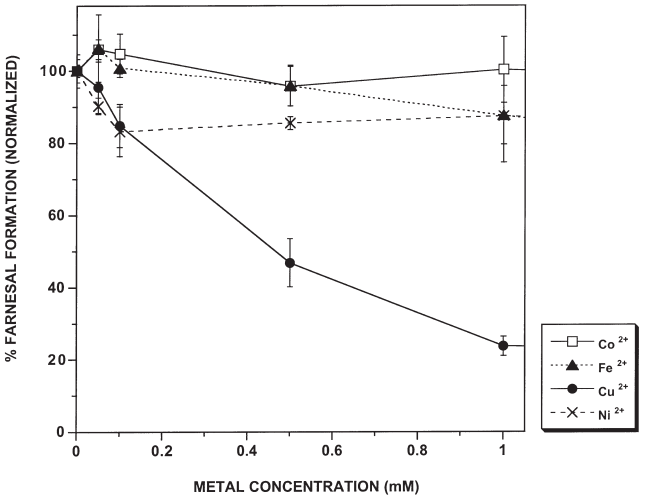

Fig. 3. Effect of metal cations on farnesol oxidation in larval *M. sexta* CC–CA homogenates. Data was obtained using 100 mM MOPS, pH 7, as described in Section 2.9 (n=2 experiments in duplicate).

metabolites formed were analyzed by radio-TLC. In the absence of any added cofactors, farnesol was cleanly oxidized to farnesal (150–250 pmol/CC–CA pair equiv/h), with <2% farnesoic acid being formed. Although it is unclear at this time whether farnesol oxidation in the corpora allata is a single enzyme process, several studies were performed to better characterize enzymatic activity. Farnesol oxidation displays conventional Michaelis–Menten kinetics (Fig. 1) with a K_m apparent of 1 μM, is localized in the cytosol (Table 2), and is maximal at neutral to slightly acidic pH (Fig. 2). In comparison, *M. sexta* whole body homogenates (with gut removed) rapidly converted farnesol to a mixture of farnesoic acid and unidentified polar metabolites, with little farnesal being detected. Farnesol metabolism in whole body homogenates appears to be mediated by several mixed-function oxidative enzymes, as demonstrated by its potent inhibition by sodium cyanide, EDTA, NEM, and pyrazole (data not shown).

Terpenol oxidation is commonly found in animal and plant systems and is often mediated by non-specific oxidative enzymes (Duester, 1996; Kjonaas et al., 1985; Croteau et al., 1978). To determine the substrate specificity of farnesol metabolism in CC–CA homogenates, we examined the ability of several structurally related and unrelated alcohols to competitively inhibit farnesol oxidation. The results of these experiments (Table 2) indicate that farnesol metabolism is not mediated by a simple insect alcohol dehydrogenase (L'Hélias, 1979; Madhavan et al., 1973; Sieber et al., 1972; Sofer and Ursprung, 1968), since simple 1° aliphatic alcohols (i.e., ethanol, octanol, dodecanol) are devoid of inhibitory activity. Farnesol oxidation is well known in animals, including insects, and is related to the shunt pathway involving the oxidative degradation of dimethylallyl alcohol (Vaidya et al., 1998; Keung, 1991; Havel et al., 1986; Landau and Brunengraber, 1985; Christophe and Popják, 1961). The inability of this latter terpenol to inhibit farnesol metabolism in CC–CA homogenates would suggest that the enzyme that oxidizes farnesol within the corpus allatum is structurally different from that found in other insect tissues.

4.2. Farnesol oxidation in larval CC–CA is not a nicotinamide-dependent process

Because terpenol oxidation is commonly associated with short or medium chain ADHs, and because prior studies using adult *M. sexta* CC–CA homogenates indicated an enhancement in farnesol metabolism upon the addition of exogenous NAD, we investigated the effect of nicotinamide cofactors in our assay. Surprisingly, neither the oxidized nor reduced forms of NAD(P) had any effect on farnesal formation (Table 3). To eliminate the possible involvement of endogenous cofactors and/or regenerative redox processes that might interfere with our enzyme assay, we performed filtration studies and treated the homogenate with NADase and YADH. None of these procedures caused any change in farnesol metabolism in larval CC–CA homogenates of *M. sexta*, thus supporting the conclusion that oxidation is not nicotinamide-dependent.

4.3. Farnesol dehydrogenase possesses an active site tyrosine

Several amino acid tritrants were studied in order to structurally characterize the active site residues of farnesol dehydrogenase (Table 4). Since all zinc-containing ADHs and many metal-dependent oxidases have active site thiol and histidine residues, *M. sexta* larval CC–CA homogenates were incubated with NEM, iodoacetamide, and diethyl pyrocarbonate. NEM had no effect on farnesol metabolism, and both iodoacetamide and diethyl pyrocarbonate were only weakly inhibitory. The effect of iodoacetamide could not be reversed upon addition of excess farnesol to the reaction medium, indicating that inhibition was not active site directed. We also incubated larval CC–CA homogenates with tetranitromethane, which reacts with tyrosine that is present in the active site of medium-chain dehydrogenases, including *Drosophila* ADH, and in certain alcohol oxidases (Mattevi et al., 1997; Li et al., 1993; Chen et al., 1993; Krook et al., 1992). This reagent was a potent inactivator of farnesol metabolism (IC_{50}=5 μM). The selectivity of tetranitromethane towards an active site tyrosine was confirmed by substrate protection studies.

4.4. Farnesol oxidation in larval CC–CA homogenates is mediated by an alcohol oxidase

To better characterize the nature of the terpenol oxidation within the corpus allatum, a series of additive

studies was performed (Table 4). As expected, pyrazole, a potent inhibitor of liver and yeast ADH, did not inhibit oxidation to farnesal in larval CC–CA homogenates. Likewise, the addition of carbon monoxide had no effect on farnesol metabolism. Although this latter result does not disprove the involvement of a metal redox center (see below), it clearly indicates that farnesol oxidation is not a heme-dependent process. To establish the possible role of quinone (e.g., TPQ; Anthony, 1996) in the oxidation of farnesol to farnesal, ethyl carbazate, hydrazine, and benzylhydrazine were tested for inhibitory potency. The former two had no effect on farnesol metabolism and benzylhydrazine was only weakly inhibitory. Thus, it appears unlikely that farnesol oxidation requires a quinone cofactor for activity.

Since the conversion of farnesol to farnesal did not appear to occur by dehydrogenation, we investigated the possible involvement of oxygen in the oxidative process (Table 5). Simple degassing of the CC–CA homogenates caused a decrease in enzyme activity, as well as more elaborate methods of O_2 depletion, including the addition of sodium dithionite and of glucose oxidase (in the presence or absence of catalase). In total, these results indicate that the enzyme(s) responsible for terpenol oxidation requires O_2 for activity, and that farnesol metabolism is mediated by one or more oxidases.

Alcohol oxidases are an important class of enzymes; these flavoproteins or metal-dependent (typically Fe(II) or Cu(II)-containing) enzymes, which can be highly substrate specific, oxidize a variety of alcohols including methanol, sugars, aromatic alcohols, and cholesterol (Goetghebeur et al., 1992; de Jong et al., 1992; Anthony et al., 1994). We examined the possible role of metals and flavin in farnesol metabolism of CC–CA homogenates. The presence of 5 mM FAD caused a modest increase in farnesal formation, which was attenuated by pre-filtration of the homogenate through a gel filtration column. The addition of high levels of several divalent cations ($FeCl_2$, $CuCl_2$, and $NiCl_2$) to CC–CA homogenates caused a significant decrease in farnesal formation (Table 5).

The ability of exogenous metals to inhibit oxidative activity suggested that farnesol oxidation might be mediated by a metal-dependent alcohol oxidase, prompting us to further examine the possible involvement of metals in the oxidation of farnesol in CC–CA homogenates. Thus, CC–CA homogenates were incubated with a series of metal chelators, including EDTA, sodium cyanide, potassium tartrate, 1,10-phenanthroline, and 8-hydroxyquinoline (Table 5). 1,10-Phenanthroline is an efficient chelator of both Fe^{2+} and Cu^{2+}, whereas 8-hydroxyquinoline binds selectively to Cu^{2+} and Fe^{3+} (Auld, 1988). We found that only 1,10-phenanthroline effectively inhibited farnesol metabolism. The corresponding 4,7-isomer of phenanthroline, which is not a bidentate ligand for metal cations, had no effect on farnesol metabolism, suggesting that metal chelation was responsible for the observed inhibition.

Several additional experiments were performed to establish the involvement of metals in farnesol oxidation within the corpus allatum. First, since high levels of exogenous metals are known to cause enzyme inhibition by non-selective processes (e.g., non-specific binding to nitrogen or sulfur ligands), we examined the effect of low concentrations of divalent metal cations on farnesol oxidation. A comparison of the effects of $FeCl_2$, $CuCl_2$, $NiCl_2$, and $CoCl_2$, indicated that only Cu^{2+} is strongly inhibitory, possibly because it can competitively displace the active site metal (Fig. 3). Since the inhibitory effect of 1,10-phenantholine suggested that metal chelation had occurred, we also examined the effect of "back-addition" of metals to phenanthroline-treated homogenates. Unfortunately, this chelator caused irreversible inhibition of farnesol metabolism, and none of the metals tested (including $FeCl_2$, $CuCl_2$, $NiCl_2$, and $CoCl_2$) provided any activity enhancement.

Although there is insufficient data at this time to unambiguously identify the type of oxidase involved in farnesol metabolism within the corpora allata, the above studies indicate the possible involvement of both FAD and a redox-active metal. Selective inhibition by 1,10-phenanthroline (but not 4,7-phenanthroline or 8-hydroxyquinoline) and the strong inhibitory effect of $CuCl_2$, but not $FeCl_2$, would suggest that a non-heme iron is required in farnesol oxidation within larval corpora allata homogenates of *M. sexta*.

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