Phytochemistry, Vol. 20, No. 10, pp. 2311–2314, 1981.
Printed in Great Britain.

0031-9422/81/102311-04 $02.00/0

MICROSOMAL AGROCLAVINE HYDROXYLASE OF *CLAVICEPS* SPECIES

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(*Revised received* 5 *February* 1980)

Key Word Index—*Claviceps* sp.; Clavicipitaceae; ergot; agroclavine hydroxylase; elymoclavine; cytochrome P-450.

Abstract— [4-^{14}C]Agroclavine was converted to elymoclavine in the presence of NADPH and the microsomal fraction from *Claviceps* sp. PRL 1980 and SD 58. There was high activity during the period of maximum rate of alkaloid production. The value of K for inhibition of activity by carbon monoxide was 0.086 ± 0.012. The maximum wavelength for reversal of carbon monoxide inhibition was 450 nm. This indicates that agroclavine hydroxylase is a cytochrome P-450 mono-oxygenase.

INTRODUCTION

A step in the pathway of ergot alkaloid biosynthesis is the hydroxylation of agroclavine to elymoclavine [1]. The hydroxyl oxygen did not come from solvent and may be concluded to be derived from molecular oxygen [2]. Alkaloid-producing strains of *Claviceps* species carried out the conversion of agroclavine to elymoclavine whereas non-producing strains did not. Other types of fungi lacked this activity [3]. Agroclavine was converted by the mammalian liver microsomal cytochrome P-450 mono-oxygenase system primarily to noragroclavine, with small amounts of setoclavine, isosetoclavine and elymoclavine [4]. The conversion of agroclavine to elymoclavine by an ammonium sulfate fraction from *C. purpurea* PRL 1980 was previously reported from this laboratory [5]. Subsequent experiments have given preparations with low activity (unpublished results).

Hydroxylation of methyl groups of intermediates in alkaloid biosynthetic pathways in plants have been studied in cell-free systems. The conversion of (−)kaur-16-ene to (−)kaur-16-en-19-ol in *Echinocystis macrocarpa* Greene [6] and the hydroxylation of nerol and geraniol in the pathway of biosynthesis of loganin in *Vinca rosea* [7] both involve cytochrome P-450 as shown by light reversal of CO inhibition [6] and by reconstitution of activity from the components of the enzyme system [7].

The addition of inducers of the liver microsomal oxygenase such as phenobarbital and polycyclic hydrocarbons to the culture medium of *Claviceps* increased alkaloid production [8,9] and the level of cytochrome P-450 [9] in the microsomal fraction. These results indicate that a broad specificity inducible cytochrome P-450 similar to the liver system is present in *Claviceps* and that it may be able to catalyse one or more steps in the pathway of ergot alkaloid biosynthesis.

γ,γ-Dimethylallyltryptophan (DMAT) hydroxylase activity, giving 4[*E*-4-hydroxy-3-methyl-Δ^2-butenyl]-tryptophan (HODMAT), has been reported in the cytosol fraction of *Claviceps* sp. [10, 11]. NADPH was required for activity. Additional properties of the enzyme have not been reported.

The following study reports a specific cytochrome P-450 mono-oxygenase that catalyses the conversion of agroclavine to elymoclavine in the microsomal fraction of *Claviceps*.

RESULTS

Cell-free agroclavine hydroxylase activity

The radioactive product was identified as elymoclavine by co-chromatography on TLC with reference elymoclavine with EtOAc–Me_2CO–DMF (5:5:1), $CHCl_3$–MeOH (8:2), and $CHCl_3$–MeOH–HOAc (75:20:5). Agroclavine hydroxylase activity was 17 600 *g* (20 min)–105 000 *g* (90 min), 49%; 5000 *g* (20 min)–17 600 *g*, 33%; 700 *g* (10 min)–5000 *g*, 11%. The 15 000–105 000 *g* fraction was used in the following studies. Earlier this laboratory reported agroclavine hydroxylase activity in the 60–80% ammonium sulfate fraction of the 100 000 *g* supernatant [5]. In this work, although activity was present consistently in the microsomal fraction, enzyme activity was not observed either in the ultracentrifuge supernatant or the 0–5% or 50–80% ammonium sulfate fractions from it. The only product observed in the TLC radiochromatogram was elymoclavine. Freezing and thawing decreased microsomal enzyme activity 30%. Storage of frozen microsomes for 2 months did not cause further loss of activity.

C. purpurea PRL 1980 cultured under the conditions described in the Experimental section (washed, large inoculum) had activities of 7, 5 and 0.3% conversion at 1, 2 and 3 days of culture time. The cells were therefore harvested at 1.5–2 days of incubation. Agroclavine hydroxylase activity had a broad pH optimum at pH 7.3–8.3. NADPH was required for activity. Per cent conversion was no addition, 3.2; 2 mM NADPH, 16.0; 2 mM NADH, 2.3; 2 mM NADPH plus 2 mM NADH, 19.8.

The microsomal fraction had no measurable hydroxylase activity toward benzphetamine or 3,4-benzo[*a*]pyrene, which are good substrates of the liver microsomal mono-oxygenase. Agroclavine hydroxylase activity was not increased by addition of inducers of liver microsomal cytochrome P-450 to the culture medium. Per cent conversion was: no addition, 39%; 2 mM benzo[*a*]pyrene added to medium, 29%; 2 mM phenobarbitol added to medium, 39%. The microsomal

fraction did not catalyse the conversion of DMAT to HODMAT.

The cytochrome P-450 inhibitor SK&F 525A at 2 mM concentration inhibited activity 35% (Table 1). Metyrapone did not inhibit activity. Liver microsomal cytochrome P-450 substrates, naphthalene, *p*-nitroanisol, and benzphetamine exhibited low or no inhibition. DMAT did not inhibit activity. KCN stimulated activity 23%. Activity is therefore cyanide insensitive. The stimulation may be due to decreased competition for intermediates in the electron transport system due to inhibition of cyanide-sensitive endogenous activity. EDTA, which chelates multivalent metal ions, did not inhibit activity.

Agroclavine hydroxylase activity in microsomes from cells harvested at different culture times

The specific activity increased during the second and third day of incubation to a maximum at 4 days (Fig. 1). Activity decreased to a low constant value which was maintained from 10 to 18 days. The period of high enzyme activity from 3 to 6 days correlates with the maximum rate of alkaloid production during this period.

Table 1. Effect of additions on agroclavine hydroxylase activity in *C. purpurea* PRL 1980

Addition 2 mM	Relative activity
None	100
SK&F 525A	65
Metyrapone	100
Benzo[*a*]pyrene*	108
Benzphetamine*	90
Naphthalene	110
p-Nitroanisole	92
DMAT*	108
EDTA†	104
KCN†	123

* No activity when used as substrate.
† 1.0 mM.

Inhibition of agroclavine hydroxylase activity by carbon monoxide

The inhibition of agroclavine hydroxylase activity at different carbon monoxide concentrations is shown in Fig. 2. The K value calculated from the data was 0.086 ± 0.012. The K value of the steroid C-21 hydroxylase was reported to be around 1.0 [12] and the kaurene hydroxylase around 9 [6]. This low value in K indicates that the heme moiety of this enzyme has an unusually high affinity for carbon monoxide.

Action spectrum for reversal of inhibition of agroclavine hydroxylase activity by carbon monoxide.

The action spectrum had a maximum at 450 nm (Fig. 3) corresponding to the maximum in the reduced-CO vs reduced difference spectrum of cytochrome P-450.

DISCUSSION

Agroclavine was converted to a single product, elymoclavine, by the microsomal fraction of *Claviceps* strains SD 58 and PRL 1980. Previous inability to observe agroclavine hydroxylase activity in the *Claviceps* particulate fraction was probably due to the fact that the cells were harvested at the later stages of alkaloid production [5] when agroclavine hydroxylase activity is low (See Fig. 1).

The maximum in the photoreactivation spectrum in the presence of CO at 450 nm shows the involvement of a cytochrome P-450 mono-oxygenase. Agroclavine hydroxylase did not competitively bind substrates of liver microsomal cytochromes P-450 or catalyse their hydroxylation. Inducers of the liver cytochromes P-450 did not induce agroclavine hydroxylase activity. Agroclavine hydroxylase is therefore not in the class of broad specificity cytochrome P-450 mono-oxygenases that act on exogenous substrates. DMAT was not a substrate with agroclavine hydroxylase-active microsomes and did not inhibit agroclavine hydroxylase activity. DMAT is therefore not hydroxylated by the agroclavine hydroxylase cytochrome P-450 and does not bind significantly to it. SK&F 525A inhibited conversion of agroclavine to elymoclavine, but the inhibition was

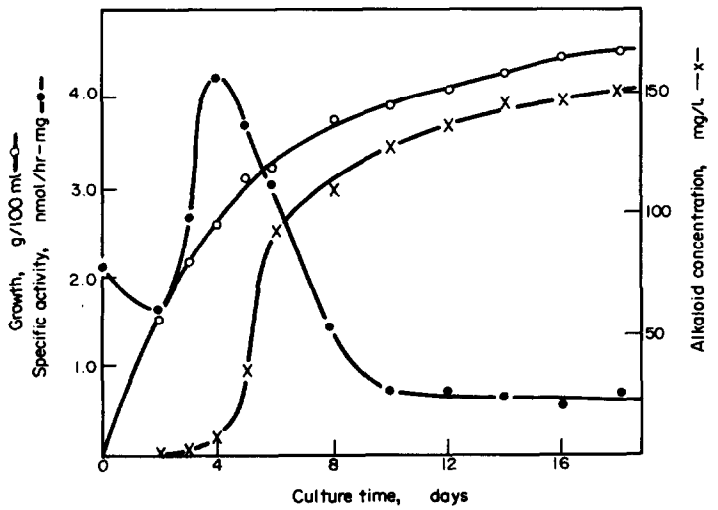

Fig. 1. Agroclavine hydroxylase activity, alkaloid production, and growth vs age of culture of *Claviceps* sp. SD 58.

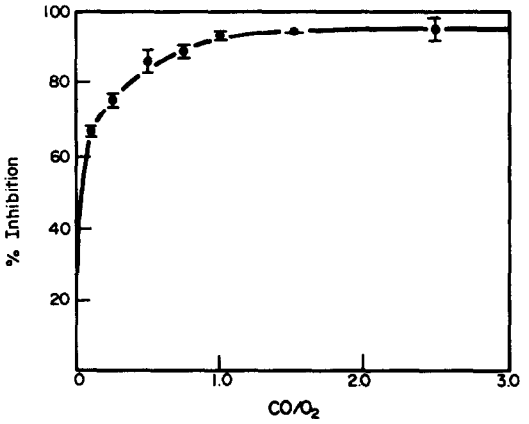

Fig. 2. Inhibition of agroclavine hydroxylase by carbon monoxide. The concentration of oxygen was 20%. Carbon monoxide was added to give the indicated $CO:O_2$ ratio. Nitrogen was added to 100%. Microsomal protein was 1.82 mg/ml. Two incubations were done at each $CO:O_2$ ratio.

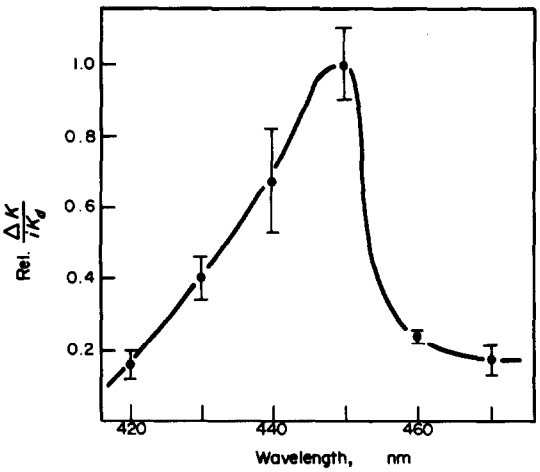

Fig. 3. Photochemical action spectrum of agroclavine hydroxylase–CO complex. The values are relative $\Delta K/i\,K_d$ values compared with $\Delta K/i\,K_d$ at 450 nm. Two observations were made at each wavelength. Protein concentration was 3.72 mg/ml. $k_{O_2}(O_2:N_2—20:80) = 12.2$ pkat/mg; $k_{CO\cdot d}(CO–O_2–N_2—5:20:75) = 3.1$ pkat/mg.

much less than that observed with the broad-spectrum type cytochromes P-450.

The requirement for NADPH and lack of inhibition by EDTA and cyanide, observed for the microsomal agroclavine hydroxylase, are properties expected of a cytochrome P-450 mono-oxygenase. In contrast to the microsomal agroclavine hydroxylase, the ammonium sulfate fraction activity was inhibited by EDTA and cyanide [5]. The agroclavine hydroxylase activity in the ammonium sulfate fraction thus appears to be different from the microsomal agroclavine hydroxylase activity. Evaluation of the role of the agroclavine hydroxylase activity in the ammonium sulfate fraction will require establishing conditions for consistently good activity (preparations have given inconsistent activity) and a systematic study of the properties of the enzyme. As discussed in this section, the microsomal enzyme appears to be the enzyme in the main pathway of ergot alkaloid biosynthesis.

Induction of agroclavine hydroxylase activity occurred just before the increase in alkaloid production and dropped just before alkaloid production levelled off (Fig. 1). There is thus a good correlation between alkaloid production and enzyme activity. There was, however, higher activity in the first 2–3 days than was indicated by the production of alkaloid. Another enzyme (or enzymes) in the pathway is apparently limiting at day 2 and is not present in appreciable quantities until day 4.

The close relationship between enzyme activity and alkaloid production, the specificity for agroclavine as substrate, and the formation of elymoclavine as the only product indicate that the microsomal agroclavine hydroxylase is a component of the ergot alkaloid biosynthetic pathway.

Cytochrome P-450 may be involved in other biosynthetic steps in ergot alkaloid biosynthesis, in ergosterol biosynthesis [13, 14], and in biosynthesis of the ergot pigments. The cytochrome P-450 mono-oxygenases of *Claviceps* sp. is a fruitful area for future study.

EXPERIMENTAL

Culture conditions. Claviceps purpurea PRL 1980 cultures were grown in a sucrose–succinic acid medium, the cells were washed in H_2O, and this inoculum was added in a ratio of 50 ml of original growth medium to 100 ml of mannitol–tryptophan–succinic acid production medium [15]. The cells were harvested after 1.5–2 days shaking at 25°. For the time course experiment *Claviceps* sp. SD 58 was grown in 100 ml of NL 406 medium modified by the omission of yeast extract and by increase in KH_2PO_4 concn to 1 g/l. in order to suppress alkaloid biosynthesis [16]. This culture was used to inoculate (2% inoculum) 100 ml of unmodified NL 406 medium [17]. The flasks were harvested at appropriate time intervals after inoculation. For the other experiments *Claviceps* sp. SD 58 was grown in a New Brunswick MF-100 fermentor with NL 406 medium. Medium (11 l.) was inoculated with 220 ml of a 5-day culture. The culture was aerated at a rate of 3 l/min and agitated at 100–200 rpm with occasional addition of antifoam A. Three days after inoculation the mycelia were harvested. The mycelia from the various cultures were washed with H_2O and with 0.1 M KPi buffer pH 7.6, 0.5 M sucrose, 10^{-3} M EDTA (buffer A), most of the buffer was removed by vacuum filtration, and the cell mat was stored at −20°. The wet wt of the packed cells was determined and used in the time course study as the measure of cell growth.

Analytical procedures. Protein content was assayed with BioRad protein dye reagent [19]. Microsomal protein was solubilized before assay by alkaline treatment [19]. Hydroxylase activity for 3,4-benzo[*a*]pyrene [20] and benzphetamine [21] in the microsomal fraction was measured by published procedures. [Sidechain 3-^{14}C]DMAT conversion to HODMAT was measured by applying the incubation mixture directly to a Si gel G plate, developing with MeOAc–isoPrOH–NH_4OH (45:35:20), scanning with the radioactivity scanner, and observing the presence or absence of a radioactivity peak corresponding to reference HODMAT. For total alkaloid determination an aliquot of the mother liquor was made basic with conc. NH_4OH. The soln was extracted with Et_2O. The Et_2O extract was back extracted with 0.1 M H_2SO_4. An aliquot of the H_2SO_4 extract was mixed with Van Urk's reagent [22] and the *A* was read. Ergotamine tartrate was used as standard. The concn was converted to mg elymoclavine by use of the appropriate gravimetric factor.

Preparation of [4-^{14}C]*agroclavine.* Mycelia from 10 ml *C. purpurea* PRL 1980 grown in production medium for 1.5 days were suspended in 10 ml production medium without tryptophan plus 5 ml DL-[sidechain 3-^{14}]tryptophan (90 μCi, 3.76 mCi/mmol). The culture was incubated for an additional 10 days. The mixture was then homogenized, the pH was adjusted to 11 with NH_4OH, and the alkaloids were extracted with Et_2O. The Et_2O layer was brought to dryness with a rotary evaporator and the residue was dissolved in MeOH. The labelled agroclavine was separated by TLC using EtOAc–Me_2CO–DMF (5:5:1) and MeOH–$CHCl_3$–NH_4OH (20:80:0.2). The radioactivity recovery was 32% and the sp. act. was 1.69 mCi/nmol agroclavine.

Preparation of microsomes. The frozen mycelia were suspended in Buffer A and homogenized at maximum speed with a VirTis '45' homogenizer for 1 min twice with a 1-min interval. The homogenate was centrifuged at 15000 *g* for 20 min and the supernatant was centrifuged at 105000 *g* for 90 min. The pellet was suspended in 0.1 M KPi buffer pH 7.6 at a concn of 5–10 mg/ml and used immediately or frozen and stored at −20°.

Assay of conversion of agroclavine to elymoclavine. The reaction mixture in a final vol. of 1 ml contained 100 μmol of KPi buffer pH 7.6, microsome suspension, and 48 nmol of [^{14}C]agroclavine (1.89 mCi/mmol). The reaction was started by the addition of 2 μmol NADPH in 0.2 ml. The mixture was shaken for 1 hr at 25°, the pH was adjusted to 11 with NH_4OH, and the alkaloids were extracted with Et_2O. The Et_2O layer was evaporated to dryness under a stream of N_2, dissolved in MeOH, and the products were separated by TLC with EtOAc–Me_2CO–DMF (5:5:1). The plates were scanned with a Packard Model 7220-21 radiochromatogram scanner. The areas under the curves of the agroclavine (R_f 0.36) and elymoclavine (R_f 0.15) peaks were measured. The per cent conversion was {area (*E*)/[area (*E*) + area (*A*)]} × 100. This was converted to nmol by: nmol *E* = (per cent conversion/100) × nmol *A* added (*E*—elymoclavine, *A*—agroclavine).

Inhibition of activity by CO. For the CO inhibition study a 30-ml Thunberg tube or a 3-ml quartz anaerobic cell was used. The vessel containing the reactants was evacuated and gassed with the desired gas mixture. The reaction was initiated by addition of 0.1 ml containing 2 μmol NADPH in 0.1 M KPi buffer, pH 7.6, from the sidearm. The vessel was then incubated at 25° in the dark for 1 hr.

Action spectrum of agroclavine hydroxylase–CO complex. The light source was a 450-W high-pressure Xe arc lamp (Oriel Optic Corp., C-60-53) with a water-cooled IR filter. The light from the exit iris diaphragm passed through an additional IR blocking filter and the interference filter (2 × 2 in. band-pass width at half-maximum less than 10 nm). The light was reflected 90° upward with a mirror. The anaerobic cuvette was laid horizontally 1 cm above the bottom of a 2 l. glass vessel, the sample being irradiated by the reflected light beam. The large vessel was filled with 7.5% $CuSO_4$ soln, which was maintained at 25° by passing H_2O through a glass tubing coil in the $CuSO_4$ soln. The light intensity was measured with a Yellow Springs Radiometer (YSI-65 A).

The principle of the determination of the photochemical action spectrum of cytochrome P-450–CO is described elsewhere [6, 12]. The partition constant *K* relates the amounts of the two gases bound to the enzyme (*E*).

$$K = \frac{E\text{–}O_2}{E\text{–}CO} \times \frac{CO}{O_2}.$$

The change in *K* relative to the dark value of *K* is equal to

$$\frac{1}{i}\frac{\Delta K}{K_d} = \frac{1}{i}\left(\frac{k_{O_2}}{k_{CO\cdot d}}\right)\left(\frac{k_{CO\cdot h} - k_{CO\cdot d}}{k_{O_2} - k_{CO\cdot h}}\right),$$

where $\Delta K = K_h - K_d$ with K_h the value of *K* with light and K_d the value of *K* in the dark, and *i* is the light intensity of the particular wavelength striking the cuvet. The rates of agroclavine hydroxylation were k_{O_2}, no CO; $k_{CO\cdot d}$, CO present, dark; $k_{CO\cdot h}$, CO present, exposed to light of the particular wavelength.

Acknowledgements—The research was supported by the Robert A. Welch Foundation Grant No. D-117. Benzphetamine was a gift from Dr. Paul W. O'Connell, The Upjohn Company. The technical assistance of M. Song and R. Messmore is acknowledged.

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