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CONVERSION OF 4-γ,γ-DIMETHYLALLYLTRYPTOPHAN TO CLAVICIPITIC ACID SOME PROPERTIES OF THE ENZYME

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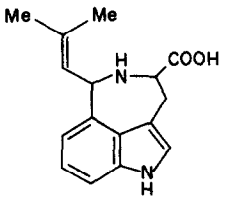
Key Word Index—*Claviceps purpurea* PRL 1980; Clavicipitaceae; ergot; enzymatic study; clavine alkaloids; 4-γ,γ-dimethylallyltryptophan; clavicipitic acid.

Abstract—The particle-bound enzyme catalysing the conversion of DL-4-γ,γ-dimethylallyltryptophan into clavicipitic acid in *Claviceps purpurea* PRL 1980 has been solubilised. The K_m value and pH optimum were determined and the substrate specificity of the enzyme and the effect of inhibitors were studied. The racemisation of L-tryptophan in the culture medium was investigated.

INTRODUCTION

A major product in cultures of *Claviceps* species which produce clavine alkaloids is the secondary amine clavicipitic acid [1]. The structure of clavicipitic acid (**1**) recently proposed by King *et al.* [2] was based on the NMR spectrum of the *N*-acetylmethyl ester.



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The cell free conversion of DL-4-γ,γ-dimethylallyltryptophan (DMAT) to clavicipitic acid has been reported [3]. The enzymic activity was located in the particulate fraction and was higher at high pH. The stoichiometry, specificity, and partial purification of the enzyme which catalyzes the conversion, are described in this report. The relation of the appearance of enzyme activity in cultures to other events of DMAT metabolism is also reported.

RESULTS

The conversion of DMAT to clavicipitic acid was 6.0% for the crude extract, 8.3% for the mitochondrial fraction, and 16.7% for the microsomal fraction [3,4]. The protein concentration was 2.6 mg/ml for all samples. Activity was therefore present in both the mitochondrial and microsomal fractions, although the specific activity was higher in the microsomal fraction. Subsequently, the cells were homogenized in 0.02M K Pi buffer pH 7.0 and the combined microsomal and mitochondrial fractions (particulate fraction) was used.

The enzyme was solubilized in 1% Triton X-100 at pH 7.0 at 25°. Protein concentrations and conversions to clavicipitic acid for the fractions were: particulate fraction, 4.0 mg/ml, 21.0%; solubilized supernatant, 1.0 mg/ml 53.1%; unsolubilized residue, 4.0 mg/ml, 16.5%.

With the solubilized enzyme, the ratio of oxygen consumed to clavicipitic acid formed (Table 1) was approximately 1:1 in the presence of the catalase inhibitor, 3-amino-1,2,4-triazole (equation 1), and approximately 0.5:1 in the presence of catalase (equation 3). These ratios are consistent with the formation of hydrogen peroxide by the enzyme.

(1) DMAT + O_2 → clavicipitic acid + H_2O_2

(2) H_2O_2 → H_2O + 1/2 O_2

(1) + (2) = (3) DMAT + 1/2 O_2 →
clavicipitic acid + H_2O

A spectrophotometric assay was developed in which hydrogen peroxide produced in the reaction was coupled to peroxidase. Guaiacol was tried as co-substrate but it was found to inhibit formation of hydrogen peroxide. The concentration of guaiacol for one-half maximum activity was 6.0mM. *o*-Phenylenediamine was subsequently used as co-substrate for peroxidase. The results which follow were obtained with the spectrophotometric assay.

With increasing protein concentrations, the rate was linear to 2.0 mg/ml and then leveled off at higher enzyme concentrations. Similar results were obtained with the radioactive assay. This suggests that the enzyme preparation contains an inhibitor of the conversion of DMAT to clavicipitic acid. The maximum rate obtained with solubilized enzyme under the assay conditions in the Experimental Section was 0.60 nkat/mg protein. The K_m for DMAT was 0.35mM under these assay conditions. The activity was determined as a function of pH. There was a plateau region from pH 6.5 to 8.5 which was 30% of the maximum activity. The activity rose to a maximum at pH 10.5 and then decreased. There was an

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Table 1. Oxygen consumption and conversion of DMAT to clavicipitic acid in a solubilized extract from *Claviceps purpurea* PRL 1980

Addition	μmol O_2	μmol ΔO_2	Clavicipitic acid %	Clavicipitic acid μmol	ΔO_2/clavicipitic acid
3-Amino-1,2,4-triazole	0.65		—	—	
3-Amino-1,2,4-triazole + DMAT	3.12	2.47	59	2.17	1.1
Catalase	0.80		—	—	
Catalase + DMAT	2.15	1.35	62.5	2.30	0.6

The reaction mixture, consisting of 7.2 mg (2.0 ml) solubilized enzyme, 30 μmol of diethyldithiocarbamic acid and either 0.30 mg of catalase or 4.0 μmol of 3-amino-1,2,4-triazole (2.6 ml total vol) was placed in the main compartment of a Warburg flask. DMAT-[^{14}C] (3.68 μmol, 0.20 μCi) was placed in the sidearm of the Warburg flask. The temperature was 28°. Two hr after addition of DMAT oxygen consumption was recorded and the reaction was stopped by lowering the pH to 5 with 0.1M HCl. The percentage of clavicipitic acid was determined as described previously [3] and was converted to μmol clavicipitic acid formed by the relationship:

$$\mu\text{mol clavicipitic acid} = \frac{\%\text{ clavicipitic acid}}{100} \times \mu\text{mol DMAT added.}$$

The radioactivity added in the incubation mixture was recovered quantitatively from the combined areas of the TLC plate (97–110%). There was a negligible amount of endogenous DMAT and clavicipitic acid in the solubilized enzyme preparation. In this experiment the microsomal fraction [3] was solubilized by incubation in 0.1% Triton X-100, 0.1M sodium carbonate pH 10.3 at 0° for 30 min.

apparent pK in the region pH 9–10. The approximate pK estimated from the pH at 0.5 × (maximum activity minus plateau activity) was 9.5.

Activity with agroclavine, DL-6-γ,γ-dimethylallyltryptophan (6-DMAT), DL-4-[4-*E*-hydroxy-3-methyl-Δ^2-butenyl]-tryptophan (*E*-HODMAT), or L-tryptophan, was less than 8% of the activity with DMAT in the spectrophotometric assay. None of these compounds at a concentration of 220 μM added with 220 μM DMAT decreased the rate significantly (activity ≥ 92% of activity with DMAT alone). Isonicotinic acid hydrazide (5.0 mM) and hydroxylamine (5.0mM) decreased activity more than 92%. Pyridoxal 5′-phosphate (24 μM) inhibited activity 30%. Clavicipitic acid (400 μM) inhibited activity 46%.

The enzyme specific activity and total protein at successive stages of purification were: solubilized enzyme 0.050 nkat/mg (170 mg), DEAE Sephadex pH 7.0 0.11 nkat/mg (64 mg), CM Sephadex 0.20 nkat/mg (14 mg), and DEAE Sephadex pH 9.0 0.25 nkat/mg (7 mg). The specific activity was thus increased five fold with a yield of 22%.

The values of $[\phi]_{365}$ were: L-tryptophan + 1.55 (*c* 0.155); isolated tryptophan 0.00 (*c* 0.094); isolated DMAT + 1.63 (*c* 0.054). The values of $[\phi]_{230}$ were L-tryptophan + 86.7 (*c* 0.00282) and isolated DMAT + 81.3 (*c* 0.00400). The solvent used was 95% EtOH–HCl, pH 1.0. Since the tryptophan isolated from 3-day cultures of *C. purpurea* PRL 1980 was optically inactive, it is evident that the L-tryptophan which was added in the culture medium was completely racemized after three days of incubation. The similar values of $[\phi]$ for L-tryptophan and isolated DMAT at 230 and 365 nm indicate that DMAT was the L-isomer only.

Clavicipitic acid isolated from *C.purpurea* PRL 1980 showed $\Delta\epsilon_{288} = -2.04$ (MeOH; *c* 0.00316). Careful examination of clavicipitic acid on silica gel TLC (solvent $CHCl_3$–MeOH–0.88M NH_3, 75:25:1) revealed two poorly separated Van Urk's positive spots of similar intensity with R_f values of 0.43 and 0.37. With the $\Delta\epsilon_{288}$ values of −2.95 and −1.09 previously reported for the two diastereoisomers [2], the above value of $\Delta\epsilon_{288} = -2.04$ for the mixture in *C. purpurea* PRL 1980 indicates 51.2 and 48.8% respectively of the two isomers, i.e. equal amounts of the isomers. It is not known which of the two assymetric centers contains both configurations. It is very likely that the configuration around the α-carbon of L-DMAT is unchanged and that clavicipitic acid exists in two forms with different configurations at C-10 as suggested by King *et al.* [2].

L-DMAT isolated from the mother liquor and the synthetic DL-DMAT were incubated in the spectrophotometric assay. With 220 μM L- or DL-DMAT and 1.35

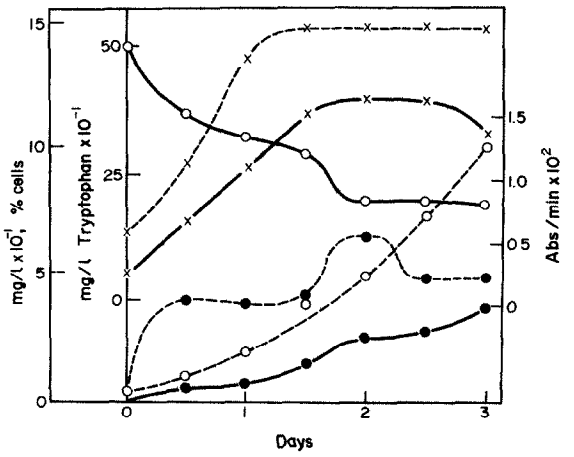

Fig. 1. Concentrations of clavicipitic acid and other metabolites and DMAT oxidase activity of *Claviceps purpurea* PRL 1980 at different culture times. See experimental section for procedures. The amount of solubilized enzyme per 3.4 ml incubation mixture was adjusted to 4.6 mg for all samples. ——●——●—— Clavicipitic acid; ····○····○···· alkaloid; ····●····●···· DMAT; ——○——○—— tryptophan; ——×——×—— DMAT oxidase activity; ····×····×···· % cells, by volume.

mg/ml solubilized enzyme, the initial rates were 0.039 nkat/mg for DL-DMAT and 0.086 nkat/mg for L-DMAT. L-DMAT was thus converted to clavicipitic acid faster than racemic DMAT. However, D-DMAT appears to also be a substrate since the absorbance approached the same value with DL-DMAT as with L-DMAT. (Both samples had an absorbance of 0.227 after 70 min.)

Changes in various parameters at 12 hr intervals after inoculation were determined (Fig. 1). The amount of tryptophan remaining after 72 hr was 40% of the amount initially present. DMAT was synthesized very rapidly immediately after inoculation with 40 mg/l. at 12 hr and remained near this level throughout the 72 hr period. The concentration of clavicipitic acid increased gradually. The DMAT oxidase specific activity increased linearly with time for 36 hr and then remained constant. Cessation of cell growth and cessation of enzyme synthesis took place at about 36 hr. There was an exponential increase in the rate of alkaloid production during the sampling period. In the 36–48 hr period there was a marked decrease in the level of tryptophan and increased levels of DMAT and clavicipitic acid.

DISCUSSION

The enzyme retained its activity after solubilization and fractionation on ion exchange column chromatography and ammonium sulfate fractionation. Binding to the membrane therefore is not necessary for the stability or activity of the enzyme. The retention of activity through a 5-fold purification procedure indicates that the reaction is catalyzed by a single enzyme and that no accessory cofactors are required.

The equation for the reaction has been established from the Warburg experiment (equation 1). The descriptive name suggested for this enzyme is 4-γ,γ-dimethylallyltryptophan:oxygen oxidoreductase (forming clavicipitic acid, hydrogen peroxide); trivial name DMAT oxidase.

The pH activity curve indicates an ionizable group with pK apparent of pH 9.5. The acidic form is about one-third as active as the basic form. The pK_2 of tryptophan is 9.4. As previously suggested [3], the pK in the pH activity curve may reflect deprotonation of the α-nitrogen of DMAT.

The solubilized enzyme was inactive with tryptophan, 6-DMAT and *E*-HODMAT. Also, these compounds were not effective competitive inhibitors of DMAT oxidation. Evidently, small differences in structure, the addition of a hydroxyl to the dimethylallyl group (*E*-HODMAT) or the transfer of the dimethylallyl group from the 4 to the 6 position on indole, markedly reduce the binding to the active site.

Inhibition by isonicotinic acid hydrazide and hydroxylamine suggests that DMAT oxidase contains pyridoxal 5′-phosphate. However, pyridoxal 5′-phosphate addition reduced activity rather than increased activity.

Since L-tryptophan is converted to a racemic mixture after 72 hr there is an active system for interconversion of L- and D-tryptophan. DMAT isolated from the mother liquor was the L-isomer. This indicates that only L-tryptophan is converted directly to DMAT. D-Tryptophan must be first converted to L-tryptophan. Heinstein *et al.* [5] similarly observed only synthesis of L-DMAT from D-tryptophan in a cell-free system. The L-DMAT is then converted to clavicipitic acid by DMAT oxidase. The availability of only L-DMAT *in vivo* is consistent with the greater activity of DMAT oxidase with L-DMAT than with DL-DMAT.

The rapid synthesis of DMAT (Fig. 1) indicates that dimethylallylpyrophosphate–tryptophan dimethylallyltransferase [5] is present in the inoculum. The rise in DMAT at 36–48 hr apparently results from formation of additional enzyme. Heinstein *et al.* [5] reported enzyme activity for DMAT formation in the inoculum of *Claviceps* sp. SD 58 and an increase in enzyme activity immediately before an increase in the amount of alkaloid.

DMAT is available (38–64 mg/l.) both for clavicipitic acid and alkaloid biosynthesis during the 72 hr period. The slow initial rate of alkaloid biosynthesis is therefore not due to a deficiency of substrate DMAT. The rate of alkaloid synthesis increased throughout but the greatest increase appears to be between 24 and 60 hr. Enzyme induction by DMAT, release of catabolite repression, or accumulation of necessary cofactors could be involved in the increase in rate of alkaloid production.

DMAT oxidase specific activity increased linearly with time until growth stopped and then remained at a high level. The change in clavicipitic acid level is consistent with the change in DMAT oxidase and DMAT levels. The increased synthesis of clavicipitic acid between 36 and 48 hr probably results from the increased amount of DMAT in this period.

The formation of clavicipitic acid is a major alternative to the biosynthesis of alkaloids in the utilization of DMAT. There is apparently no feedback control of clavicipitic acid formation by alkaloids since agroclavine did not inhibit DMAT oxidase significantly. DMAT oxidase and the alkaloid biosynthetic pathway may have developed to remove DMAT under different conditions. This is reflected in the contrasting properties of the two pathways. DMAT oxidase is membrane bound, is produced during the rapid growth phase, and has a high pH optimum. The alkaloid biosynthetic pathway components are probably soluble [6], are produced during the phase of slow or no growth, and the pH optimum is near 7 [6,7]. The production of clavicipitic acid rather than agroclavine or elymoclavine at higher pH would be advantageous to the fungus, since the production of the basic alkaloids would tend to further increase the pH.

EXPERIMENTAL

DMAT and DMAT-[^{14}C] were synthesized as described previously [8]. 6-DMAT was synthesized by the same procedure from the 4-bromo-6-nitrophenylpyruvic acid produced during the synthesis of DMAT [9]. Catalase, 2× crystallized from beef liver, approx. 30000 units/mg, and horseradish peroxidase, approx. 60 purpurogallin (20-sec) units/mg were from Sigma Chemical Company. *Claviceps purpurea* PRL 1980 was maintained on slants of a sucrose–succinic acid–yeast extract medium [10] containing 2% agar under sterile mineral oil. Sucrose–succinic acid–yeast extract medium was inoculated from a slant and incubated 4–5 days on a rotary shaker at 24°. The medium was inoculated from this culture and incubated for 3 days. The mycelium was washed with two vols sterile H_2O and resuspended in an equal vol of H_2O. The mycelial suspension was used to inoculate biotin-free mannitol–tryptophan–succinic acid medium [11] supplemented with 3 mM niacinamide. Twenty ml of culture was used to inoculate 100 ml alkaloid production medium. Cultures of *Claviceps purpurea* PRL 1980 (2–3 days old) containing about 80 mg alkaloids per l. (about 40% of maximum) and showing a pink

colour were harvested. The culture was filtered on a Buchner funnel and the washed cells homogenized at 0° in 0.02M K Pi buffer pH 7.0 with a Virtis 45 homogenizer for 2 min at high speed (30 sec at a time with 5 min intervals to avoid increase in temp). Homogenate was centrifuged at 800*g* for 10 min to remove cell debris. The supernatant (crude extract) was centrifuged at 100000*g* for 60 min. Residue was resuspended in 0.02M K Pi buffer pH 7.0 (particulate fraction). The particulate fraction was incubated for 90 min in 1% Triton X-100, 0.02M K Pi buffer pH 7.0, at 25°. The supernatant after centrifugation at 100000*g* for 60 min (solubilized enzyme) was used in the Warburg experiment and the spectrophotometric assays. In the determination of the distribution of activity between the mitochondrial and microsomal fractions, the mycelium was homogenized in 0.5M sucrose-0.2M Na Pi buffer pH 7.5 and fractionated as previously described [4]. In the radioactive assays [3] DMAT-[^{14}C] (0.08 μCi/mg), 0.1M Na_2CO_3 pH 10.3, and enzyme in a final vol of 4.0 ml were incubated for 20 min at 26°. The mixture was fractionated with Dowex 50 resin and by TLC. Regions of the chromatogram were assayed by liquid scintillation counting as described previously [3]. The percentage conversion of DMAT to clavicipitic acid is the radioactivity in the clavicipitic acid region of the TLC plate divided by the total radioactivity from the TLC plate times 100. In the spectrophotometric assay coupled to peroxidase, diethylidithiocarbamate [3] was omitted from the spectrophotometric assay because it inhibited the peroxidase reaction. To insure that peroxidase activity was not limiting, an assay was run in the absence of DMAT and enzyme but with added H_2O_2 under the same conditions of pH, addition of inhibitor, etc. Unless otherwise indicated the incubation mixture contained 220 μM DMAT, 2 mM *o*-phenylenediamine, 0.10 mg horseradish peroxidase, 350 μM 3-amino-1,2,4-triazole, 4.6 mg solubilized enzyme and 0.1M Na_2CO_3 pH 10.3 in a final vol of 3.4 ml. The reaction was started by the addition of substrate and the absorbance change at 28° was measured at 430 nm. After incubation of *o*-phenylenediamine, peroxide, and excess H_2O_2, the log ϵ value was 3.43 at 430 nm, pH 10.3. The enzyme activity for conversion of DMAT to clavicipitic acid was partially purified by ion exchange column chromatography. Solubilized enzyme was applied to a DEAE Sephadex column and eluted with a soln which contained 0.02M K Pi buffer pH 7.0, 10% glycerol, 0.1mM EDTA. Activity was in the breakthrough peak. Active fractions were applied to a CM-Sephadex column and eluted with 0.02M K Pi buffer pH 7.0, 10% glycerol, 0.1mM EDTA. Activity was in the breakthrough peak. The active fractions were combined and the protein was precipitated with 80% ammonium sulfate and dialyzed overnight against eluting buffer soln. The resulting preparation was applied to a DEAE Sephadex column equilibrated with 0.02M Tris–HCl pH 9.0, 0.1mM EDTA. Stepwise elution was carried out with this buffer to which had been added 0.02, 0.10, and 0.20M NaCl. The enzyme activity was in the 0.2M NaCl fraction. The following procedure was used for the fractionation of tryptophan, DMAT, and clavicipitic acid from the mother liquor. The mother liquor (10 l.) of 2–3 day old cultures of *C. purpurea* PRL 1980 was adjusted to pH 4.0 by addition of dilute HCl. Dowex 50 × 4 (100)(H^+) cation exchange resin (200 g per l.) was added [5]. The mixture was washed with distilled H_2O and the resin was collected by centrifugation. The resin was extracted with 5% ammonia and the extract was conc on a rotary evaporator at 50°. The conc soln was adjusted to pH 6.0 and applied to a Sephadex G-10 column that had been equilibrated with 0.02M ammonium acetate buffer pH 6.0 [12]. The same buffer was passed through the column and fractions were lyophilized. Clavicipitic acid was purified from the later fraction from the Sephadex G–10 column by PLC with MeOAc–*iso*–PrOH–30% NH_3 (45:35:20) and then with $CHCl_3$–HOAc–H_2O (8:4:1). The earlier fractions which contained DMAT and tryptophan were put on a Dowex 50 × 4 (100) ion exchange column equilibrated with 0.06M ammonium acetate buffer pH 4.5. The same buffer was passed through the column. The buffer was then changed to 0.10M ammonium acetate buffer pH 6.5. The eluate from each step was lyophilized and dissolved in EtOH:10% ammonium (95:5). The first step contained primarily tryptophan and the second step contained primarily DMAT. The fractions with tryptophan and DMAT were successively fractionated on TLC with $CHCl_3$–MeOH–HOAc (10:5:1) and with MeOAc–*iso*–PrOH–30% NH_3 (45:35:20). The solvent for the optical rotation measurement was 95% EtOH–HCl pH 1.0. For tryptophan in this solvent λ_{max} (log ϵ):280 (3.64). It was assumed that ϵ for DMAT was the same as tryptophan. The optical rotation at 365 nm was measured with a one ml cell with 10 cm pathlength. ORD and CD measurements were with a JASCO J-20 Automatic Recording Spectropolarimeter. The instrument was calibrated with androsterone in dioxane. In the time-course expt culture vol was 1.5l. in a 4l. flask. The first sample (0 days) was an aliquot of the inoculum. Subsequent 250-ml aliquots were removed every 12 hr for analysis. For measurement of cell growth, the culture flask was vigorously shaken and 15 ml was transferred to a conical graduated centrifuge tube. The suspension was centrifuged at 2000 rpm for 5 min and the level of mycelium sediment in the tube was measured. The % by vol of cells was the fraction of packed cells per total vol times 100. After fractionation of the mother liquor with Dowex 50 as described above, TLC was used to determine DMAT, tryptophan and clavicipitic acid. Regions of amino acids were located by comparison with standards which were spotted on one side of the TLC plate. The regions of the amino acids were scraped, and the Si gel was transferred to 25-ml Erlenmeyer flasks. MeOH–HOAc–H_2O (2:1:2) (3 ml) was added to the flasks which were shaken for 10 min. Van Urk's reagent (3.0 ml) was added and the mixture was again shaken for 10 min. The mixture was then centrifuged for 15 min at 10000*g*. The absorbance of the supernatant was immediately read at 550 nm [13]. Tryptophan was used as reference. Correction was made for differences in molecular weight. Molar extinction coefficients of clavicipitic acid and DMAT were assumed to be the same as tryptophan.

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