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0014-4754/85/081048-04$1.50 + 0.20/0

Role of the Δ 8 double bond of agroclavine in lysergic acid amide biosynthesis by *Claviceps purpurea*

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Summary. Agroclavine, given to actively-growing sclerotial tissue of a strain of *Claviceps purpurea* which can not normally elaborate ergot alkaloids, was transformed by this tissue into lysergic acid amide with overall efficiency of approximataly 40%. By contrast, festuclavine (8,9-dihydro-agroclavine) was not transformed, indicating specificity in the mechanism of lysergyl biosynthesis.
Key words. Agroclavine; festuclavine; lysergic acid amide; *Claviceps purpurea;* ergot alkaloids; biosynthesis.

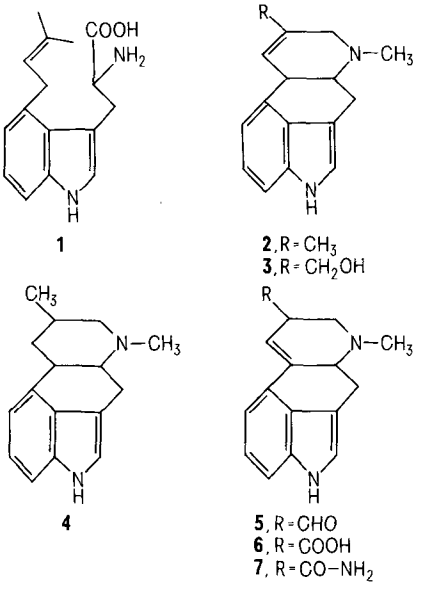
The biosynthetic pathway leading to the ergot alkaloids is fairly well understood though much mechanistic detail remains unclear[1]. A strain of *Claviceps purpurea* which, as a parasite, elaborates trace amounts of dimethylallyltryptophan (**1**), but no ergoline alkaloids, has been shown to metabolize exogenous agroclavine (**2**) or lysergic acid (**6**) to lysergic acid amide (**7**) only[2]. This system is therefore suitable for exploring certain aspects of the biosynthesis of the lysergyl nucleus in parasitic sclerotial tissue.

The most recently proposed speculative scheme for the formation of lysergyl derivatives from clavine alkaloid intermediates[1] involves an activated lysergic acid and excludes free lysergic acid as a direct intermediate[3]. Biosynthesis of the activated lysergic acid from elymoclavine (**3**) is deduced to be via the enol form of Δ 9 lysergaldehyde (**5**) in the formation of which the Δ 8 double bond migrates to the Δ 9 position giving a system conjugated with the aromatic ring. Since in one ergot fungus (*Sphacelia sorghi*) the alkaloids are all dihydrogenated with respect to positions 8,9 and 9,10 of the ergoline nucleus[4], the question arises as to whether, in all of the other ergot fungi producing lysergyl derivates, the Δ 8 is essential in order to stabilize the enol form for activation by coenzyme A.

Sclerotia of the alkaloid-free strain of *C. purpurea* were therefore produced on rye in 1983 and, while actively growing 35 days after inoculation, the distal and proximal 3 mm of each sclerotium was excised and the remaining tissues combined and homogenized briefly in 0.01 M phosphate buffer, pH 6.5. The homogenate was subdivided into three parts and incubated (1 g tissue: 10 ml buffer) with gentle agitation for 24 h at 27 °C as indicated (table). The crystalline substrates, agroclavine and festuclavine (**4**) (8,9 dihydro-agroclavine), were prepared from submerged fermentation of *C. fusiformis*[5] and *Sphacelia sorghi*[6], respectively. After incubation with the substrates the cells were separated from the supernatant by centrifugation, washed and lyophilized. Dry cells were made alkaline with aq. $NaHCO_3$ and exhaustively extracted with diethyl ether[2]. Alkaloids were extracted from the ether with 2% tartaric acid, a portion made alkaline with NH_4OH, extracted exhaustively with chloroform, evaporated to dryness and the alkaloids evaluated spectrophotometrically in methanol at 284 nm and 311 nm, λmax for the clavine alkaloids and lysergic acid amide, respectively. A portion of the extract was examined on silica gel chromatograms developed in chloroform:methanol (4:1) and by HPLC in a Waters Novapak column using acetonitrile:0.01 M ammonium carbonate (4:6) with UV detection at 284 nm. The expected occurrence of lysergic acid amide was confirmed by both chromatographies, as was also the spectrophotometrically-assayed relative abundance of agroclavine and lysergic acid amide.

Lyophilized supernatants from the incubations were also examined by spectrophotometry and chromatography; only given alkaloid was detected.

As expected, tissue incubated with or without agroclavine (table) confirmed previous findings that the tissue was intrinsically alkaloid-free[7]. Additionally, the efficiency of transforming agroclavine into lysergic acid amide may be implied from the calculation that in one day the tissue transformed an amount of alkaloid equivalent to about 10% of that commonly elaborated in ergot sclerotia over several weeks of parasitism. The contrasting failure of such metabolically-active tissue to utilize festuclavine for alkaloid biosynthesis may therefore reflect either a mechanistic requirement for Δ 8 in the biosynthesis of the lysergyl nucleus



from clavine precursors, or a high degree of specificity of, for example, the agroclavine hydrolase enzyme with respect to the unsaturation of ring D of the ergoline nucleus of its substrate. While the strain of *C. purpurea* selected for this experiment is unusual in forming only the simple amide of lysergic acid as an end product, by a mechanism which is itself of interest, it seems unlikely that the organism's biosynthetic pathway to the lysergyl nucleus would be atypical. Further, the reported formation of dihydroergotamine from given dihydrolysergic acid by *C. purpurea*[1] implies that there is no general high enzymic specificity with respect to simple analogues of normal substrates where the molecular conformation is not altered. Therefore it is suggested that the present experimental finding supports a mechanistic requirement for the Δ 8 of agroclavine and elymoclavine in lysergyl biosynthesis.

Comparative acceptance of agroclavine and festuclavine as substrates for lysergyl biosynthesis by *C. purpurea*

Fresh weight of sclerotial tissue incubated	Alkaloid substrate given	Cell-associated alkaloids after incubation % of given substrate	Alkaloids detected and quantified	
6 g	None	0	None	
6 g	Agroclavine 2 mg	60	Lysergic acid amide	63%
			Agroclavine	37%
12 g	Festuclavine 4 mg	48	Festuclavine	100%

While this may well apply to most organisms producing ergot alkaloids, the exception is *S. sorghi* which elaborates as principal alkaloid the only naturally-occurring dihydrogenated cyclic tripeptide ergot alkaloid, dihydroergosine, from a series of dihydrogenated precursors including festuclavine and dihydrolysergic acid[4]. However, since in axenic culture *S. sorghi* does not accept agroclavine into ergot alkaloid biosynthesis[4], specificity is also evident though for a mechanism which may be different from that proposed for other ergot fungi.

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0014-4754/85/081051-02$1.50 + 0.20/0

Comparative analysis of phospholamban phosphorylation in crude membranes of vertebrate hearts

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Summary. Phospholamban, a sarcoplasmic reticulum phosphoprotein, is present in the hearts of mammalian, avian, amphibian, and fish species. Phylogenetic changes are indicated by marked differences among species in cardiac phospholamban content and by the absence of Ca^{2+}/calmodulin-dependent phospholamban phosphorylation at an early developmental stage.
Key words. Cardiac muscle; phylogenesis; sarcoplasmic reticulum; phospholamban; protein kinase; Ca^{2+}-transport.

An important cellular reaction in the response of heart muscle to catecholamines is the phosphorylation of phospholamban in membranes of the sarcoplasmic reticulum[1]. Following β-adrenergic stimulation the protein is phosphorylated by a cyclic AMP-dependent protein kinase (cAMP-PK)[2]. Phospholamban phosphorylation is catalyzed also by a membrane-bound Ca^{2+}/calmodulin-dependent protein kinase (Ca-PK)[3]. The latter reaction possibly plays a role in the feedback control of heart cells in the presence of high intracellular Ca^{2+}. The present paper reports a comparative quantitative evaluation of phospholamban phosphorylation in crude membranes of various vertebrate hearts.

Materials and methods. Crude cardiac membranes were collected quantitatively by high speed centrifugation from homogenates of freshly excised and frozen hearts[4]. Human heart membranes were prepared from a small piece of ventricle tissue that had been removed 4 h post mortem. Membrane phosphorylation was carried out at 30 °C with 1 mg membrane protein per ml 40 mM histidine-HCl (pH 6.8), 0.12 M KCl, 10 mM $MgCl_2$, 15 mM NaF and 0.3 mM /γ-^{32}P/ATP (Amersham) of specific activity 25 mCi/nmole. Phosphorylated membranes were solubilized in 5% SDS, 1% β-mercaptoethanol, 0.1 mM EDTA, 50 mM Tris-H_3PO_4(pH 6.8), and separated in a SDS-urea-polyacrylamide system[5]. The concentrations of acrylamide and N, N'-methylenebisacrylamide were 12.5% and 0.83%, respectively. Following electrophoresis, gels were stained with Coomassie brilliant blue, destained, dried and autoradiographed with ORWO HS 11 film. M_r weight markers were human albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,500), cytochrome c (12,800), aprotinin (6500), and glucagon (3480). Oxalate-dependent Ca^{2+}-uptake was measured at 37 °C in 40 mM imidazol-HCl (pH 7.0), 100 mM KCl, 5 mM $MgCl_2$, 5 mM Na_2ATP, 10 mM K-oxalate, 10 mM NaN_3, 2 µM $NaVO_3$, 0.2 mM EGTA, 75 µM $^{45}CaCl_2$ (Amersham) of specific activity 15 µCi/µmole and 40 to 80 µg membrane protein[4].

Results and discussion. The identification of phospholamban in crude heart membranes is facilitated by the high phospholamban content, the effective phosphorylation of the protein by cAMP-PK and Ca-PK, and the unique behavior of phospholamban in SDS-polyacrylamide gel electrophoresis. There occurs a characteristic change in electrophoretic mobility consistent with dissociation of the phosphoprotein into subunits after heat treatment of solubilized phospholamban[6]. The M_r of phospholamban oligomer is 20,000–24,000. Estimates of the M_r of dissociated subunits vary between 6000[4] and 12,000[3]. Lowest M_r values of about 6000 are observed in SDS-urea-polyacrylamide systems adapted for analysis of small polypeptide chains[5]. Such a system has been used in the present work.

The figure demonstrates the electrophoretic separation of phosphoproteins contained in crude membranes of human, dog, frog,