Impaired Regulation of Aromatic Amino Acid Synthesis in a Mutant Resistant to p-Fluorophenylalanine

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SUMMARY

Coprinus lagopus is inhibited by the analogue of phenylalanine, p-fluorophenylalanine (pFPA). Mutant strain F, selected for growth on pFPA, is a slow grower which is not inhibited by the analogue. Over a prolonged period of growth, the amount of $^{14}$C$\text{pFPA}$ incorporated into the trichloroacetic acid-insoluble fraction of resistant F, mycelium is significant, but is less than 20% of the amount incorporated into similar fractions of the mycelium of the parental sensitive strain TCI. Resistant F, grown on minimal medium, excretes into the medium p-hydroxyphenylpyruvic acid, phenylpyruvic acid and a number of unidentified phenolic compounds. The sensitive TCI strain grown on minimal medium does not excrete, but when grown on media supplemented with phenylalanine or shikimate excretes traces of these compounds. The total biosynthesis of phenylalanine over 24 h by sensitive strain TCI is greatly reduced by the presence of exogenously supplied phenylalanine. No such reduction in synthesis is observed in the resistant mutant F,. It is inferred that the mutation impairs the regulation of phenylalanine biosynthesis, probably by the loss of feedback control at either the 3-deoxy-d-arabino-heptulosonate 7-phosphate (DAHP) synthetase or chorismate mutase.

INTRODUCTION

pFPA is a well-known toxic analogue of phenylalanine. Mutants resistant to the analogue have been selected in several species of bacteria and fungi (for reviews see Richmond, 1962; Fowden, Lewis & Tristram, 1967). In Escherichia coli, mutants resistant to pFPA have been found which excrete phenylalanine and are derepressed for the chorismate mutase P-prephenate dehydratase (Im & Pittard, 1971).

The present account describes a similar resistant mutant in a basidiomycete fungus Coprinus lagopus which secretes p-hydroxyphenylpyruvic and phenylpyruvic acids.

METHODS

Media. Minimal medium (MM) was that of Fries (1953) with the addition of magnesium sulphate, 0.5 g/l (Lewis, 1961). Supplements to minimal medium were added as sterile solutions, to give the required final concentration. Stocks were maintained on minimal medium with or without minimal supplements and with and without pFPA according to genotype. Current stocks were on Petri dishes at 4 °C, long-term reference stocks were kept both under oil and freeze-dried and stored at 4 °C. The resistant strain F, was isolated by L. A. Casselton (unpublished) as a mutant of the stock TCI.

Growth tests. Radial growth of strains on any solid medium was measured at 37 °C for
standardized inocula from cultures on the same medium, over a period of three days. Dry weight was measured by drying mycelium samples at 55 °C to constant weight.

**Liquid cultures.** Liquid cultures for the purpose of examining the growth medium were 250 ml cultures shaken at 37 °C for 3 days, then harvested by filtration. Each yielded 5 to 10 g wet weight mycelium.

**Chromatography.** Growth medium samples for chromatography were reduced to a small volume in a current of cool air. (a) Amino acids. Samples were desalted on Zeo-Carb 225 (Dunnill & Fowden, 1965), dried down, redissolved in water, and run as a descending system on Whatman no. 3 paper: direction 1, n-butanol-glacial acetic acid–water, 90:10:29 (by vol.); direction 2, phenol/ammonia (atmosphere in tank saturated with ammonia). (b) Keto-acids. Samples were acidified with 0.1 vol. glacial acetic acid, extracted three times with 1 vol ether, evaporated to remove the ether and acetic acid, then made up to known volume. Two-dimensional chromatography of free keto-acids was carried out on cellulose: direction 1, benzene–methanol–acetic acid, 45:8:4 (by vol); direction 2, aqueous acetic acid (6 %, v/v). For chromatography of dinitrophenolic-keto acids, phenylhydrazones were prepared by the method of Cavallini & Frontali (1954). The solvent system was t-amyl alcohol–ethyl alcohol–water, 50:10:40 (by vol.) (upper phase). Hydrogenated samples of phenylhydrazone preparations were prepared by hydrogenation in acid solution with a platinum catalyst. (c) Phenolic compounds. Growth medium was extracted as described in (b) for keto-acids. Chromatography was on cellulose–silica plates and solvent systems were: 1) 6 % acetic acid; (2) benzene–acetic acid–water, 6:7:3 (by vol.) (upper phase); (3) butanol–ammonia–water, 90:3:7 (by vol.).

**Measurement of incorporation of 14C compounds.** Mycelial samples from small-scale liquid cultures were harvested by filtration on to washed Oxoid discs, then each was washed once with 5 ml sterile minimal medium, and twice with 5 ml sterile distilled water. Each sample was then transferred on the Oxoid disc to a centrifuge tube and fractionated: (i) A few drops of toluene were added for 30 min then removed by evaporation. (ii) Ice-cold 10 % trichloroacetic acid (TCA) solution (2 ml) was added, and the sample homogenized, then centrifuged. (iii) The pellet was washed twice with 2 ml TCA. The pooled supernatants were the TCA-soluble fraction. (iv) The pellet was resuspended in 10 ml TCA and incubated at 60 °C for 30 min. The ‘hot TCA supernatant’ was removed by centrifugation. (v) Pellets were washed in acetone and the washings discarded. (vi) The TCA-insoluble fraction was washed in formic acid. (vii) Protein hydrolysates were prepared by hydrolysis of the sample as in (vi). The acid was removed from each TCA soluble fraction by extracting four times with 1.5 ml portions of diethyl ether. All TCA-soluble fractions and protein hydrolysates were desalted before chromatography.

**Radioactive counting.** In time-course experiments, portions of whole mycelium, TCA-soluble or TCA-insoluble fractions were dried on to a planchet and counted with a thin end-window Geiger–Muller tube (Mullard Mx123) connected to a 1700 scale (Isotope Development Ltd.; counting efficiency 4 %), or on a Packard scintillation counter. Compounds separated in paper chromatography were counted by cutting out the entire spot on the paper, drying it, and transferring the paper, cut into small pieces, directly into scintillation fluid (Blair & Segal, 1962). For all samples counting efficiency was 80 to 86 %.

**Glucose estimation.** Glucose was determined by the method of Marks (1959).
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Fig. 1. Distribution of radioactivity in fractions of Coprinus mycelium TCI and F7 after growth on \([^{14}C]pFPA\) for 12 h. Liquid cultures (2 ml) of each strain were grown in quadruplicate for 20 h on unsupplemented MM. 1.25 × 10^{-4} \text{M-^3[^{14}C]} \text{DL-pFPA} (1 \mu\text{Ci; 0.02 ml}) was then added to each culture, and incubation was continued for a further 12 h. [I], TCA soluble, ether insoluble fraction; [II], TCA soluble, ether soluble fraction; [III], cold TCA insoluble, hot TCA soluble fraction; [IV], cold TCA insoluble, hot TCA insoluble fraction.

RESULTS

Growth rate and pFPA utilization

The mutant strain F7, with 4.7 mm radial growth/24 h has a growth rate about two-thirds that of its parental wild-type TCI with 7 mm/24 h. The growth of TCI is completely inhibited by DL-pFPA at 10^{-4} \text{M}, at which concentration the growth of F7 is unaffected. Strain F7 has sparse growth with little or no aerial mycelium, and produces few oidial spores.

Incorporation of \([^{14}C]\text{phenylalanine}\) and pFPA

Over an extended growth period of 26 h on MM supplemented with (U-^{14}C)L-phenylalanine, the amount of phenylalanine incorporated into the protein of F7 is less than 5% of the amount incorporated by TCI. There is also a corresponding difference in the amount taken up from the medium.

After 12 h growth in liquid culture with [3-^{14}C]DL-pFPA, TCI mycelium contained three times as much radioactivity in the TCA-soluble fractions and eight times as much in the TCA-insoluble fractions as did F7 (Fig. 1). That the radioactivity in the fractions was due to \([^{14}C]pFPA\) was proved by chromatography and counting the appropriate spots.

The ATP pyrophosphate exchange stimulated by phenylalanine or pFPA was compared in the appropriate (40 to 60%) ammonium sulphate protein fractions from four Coprinus strains. Strain F7 was found to exhibit activity at similar levels to wild-type TCI, with either phenylalanine or pFPA as substrate. The reduced incorporation of pFPA into F7 protein is therefore unlikely to be caused by an altered phenylalanyl tRNA synthetase.

Excretion of aromatic keto acids, amino acids and phenolic compounds

The F7 culture medium had a characteristic odour which indicated the excretion of compounds into the medium.

The following tests were used to show that the excreted compounds included phenylpyruvic acid and p-hydroxyphenylpyruvic acid: (i) The ferric chloride test. Addition of ferric chloride to F7 medium gave a dark olive green colour which persisted for 1 h. (ii) Dinitrophenolic (DNP) compounds extracted from F7 growth medium were chromatographically identical with DNP-phenylpyruvic acid and DNP-hydroxyphenylpyruvic acid (from Sigma.
and Kodak Eastman). (iii) Hydrogenation of the DNP compounds yielded phenylalanine and a compound which is the result of hydrogenation of phenylalanine. It is ninhydrin positive and has identical \( R_p \) values to a hydrogenation product of authentic phenylalanine. (iv) The presence of \( p \)-hydroxyphenylpyruvic acid was confirmed by thin-layer chromatography of the free keto-acids, and u.v. spectroscopy.

A comparison of the size and intensity of DNP-keto acid spots from \( F_7 \) medium with those of the markers, leads to an estimate of 100 to 300 \( \mu \)g of phenylpyruvic acid and of \( p \)-hydroxyphenylpyruvic acid excreted per gram fresh weight mycelium. Similar tests on growth medium samples of the wild-type strain \( TC_1 \), and three other genetically distinct \( pFPA \) resistant mutants, gave no evidence of the presence of these keto-acids.

Strain \( F_7 \) was found to excrete u.v.-fluorescent compounds into the growth medium, while such compounds were not excreted by any other strain. Initial examination of an acid-ether extract by thin-layer chromatography, carried out by Dr J. B. Harborne, indicated that there was one major component, running with a high \( R_p \) value in solvents 1, 2 and 3 (see Methods), and at least eight minor components; all were u.v. fluorescent and gave a positive reaction with Folin's reagent. None of these compounds could be identified by their u.v. absorption or by mass spectral analysis.

### Glucose consumption

In liquid medium with 10, 5 or 2.5 mg glucose/ml over a period of 65 h, the mutant \( F_7 \) and \( TC_1 \) showed identical utilization of the glucose in the medium.

#### Utilization of exogenously supplied phenylalanine

The identification of \( F_7 \) as an excretor of compounds related to the aromatic amino acids suggests that in this strain control of biosynthesis of aromatic amino acids may have been lost. A clear difference should then be observed between \( F_7 \) and the \( pFPA \)-sensitive strain \( TC_1 \), when each is grown on an exogenous supply of phenylalanine. The phenylalanine incorporated by \( TC_1 \) should be all, or almost all, derived from the exogenous supply, whereas that incorporated from \( F_7 \) should be almost wholly derived from the main carbon source, i.e. glucose (Roberts et al. 1955; Clark & Rowbury, 1964).

Cultures of \( F_7 \) and of \( TC_1 \) were grown on MM containing \([^{14}C] \)glucose supplemented with varying amounts of non-radioactive phenylalanine for 24 h, then each culture was harvested, washed, fractionated, and the total radioactivity present in the protein hydrolysate from each culture determined. The phenylalanine present in each fraction was then isolated by chromatography and a sample taken for determination of radioactivity. The variation in activity of phenylalanine as compared with the total activity of each protein hydrolysate fraction with exogenous phenylalanine concentration is shown in Table 1.

For \( TC_1 \) the amount of phenylalanine synthesized from the glucose (i.e. \( ^{14}C \)-labelled) decreases sharply with increasing exogenous supply of phenylalanine. No such trend is observed for \( F_7 \).

It might be expected that if reduction of endogenous phenylalanine synthesis in \( TC_1 \) is due to a control mechanism operating on the phenylalanine biosynthetic pathway, no endogenous synthesis would occur at all in the presence of exogenous phenylalanine, rather than the reduction in synthesis observed. However, analysis of the growth medium of \( TC_1 \) after \([^{14}C] \)phenylalanine feeding experiments of the type described above indicated that after 24 h growth of \( TC_1 \) with an initial phenylalanine concentration of \( 10^{-4} M \), very little phenylalanine (1 to 2 % of the initial amount) would be left in the medium. Doy & Halsall (1968) have pointed out that for an aromatic amino acid-deficient mutant of Neurospora with an
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Table I. Effect of exogenously supplied phenylalanine on the synthesis of phenylalanine from [14C]glucose

The specific activity of each phenylalanine sample could not be accurately estimated owing to the minute amount of phenylalanine present in some samples. Therefore radioactivity in phenylalanine is expressed as a percentage of the total radioactivity in the protein hydrolysate. The use of this mode of expression rests on the assumption that for any one set of values, e.g. TCI protein hydrolysate, the amount of phenylalanine present will be a constant proportion of the total amount of amino acids in a sample of protein hydrolysate, regardless of the level of phenylalanine exogenously supplied.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Exogenous phenylalanine concn (M)</th>
<th>14C in total amino acids (A) (d.p.m.)</th>
<th>14C in phenylalanine (B) (d.p.m.)</th>
<th>B:A (%)</th>
</tr>
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<tbody>
<tr>
<td>TCI</td>
<td>0</td>
<td>(43 200)</td>
<td>(947)</td>
<td>(1:74)</td>
</tr>
<tr>
<td>Protein</td>
<td>10^-6</td>
<td>246 000</td>
<td>6200</td>
<td>2:52</td>
</tr>
<tr>
<td>Hydrolysate</td>
<td>10^-6</td>
<td>162 200</td>
<td>1519</td>
<td>0:94</td>
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<td>F7</td>
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<td>332 400</td>
<td>878</td>
<td>0:26</td>
</tr>
<tr>
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<tr>
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</tr>
<tr>
<td></td>
<td></td>
<td>183 300</td>
<td>2672</td>
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</table>

unaltered DAHP synthetase, growth on supplemented medium continues (i.e. aromatic amino acids are not exhausted) for a considerable period of time after DAHP begins to accumulate, i.e. after feedback inhibition has ceased to operate. It is therefore likely that towards the end of the growth period endogenous synthesis, though possibly completely inhibited in TCI at an earlier stage, would have started even in the sample with the maximum initial endogenous phenylalanine concentration.

DISCUSSION

In all organisms so far studied with respect to aromatic amino acid biosynthesis, phenylalanine is the product of a branched biosynthetic pathway, and in each case its synthesis is controlled at several points, as expected for a pathway from which intermediates are channelled in several directions (Gibson & Pittard, 1968).

The whole of the aromatic amino acid biosynthetic pathway has not been studied in Coprinus; however, it has been reported (Ahmed & Giles, 1969) that the five enzymes involved in the conversion of DAHP to 3-enol pyruvyl shikimic acid 5-phosphate (the precursor to chorismic acid) exist in Coprinus lagopus as a molecular aggregate similar to that reported for Neurospora crassa and other fungi. Control of this system has not, however, been studied in Coprinus. The evidence so far obtained from work with Neurospora and Saccharomyces would indicate that repression plays a negligible part in the control of aromatic amino acid biosynthesis in fungi (Lingens, Goebel & Uessler, 1967; Doy, 1967, 1968; Baker, 1966). Feedback inhibition operates in these organisms at several points on the pathway. In Escherichia coli there is a similar emphasis on feedback inhibition for aromatic amino acid biosynthesis (Im & Pittard, 1971).

As F7 excretes not only phenylpyruvic acid, the immediate precursor of phenylalanine on the biosynthetic pathway, but also p-hydroxyphenylpyruvic acid which is the immediate precursor of tyrosine, it might be deduced that loss of biosynthetic control is related to an enzyme situated on the pathway before the branch point of phenylalanine and tyrosine.
biosynthesis. This would indicate that the lesion in \( F \) is loss of feedback control of either the DAHP synthetase (the first enzyme on the aromatic amino acid biosynthetic pathway) or at chorismate mutase (the first enzyme after the branch point of the tryptophan pathway from that of phenylalanine and tyrosine).

Little is known of the effect of analogues on chorismate mutase activity. *In vitro* inhibition of *Escherichia coli* DAHP synthetase activity by pFPA has been reported (Smith, Ravel, Lax & Shive, 1962, 1964). A mutant of Neurospora isolated by De Busk, which is resistant to pFPA, has a DAHP synthetase which is no longer inhibited by phenylalanine (C. H. Doy, personal communication).

When \( F \) is present in a dikaryon with a pFPA-sensitive strain it is semi-dominant, as would be expected for a mutation involving loss of biosynthetic control.

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REFERENCES


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