Mutants of the Arginine-proline Pathway in *Aspergillus nidulans*

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SUMMARY

Proline suppressor mutant *su-19* was analysed for arginase and ornithine δ -transaminase (OTA) activity. Both enzymes are constitutively produced by this mutant. Five other suppressor loci not linked with *su-19* or to each other are involved in regulation of the two enzymes.

A mutant showing no OTA activity was isolated. Properties of this mutant and its effect in various strains of the *pro su* type were studied.

INTRODUCTION

Mutants of one (*su-19*) out of three proline suppressor loci studied previously in *Aspergillus nidulans* showed the constitutive synthesis of ornithine δ -transaminase (OTA), the enzyme involved in the conversion of ornithine to glutamic γ -semialdehyde (GSA) (Weglenski, 1966, 1967). It was suggested that due to high level of the OTA in *su-19* strain, ornithine is diverted from the arginine pathway and utilised for GSA and proline synthesis. Thus, mutations in the *su-19* locus were considered responsible for opening the alternative route of the proline synthesis which by-passes the metabolic block (between glutamate and GSA) in the major proline pathway. Assays for arginase showed that the activity of this enzyme is also higher than in the wild strain. However, the arginase results were not reliable as the assay procedure applied does not work well in *A. nidulans* (cf. Weglenski, 1967); moreover, all assays were carried out with strains of the *pro su* type and not of the *pro*⁺ *su* type. As previously shown (Weglenski, 1967), the presence of the *pro* mutation strongly influences the activity of the enzymes metabolizing arginine. The relations between the pathways of arginine and proline synthesis are shown in Fig. 1.

Fig. 1. *Aspergillus nidulans*: the pathway of proline and arginine synthesis. OTC, ornithine transcarbamylase; OTA, ornithine δ -transaminase.

The aim of the experiments presented in this paper was: (1) to obtain the *pro*⁺ *su-19* strain and to test if the *su-19* mutation affects only OTA or both OTA and arginase synthesis, (2) to test if other loci are involved in regulation of arginase and/or OTA synthesis, (3) to select OTA deficient mutants and to construct a strain blocked both in the major and in the alternative pathway of proline synthesis, which would make it possible to test decisively whether the opening of the alternative pathway is responsible for the suppression phenomenon in the case of *su-19* and other suppressor loci.

METHODS

Media and standard techniques used in this work were as in Pontecorvo *et al.* (1953).

Material. All proline suppressor mutants except *su-1* were obtained as spontaneous mutants from the *pro-6 pab-9 bio-1* or the *pro-6 ade-9 y phe-2* strains (Weglenski, 1966). Strain *pro-1 su-1* was obtained from the Department of Genetics, Glasgow University.

Isolation of mutants. Conidia of the *pro-6 su-19 pab-9 bio-1* strain were treated for 1 hr with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (500 µg./ml.). After removing mutagen conidia were resuspended in water and plated on the medium containing all required supplements. Colonies obtained were tested by needle inoculations for growth on a prolineless medium.

Isolation of the pro⁺ *su-19* strain. Locus *su-19* maps on chromosome I between *pro-1* (an allele of *pro-6*) and *ade-9* showing with these two loci about 6 and 4% recombination respectively. To obtain the *pro*⁺ *su-19* strain the following cross was made:

$$\begin{array}{ccccccc} \textit{pro-6 su-19} & + & \textit{pab-9} & + & \textit{bio-1} & & \\ + & + & \textit{ade-9} & + & \textit{y} & + & \end{array} .$$

From the progeny of this cross colonies of the *pab bio* phenotype were selected and tested in heterokaryons against the *pro-6 ade-9 y phe-2* strain. By this method *pro-6* can be distinguished from the *pro*⁺ colonies since those belonging to the former class will not form heterokaryons which can grow on the prolineless medium. To the latter class belong colonies of the *pro*⁺ *su*⁺ *pab-9 bio-1* and *pro*⁺ *su-19 pab-9 bio-1* genotypes, which can be distinguished after crossing them with the *pro-6 su-19* strain. If a colony used in the cross was *pro*⁺ *su-19*, no proline requiring progeny will be obtained.

Methods of culture and harvesting mycelium and of enzyme extraction and assay for OTA were as previously described (Weglenski, 1967).

Assay of arginase. 0.2 ml. of enzyme extract (equiv. 0.3 mg. protein) in 0.1 M. potassium phosphate buffer (pH 7.0) was preincubated at 37° for 1 hr with 0.1 µmole of MnCl₂. After preincubation 50 µmole of arginine-HCl, pH 10.0, were added and the reaction mixture was incubated for 20 min. at 37°; total volume of reaction mixture 1.0 ml. The reaction was stopped by adding 0.2 ml. 0.2 M-HCl. Urea formed in the reaction was split to ammonium by adding an excess of urease. Ammonium was estimated by the Conway microdiffusion method (Conway, 1962).

Determination of protein. Protein estimations were made according to the Lowry, Rosebrough, Farr & Randall (1951) method, with crystalline bovine serum albumin as a standard.

Abbreviations used: *pro* = proline; *ade* = adenine; *pab* = p-aminobenzoic acid; *phe* = phenylalanine; *bio* = biotin; *y* = yellow conidia; *su* = suppressor.

RESULTS

su-19 and other mutants constitutive for arginase and OTA synthesis

Activities of arginase and OTA were compared in *pro-6 su-19*, *pro⁺ su-19* and wild strains. The high level of synthesis of both enzymes results from the suppressor mutation and is not influenced by the presence or absence of the *pro-6* mutation (Table 1). Several other suppressor mutants were tested for arginase and OTA activities. Five suppressor mutants showed constitutive levels of both enzymes (Table 1). All these mutants are recessive and they complement with *su-19* and with each other. Crosses were made of all possible pairs of these suppressors. No linkage between suppressor loci was observed. One suppressor mutant which we studied, *su-1*, was isolated and mapped by Forbes (1956) in linkage group III. Two other mutants, *su-11* and *su-22* were assigned to linkage groups V and III respectively by means of mitotic haploidisation (Sawicka, personal communication).

Table 1. *Aspergillus nidulans*: specific activities of arginase and ornithine δ -transaminase (OTA) in wild, *ota-1* and various suppressor strains

Strain	Arginase activity (μ g. N-NH ₃ /mg protein/min.)	OTA activity (m μ mole ornithine/mg. protein/min.)
Wild	4.0	12
Wild, induced by arginine	39.1	About 200
<i>pro-6 su-19</i>	28.5	144
<i>pro⁺ su-19</i>	27.8	145
<i>pro-6 su-11</i>	26.7	163
<i>pro-6 su-22</i>	25.8	134
<i>pro-6 su-1</i>	23.8	128
<i>pro-6 su-30</i>	24.2	150
<i>pro-6 su-35</i>	26.5	171
<i>pro-6 su-19 ota-1</i>	30.2	About 1
<i>pro⁺ su⁺ ota-1</i>	3.8	About 1

Table 2. *Aspergillus nidulans*: crosses involving the *ota-1* mutant

Cross	Number of colonies tested	Number of <i>pro</i> colonies	% <i>pro</i>
<i>pro-6 su-19 ade-9 + ota-1</i>			
+ + + <i>pab-9</i> +	251	42	17
<i>pro-6 + pab-9 + ota-1</i>			
<i>pro-6 ade-9 + su-6 +</i>	225	168	75

Isolation and characterisation of the ota-1 mutant

The mutant isolation procedure applied to the *pro-6 su-19* strain provided several mutants unable to grow on the prolineless medium. They could represent: (1) back mutations in the *su-19* locus, (2) mutations in the major pathway of proline synthesis resulting in metabolic blocks between GSA and proline, (3) mutations in the alternative pathway of proline synthesis. Mutants of the first class can be easily distinguished as their growth responses are the same as of the *pro-6* strain which grows on proline or arginine. Only one mutant which does not respond to arginine, but still grows on

proline, was found; it will be henceforth referred to as *ota-1*. In order to distinguish whether this mutant belongs to the second or to the third class, it was crossed to the wild strain. The *pro-6* and *su-19* loci are closely linked, thus if the *ota-1* mutation is concerned only with the supplementary proline pathway, 25 % of proline requiring colonies should result from this cross rather than the 50 % which would be expected if *ota-1* blocks the major proline pathway. The result (Table 2) is close to the former figure. To test the third possibility, i.e. that the *ota-1* mutation causes a metabolic block in the alternative pathway of proline synthesis, arginase and OTA activities were assayed. Strains carrying the *ota-1* mutation show no OTA activity, while arginase activity remains unaffected (Table 1). The *pro-6 su-19 ota-1* strain was tested in a heterokaryon against the *pro-6 su-19 ota⁺* strain; *ota-1* appeared to be recessive to the *ota-1* allele.

Effects of the ota-1 mutation in the pro-6 su-6 strain

Mutations in the *su-6* locus result in partial deficiency of ornithine transcarbamylase, the enzyme which converts ornithine to citrulline (Weglenski, 1967). It was suggested that in suppressor strains of this type ornithine accumulates and becomes available for transamination to GSA. If this hypothesis is correct, the *ota-1* mutation introduced into the *pro-6 su-6* strain should cancel the effects of the suppressor on the proline mutation. The *pro-6*, *su-6* and *ota-1* loci are not linked; thus when *ota-1* cancels the *su-6* effect, one can expect 75 % of proline requiring colonies among progeny of the cross between *pro-6 su-6* and *pro-6 ota-1* strains. The results of this cross (Table 2) are in full agreement with this supposition.

DISCUSSION

The results presented in this paper show that the synthesis of arginase and OTA is controlled by the same regulatory system. Similar observations were made with *Bacillus subtilis* by De Hauwer, Lavallo & Wiame (1964). These authors demonstrated the co-ordinate induction of arginase and OTA together with arginine permease and isolated mutants constitutive for all three enzymes. In terms of Jacob & Monod (1961) operon model, *su-19* should be considered as a regulatory gene, while *ota-1* as the structural gene for OTA. However, the presence of five suppressor mutations mapping in different loci but resulting in the same effects as the mutation in the *su-19* locus suggests that the genetic regulation of arginine break-down enzymes is complicated and can not be explained by the previously proposed models.

From properties of the *ota-1* mutant it can be concluded that the arginine-ornithine-GSA pathway is the only one by which arginine (and ornithine) can be utilised for proline synthesis. The results clearly show that the proline suppressors observed in this study cause the opening of this particular pathway. However, the proline suppressor mutants not linked to the *pro-6* locus were found, which are active in the strain *pro-6 ota-1* being blocked both in major and alternative pathway of proline synthesis. The presence of suppressors of this type suggests a possibility for existence of a third pathway of proline synthesis in *Aspergillus nidulans*. Genetical and enzymological analysis of these mutants is now in progress.

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