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Use of Analogues and the Substrate-Sensitivity of Mutants in Analysis of Purine Uptake and Breakdown in *Aspergillus nidulans*

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Aspergillus mutants resistant to various purine analogues (purine, 8-azaguanine, 2-thioxanthine, and 2-thiouric acid) are defective in at least one step of purine uptake or breakdown. The properties of these mutants show that there are two uptake systems for purines, one which mediates the uptake of hypoxanthine, guanine, and adenine, and the other, xanthine and uric acid. Allantoinase-less strains are sensitive to the toxic effects of allantoin accumulation. They are severely inhibited when grown in the presence of naturally occurring purines. Mutant strains derived from these, resistant to naturally occurring purines, may be isolated. These are either wild-type revertants, or carry a second metabolic block in the uptake or breakdown of purines. The properties of these double mutants confirm the interpretation of the nature of the analogue-resistant mutants.

Mutants defective in the pathway of purine breakdown in the ascomycete *Aspergillus nidulans* have been described (5). They were selected for their inability to grow on hypoxanthine as a nitrogen source. This paper deals with the detailed analysis of the steps involved in the uptake of purines and the earlier stages of their breakdown. Work on mutants concerned with the regulation of this pathway and the induction and repression of the enzymes concerned will be published elsewhere (Scazzocchio and Darlington, *in preparation*).

The breakdown of purines in *A. nidulans* proceeds via the well-known pathway (5):

$$\text{Hypoxanthine} \xrightarrow{hx} (\text{Xanthine}) \longrightarrow$$

$$\text{Uric acid} \xrightarrow{uaZ} \text{Allantoin} \xrightarrow{alX} \text{Allantoate} \longrightarrow$$

Mutants lacking xanthine dehydrogenase alone (*hx* mutants), urate oxidase alone (*uaZ*), and allantoinase alone (*alX*) are known. These have been characterized by growth tests, enzyme assays, and genetic mapping. The *hx* mutants will grow on xanthine as nitrogen source, since there is an alternative pathway of conversion of xanthine to uric acid (Darlington and Scazzocchio, *unpublished data*). Mutants resistant to the effects of various purine analogues and defective in purine uptake or breakdown are described here.

Naturally occurring purines are toxic to strains lacking allantoinase. Double mutants derived from these, resistant to one or more of the purines, may be isolated. These strains all carry a block at some earlier stage of purine uptake or breakdown, which prevents the toxic accumulation of allantoin. Analysis of these strains confirms the interpretation of the nature of mutants resistant to the various analogues. A similar system for the selection of forward mutants has been used in the arabinose pathway in *Escherichia coli* (7). Further work on a class of mutants having low levels of xanthine dehydrogenase and urate oxidase, *ua*Y (5), is reported here.

MATERIALS AND METHODS

Techniques and media for the growth of *A. nidulans* were largely those described by Pontecorvo et al. (9). All the strains described here were derived from bi_1, a biotin-requiring auxotroph, wild type with respect to purine breakdown. It was obtained from the Department of Genetics, University of Glasgow. The production of mutants, and some of their properties, have been described (5). Since that publication, these mutants have been subdivided on the basis of complementation tests and genetic mapping, but are otherwise numbered identically; e.g., hx_{13} is now hxB_{13}.

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8-Azaguanine, 2-thioxanthine, and purine were obtained from the Sigma Chemical Co., St. Louis, Mo. 2-Thiouric acid was a gift of Professor Bergmann, Department of Pharmacology, Hebrew University of Jerusalem. In testing the effects of these compounds on *A. nidulans*, concentrations which gave a clear-cut difference between sensitive and resistant strains were chosen. Diethyl sulfate mutagenesis was carried out as described previously (5), and nitrosoguanidine mutagenesis was carried out as described by Adelberg, Mandel, and Chen (1), with 300 μg (per ml) of nitrosoguanidine.

RESULTS

8-Azaguanine. 8-Azaguanine inhibits the growth of *A. nidulans* on any nitrogen source, as has been reported previously (4, 8). Mutants resistant to its toxic effect (*azg* mutants) may be isolated (as sectors which form after 4 days of incubation) from strains inoculated onto medium containing 0.0017 M ammonium tartrate as nitrogen source and 0.1 mg (per ml) of 8-azaguanine. These mutants all have the same properties. They grow well on uric acid and xanthine as nitrogen sources, but poorly on hypoxanthine, adenine, and guanine. They have wild-type xanthine dehydrogenase activity. These properties suggest that they lack a system responsible for the uptake of hypoxanthine, adenine, and guanine. This is supported by the observation that these compounds protect wild-type *A. nidulans* from the inhibitory effects of 8-azaguanine.

Purine. Purine is also inhibitory to *A. nidulans*. Resistant sectors may be selected as described for 8-azaguanine, by use of 0.25 mg of purine per ml. Several different types of mutant are found, but some are cross-resistant to 8-azaguanine. This group has properties identical to the azaguanine-resistant mutants just described.

2-Thiouric acid. This compound is not toxic to *A. nidulans*. However, at a concentration of 0.1 mg/ml in the presence of 0.0017 M ammonium tartrate, it inhibits the formation of the green conidial pigment normally produced by the wild-type strain. On this medium, the wild type produces yellow conidia identical in appearance and pigment content to those produced under normal conditions by a yellow conidial (*y*) mutant (Scazzocchio, *in preparation*).

Mutants resistant to 2-thiouric acid produce green conidia on this medium. They fall into three classes.

(i) These mutants grow well on hypoxanthine and allantoin as nitrogen sources but poorly on uric acid and xanthine. They are therefore probably defective in a system responsible for the uptake of xanthine and uric acid. These strains will be called *uap* mutants.

(ii) These mutants (*ua*Y) are not inducible for xanthine dehydrogenase and urate oxidase but have normal uninduced levels (5). Neither mutants lacking xanthine dehydrogenase alone (*hx*) nor mutants lacking urate oxidase alone (*ua*Z) are resistant to 2-thiouric acid. This is consistent with the hypothesis that these mutants are defective in uric acid uptake in addition to xanthine dehydrogenase and urate oxidase. Further work on the nature of this mutation will be reported elsewhere.

(iii) There are mutants resistant to 2-thiouric acid which have no apparent defect in purine breakdown. They grow as well as the wild type on hypoxanthine, uric acid, or allantoin. Formation of the green pigment in these strains is presumably no longer subject to inhibition by 2-thiouric acid.

2-Thioxanthine. This analogue has the same effect on wild-type *A. nidulans* as does 2-thiouric acid. All mutants resistant to 2-thiouric acid are also resistant to 2-thioxanthine. In addition, all mutants lacking xanthine dehydrogenase are resistant, though sensitive to 2-thiouric acid. 2-Thioxanthine must be taken up by the same uptake system as 2-thiouric acid, since the same class of uptake mutant is resistant to both. However, 2-thioxanthine must undergo conversion to 2-thiouric acid in order to exert its effect. In vitro work has shown that it is a substrate for *A. nidulans* xanthine dehydrogenase. Resistance to 2-thioxanthine has been used as a system for studying mutagenesis in *A. nidulans* (Alderson and Scazzocchio, Abstr. IX Intern. Congr. Microbiol., Moscow, 1966, Abstr. A3/18).

Substrate sensitivity. The growth of *al*X mutants is severely inhibited on medium containing nitrate (0.01 M) as nitrogen source in the presence of an excess of any purine or allantoin (1 mg/ml if solubility permits, otherwise leaving a reservoir in the solid phase). The *ua*Z mutant is similarly inhibited, though not by allantoin, which it is able to break down. The wild type is not inhibited on these media; therefore, inhibition must be due to the accumulation of allantoin or uric acid, respectively, at the metabolic block. Strains derived from *bi* alX_4, which are resistant to the toxic effects of exogenous hypoxanthine, uric acid, or allantoin, may be selected by plating conidia treated with nitrosoguanidine on media containing 0.01 M sodium nitrate plus 1 mg (per ml) of the appropriate purine or allantoin. Revertants were eliminated by a preliminary screening on allantoin as nitrogen source.

Except for the *al*X *hx* double mutant, which was isolated from a cross between bi_1 alX_4 and a strain carrying *hx* B_{13}, representatives of all the types shown in Table 1 were found among the

TABLE 1. *Properties of double mutants*[a]

Double mutant	Resistant to	Sensitive to
1) *alX-azg*	Hypoxanthine, adenine, guanine	Xanthine, uric acid, allantoin
	8-Azaguanine, purine	2-Thioxanthine, 2-thiouric acid.
2) *alX-hx*	Hypoxanthine, adenine	Xanthine, uric acid, guanine, allantoin
	2-Thioxanthine	8-Azaguanine, purine, 2-thiouric acid
3) *alX-uaY*	Hypoxanthine, xanthine, uric acid	Allantoin
	2-Thioxanthine, 2-thiouric acid	8-Azaguanine, purine
4) *alX-uap*	Uric acid, xanthine	Hypoxanthine, allantoin
	2-Thioxanthine, 2-thiouric acid	8-Azaguanine, purine
5) *alX-alp*	Allantoin	Hypoxanthine, xanthine, uric acid
		Purine, 8-azaguanine, 2-thioxanthine, 2-thiouric acid

[a] Naming of mutants: *azg* = azaguanine-resistant, hypoxanthine permeaseless; *hx* = xanthine dehydrogenaseless; *uaY* = having noninducible xanthine dehydrogenase, urate oxidase, and a defect in uric acid uptake; *alX* = allantoinaseless; *uap* = uric acid permeaseless; *alp* = allantoin permeaseless. The compounds are subdivided so that the naturally occurring purines and derivatives, to which each mutant group is resistant or sensitive, appear on the first line; the analogues, on the second line.

strains derived from alX_4, which were resistant to hypoxanthine, uric acid, or allantoin. In addition, azaguanine-resistant mutants were isolated from an alX_4 strain as sectors on azaguanine medium. Their properties were identical to those of mutants selected by hypoxanthine resistance.

Representatives of each class of double mutant were outcrossed, and strains carrying the single mutation, in the absence of *alX*, were isolated. These all had the expected properties. One class, however, in which the double mutant is resistant only to allantoin, has not yet been described. This is consistent with a defect in allantoin permeation. As expected, single mutant strains, which grow well on hypoxanthine and uric acid but poorly on allantoin, can be derived from these.

The nitrogen source used in these experiments and its concentration are very critical. In general, nitrate (0.01 M) or ammonia at limiting concentrations (0.0017 M) were used. At higher concentrations of ammonia (0.0125 M), the effect of the analogues described on the wild-type, and of purines on *uaZ* and *alX*, strains was markedly reduced. Except in the case of 8-azaguanine, against which ammonia protects only partially, mutants and wild types are indistinguishable under these conditions. This is consistent with the hypothesis that ammonia represses, or inhibits, the enzymes and uptake systems involved. Ammonia repression has been observed in the case of xanthine dehydrogenase (Scazzocchio and Darlington, *in press*) but has not been directly demonstrated with any of the uptake systems.

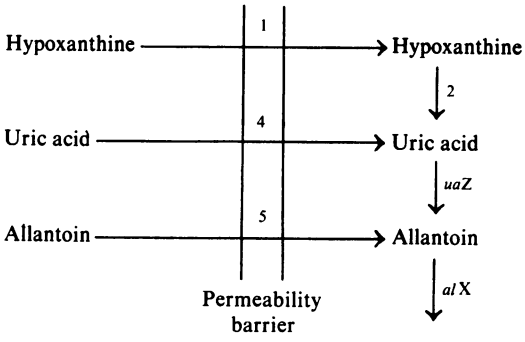

FIG. 1. *Mutational steps involved in purine uptake and breakdown.*

DISCUSSION

The scheme shown in Fig. 1 accounts for these results. Thus, resistance to azaguanine and purine is acquired by the loss of step 1; resistance to 2-thioxanthine but not to 2-thiouric acid, by the loss of step 2; and resistance to 2-thiouric acid, by the loss of step 4. Block 3, the *uaY* mutation,

has the same effect as a triple block (2 + 4 + *uaZ*). Xanthine is not included in this scheme; it is taken up by the same uptake system as uric acid, to which it may be converted by a pathway independent of xanthine dehydrogenase (Scazzocchio and Darlington, *unpublished data*).

The active uptake of purines has been demonstrated in a number of microorganisms. The yeast *Candida utilis* (10) and the alga *Chlorella pyrenoidosa* (2) have been shown to be able to take up and oxidize purines. The results given above show that two independent uptake systems of low specificity are involved in these processes in *A. nidulans*. One mediates the uptake of hypoxanthine, adenine, and guanine; the other, of xanthine and uric acid. From studies on the inhibition of growth of a guanine-requiring mutant of *Bacillus subtilis* by various purines, Demain (6) concluded that, as in *A. nidulans*, xanthine is taken up by a system independent of that responsible for the uptake of adenine, hypoxanthine, and guanine.

Preliminary direct experiments on the uptake of uric acid suggest that it is an inducible process in *A. nidulans*. Some of the enzymes involved in purine breakdown have been shown to be inducible in *C. utilis*, and probably repressible by ammonia (10). Our results on the effect of ammonia are consistent with repression of purine uptake. Berlin and Stadtman (3) have evidence suggesting that the rate of purine uptake in *B. subtilis* is regulated by the activity of pyrophosphorylases which remove purines from the intracellular pool. Such a mechanism is unlikely to operate in *A. nidulans*, however, in view of the rapid removal of intracellular purines by breakdown.

The uptake mutants are all to some extent leaky. Passive uptake may be responsible for this, at least in part. However, it is also possible that two or more active systems may contribute to the total rate of entry of each metabolite, though the existence of recognizable mutants demands that one system should predominate in each case. One possibility is that some hypoxanthine enters by the uric acid system, and some uric acid by the hypoxanthine system. However, a double mutant, *azg - uap*, grows no worse on hypoxanthine than a single *azg* mutant and no worse on uric acid than a single *uap* mutant. There may be other uptake systems responsible in part for the uptake of hypoxanthine and uric acid.

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