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Specific Regulatory Interconnection Between the Leucine and Histidine Pathways of *Neurospora crassa*

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Leucine auxotrophs of *Neurospora* fall into two discrete categories with respect to sensitivity to the herbicide, 3-amino-1,2,4-triazole. The pattern of resistance corresponds exactly to the ability to produce the leucine pathway control elements, α-isopropylmalate and the *leu-3* product. An analysis of the regulatory response of the production of enzymes of histidine biosynthesis to α-isopropylmalate implicates the control elements of the leucine pathway as important components of the mechanism governing the production of the target enzyme of aminotriazole inhibition, imidazoleglycerol-phosphate dehydratase (EC 4.2.1.19). The evidence suggests that the regulatory interconnection between the two pathways is direct and is independent of other general integrating regulatory mechanisms which appear to be operative in both pathways. A general method for isolating *leu-1* and *leu-2*, as well as other regulatory mutants, is described, which takes advantage of the specificity of the resistance to the inhibitor. Use of analogous systems is prescribed for the analysis of other regulatory interconnections which, like this one, might not be anticipated directly from structural or biosynthetic considerations.

3-Amino-1,2,4-triazole (aminotriazole) is an herbicide with a broad spectrum of inhibitory effects. It has been found to inhibit catalase (EC 1.11.1.6; 10, 20), purine biosynthesis (15), and mitochondrial protein synthesis in *Neurospora* (17). Aminotriazole, however, is best known as a specific inhibitor of imidazoleglycerol-phosphate dehydratase (IGP dehydratase), an enzyme of histidine synthesis in bacteria (13) and fungi (16). Because aminotriazole inhibition is almost completely reversed by histidine and because growth at inhibitory concentrations results in derepression of the enzymes of histidine synthesis, it has been tacitly assumed that imposition of histidine auxotrophy by aminotriazole is the primary cause for the cessation of growth of bacteria and fungi.

It was quite a surprise to learn that certain leucine auxotrophic *Neurospora* strains are resistant to aminotriazole, whereas others are not (24). It became clear that the pattern of resistance displayed by the *leu* mutants corresponds exactly to the pattern of production of the positive regulatory element of the leucine biosynthetic pathway, namely the α-isopropylmalate (αIPM)–*leu-3* complex, which is required for above basal expression of the three *leu* structural genes (11) and the major inducible amino acid permease (22). The regulatory role of the αIPM–*leu-3* complex has been shown to extend to the isoleucine and valine pathways, where it has been found also to play a crucial role in the response to pathway-specific signals (22).

The involvement of leucine pathway regulatory signals in the regulation of isoleucine and valine synthesis is not unexpected since the valine pathway provides the main precursor for leucine biosynthesis. The relationship between histidine and leucine biosynthesis, however, seems no closer than that of any random pair of amino acids. As a consequence, our attention was directed toward the possibility that the two might be indirectly related via a general regulatory system that has been referred to in *Neurospora* as "cross pathway regulation" (7) and as an overtly similar general amino acid control system in yeasts (9). Cross pathway regulation is observed as the simultaneous derepression of synthesis of enzymes of several different pathways, for example, those of histidine, arginine, and tryptophan biosynthesis, when growth of the organism is limited by depletion of any of the end products. Barthelmess (4), in fact, found that mutants deficient in cross pathway control display an increased sensitivity to aminotriazole. It seemed likely that the pattern of aminotriazole resistance of the different *leu* mutants might reflect the participation of leucine pathway-specific signals in cross pathway control. However, it is clear from the results reported below that, instead of involving a control mechanism responding to a more general set of signals, the systematic differences in the responses of *leu* mutants to aminotriazole reflect the involvement of a specific regulatory connection between the histidine and leucine pathways in which leucine pathway-specific signals participate in the control of the production of the *his* enzyme IGP dehydratase, the target of aminotriazole inhibition.

Although the relationship between *his*, *leu*, and aminotriazole resistance was discovered by chance, it seems very likely that the mutant selection procedure developed for its study will be useful, not only for the isolation of specific *leu* auxotrophic and regulatory mutants, but also as a general method for detecting regulatory interactions which cannot be deduced easily from known biosynthetic relationships.

MATERIALS AND METHODS

Biological. The sources of the mutant strains used have been described previously (11, 25).

For enzymology, mycelia were grown as previously described (12) with aeration in 1 liter of Vogel minimal medium (8), supplemented as indicated. Inocula consisted of mycelia which had been fully grown in 5 ml of minimal medium containing 10 μg of leucine per ml. Cultures were harvested

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by filtration, washed with water, pressed between paper towels, and weighed. One gram was immediately added to 10 ml of ice-cold 0.1 M Tris-hydrochloride (pH 8.1) and frozen at −70°C until used.

Selection for auxotrophic aminotriazole-resistant mutants was done by using conidia of either *inl* 89601A or wild-type STD8A. Conidia were washed twice with water, adjusted to 2×10^7/ml, and irradiated with UV light to 10 to 20% survival. They were then plated on sorbose medium containing 25 μg of inositol per ml, aminotriazole, and amino acid supplements as indicated in Table 2 and incubated at 34°C for 4 days. A fairly large background of very diffuse colonies was obtained at the usual plating concentration of 1×10^5 to 2×10^6 conidia per plate. This was especially apparent in arginine-supplemented medium, hence the increase in the aminotriazole concentration of the plating medium. In general, only well-defined, reasonably dense colonies were isolated onto aminotriazole-supplemented medium. At least four isolates were derived from cultures obtained from each original colony after streaking on medium without the inhibitor. These were tested for auxotrophy and resistance to aminotriazole. About half of the original isolates grew poorly, if at all, after the first subculture on aminotriazole and did not grow well in the absence of the inhibitor.

Growth rates were measured by the linear progression method of Ryan et al. (27), generally on 2 mM leucine-supplemented medium at 34°C.

Extract preparation. The mycelia were homogenized with a Tekmar Tissuemizer and intermittantly sonicated for a total of 45 s at 20 kc and 130 W. The solution was then centrifuged at 30,000 × *g* for 18 min. The supernatant was assayed for ATP phosphoribosyltransferase (EC 2.4.2.17) and isopropylmalate isomerase (IPM isomerase EC 4.2.1.33), and the other enzymes were assayed after desalting by passage through a Sephadex G-25 column (1 by 21 cm) equilibrated with 0.1 M Tris-hydrochloride (pH 8.1).

Enzyme assays. ATP phosphoribosyltransferase was assayed as described by Martin (21).

The IGP dehydratase reaction mixture was made up fresh within an hour of the assay and contained 0.043 μmol of $MnCl_2$, 0.43 μmol of dithiothreitol, and 2.0 μmol of imidazoleglycerol-phosphate in 0.45 ml of 0.1 M Tris-hydrochloride (pH 8.1). The reaction was started by the addition of 0.1 ml of enzyme and was run at 34°C; the blank contained no enzyme. After sampling for 60 min at 15-min intervals, the reaction was stopped by adding 0.7 ml of 1.5 N NaOH. Enzyme was then added to the blank, the incubation was continued for 30 min at 34°C, and the absorbance at 290 nm was measured.

Histidinol phosphatase (EC 3.1.3.15) was assayed in 0.9 ml of 0.1 M Tris-hydrochloride (pH 7.5) containing 0.5 μmol of histidinol phosphate. The reaction was started by the addition of 0.1 ml of enzyme, diluted 1:80 in 0.1 M Tris-hydrochloride (pH 7.5), and run at 34°C; the blank contained no enzyme. Samples were taken at 15-min intervals for 60 min, and the reaction was stopped with 1.5 ml of a solution of 6 parts 0.42% ammonium molybdate in 1 N H_2SO_4 to 1 part 10% ascorbic acid. Enzyme was added to the blank, and all were incubated at 45°C for 20 min. The absorbance at 820 nm was measured.

Histidinol dehydrogenase (EC 1.1.1.23) was determined by measuring the change in absorbance at 340 nm in a reaction mixture containing 1 μmol of $NaHCO_3$, 1 μmol of histidinol, 1.1 μmol of NAD, and 0.02 ml of enzyme in 1.0 ml of 0.1 M Tris-hydrochloride (pH 9.0) at 34°C. No manganese requirement was apparent in these preparations.

IPM isomerase was assayed as described by Gross (11).

Amino acid analysis. Intracellular amino acid levels were determined by the method of Barthelmess et al. (5), with minor modifications. Lyophilized mycelia (25 mg) was added to 5.0 ml of water and homogenized with a Tekmar Tissuemizer. The homogenate was sonicated at 20 kHz for 35 to 55 s at 100 to 130 W and centrifuged for 18 min at 30,000 × *g*. A sample of the supernatant was taken for protein determination, and trichloroacetic acid was added to the remainder to a final concentration of 10%. The solution was again centrifuged for 18 min at 30,000 × *g*, and the supernatant was ether extracted to neutrality. This solution was lyophilized and analyzed with a Beckman 6300 amino acid analyzer.

Protein determination. Protein was determined by the method of Lowry et al. (19).

Chemicals. αIPM and βIPM were isolated as described by Calvo and Gross (6). Imidazoleglycerol-phosphate was made by the method of Ames (3). All other chemicals were obtained from standard commercial sources.

RESULTS

Aminotriazole inhibition and leucine pathway control elements. The effects of aminotriazole on the rates of growth of several different auxotrophic and related strains are recorded (Table 1), and some general characteristics of the growth responses of sensitive and resistant strains to aminotriazole are illustrated (Fig. 1). Table 1 clearly shows that *leu* auxotrophic mutants fall into two well-defined categories, the highly resistant *leu-1* (βIPM dehydrogenase [EC 1.1.1.85] deficient) and *leu-2* (IPM isomerase deficient) mutants and the *leu-4* (αIPM synthetase [EC 4.1.3.12] deficient) and *leu-3* mutants which are 20- to 80-times more sensitive. *leu-3* and *leu-4* mutants are deficient in the production of the αIPM–*leu-3* regulatory complex which is necessary for switching on synthesis of the enzymes of the leucine pathway, whereas *leu-1* and *leu-2* mutants accumulate αIPM during growth and produce the normal *leu-3* product. The leucine pathway is difficult to repress in *leu-1* and *leu-2* mutants even when they are grown in the presence of a great excess of leucine. Feedback inhibition of the αIPM synthetase, though extensive, is insufficient to eliminate production of a small amount of αIPM which, in these strains, is retained because catabolism of the compound cannot occur unless it is first converted to leucine (12). Consequently, manipulation of the leucine concentration in which *leu-1* and *leu-2* mutants are grown has little effect on the lag period before maximum growth rate is attained. In those strains in which there is no constraint on αIPM catabolism, raising the internal concentration of the inducer by elimination of feedback control of its production, as in the case of the feedback-insensitive mutant *leu-4*^{FLR92}, results in increased aminotriazole resistance (Table 1), as well as an increase in the production of the leucine biosynthetic enzymes (11).

In general, the growth responses of the various strains show a near-perfect correlation between the status of the leucine pathway control system and resistance to aminotriazole. This correlation extends even to the subtle differences among the *leu-3* regulatory products produced by the different *leu-3* mutant alleles. The R156 *leu-3* product is less effective than that of R203 or R229 in provoking IPM isomerase and βIPM dehydrogenase production. The data obtained indicate that *leu-3* R156 is more sensitive than the others to aminotriazole inhibition. R229-R21, which contains an intracistronic suppressor of *leu-3* R229 that leads to constitutive low-level expression of the leucine pathway (25), is more resistant to aminotriazole than are strains

TABLE 1. Growth rate inhibition by aminotriazole

Locus, phenotype	Allele	0.5 mM αIPM	Aminotriazole (mM) yielding 50% inhibition	Histidine plus aminotriazole maximum growth rate[a] (%)
leu-1	D221		20.0	
leu-2	D3		20.0	
leu-3	R156		0.25	88
leu-3	R229		0.5	
leu-3	R203	±	0.5	
leu-3^{cc}	R229-R21		2.0	
leu-4	R59		1.0	90
Wild type	STD8A		2.0[c]	85
leu-4^{fir} [b]	*leu-4*^{FLR92}		10.0[c]	
leu-1, *leu-4*, ut$^+$	D221, R59, *ipm-1*, *ipm-2*	−	1.0	
		+	>4.0	
leu-2, *leu-4*, ut$^+$	α6α, R59, *ipm-1*, *ipm-2*	−	1.0	
		+	>4.0	
leu-3, *leu-4*, ut$^+$	R203, R59, *ipm-1 ipm-2*	−	1.0	
		+	1.0	
leu-4, ut$^+$	R59, *ipm-1*, *ipm-2*	−	1.0	
		+	2.0	

[a] The medium contained 0.5 mM histidine and 10 mM aminotriazole.
[b] Produces an αIPM synthetase that is insensitive to feedback inhibition (*fir*) by leucine.
[c] With or without 2 mM leucine.

bearing only the original R229 allele. In analogous fashion, the wild-type strain is considerably more resistant to aminotriazole than are *leu-3* and *leu-4* mutant strains but is more sensitive than the aforementioned nonauxotrophic feedback-insensitive strain, *leu-4*^{FLR92}. Finally, and perhaps most informative, strains which are permeable to αIPM (designated ut$^+$, a phenotype determined by the two genes *ipm-1* and *ipm-2* [26]) and produce a functional *leu-3* product become more resistant to aminotriazole when αIPM is provided. This response is demonstrated clearly by the multiple mutants *leu-1*, *leu-4*, ut$^+$ and *leu-2*, *leu-4*, ut$^+$, as well as by the *leu-4*, ut$^+$ strain itself, in contrast to *leu-3*, *leu-4*, ut$^+$. In fact, in the absence of αIPM they are just as sensitive to aminotriazole as are the *leu-4* mutants from which they were derived. Resistance of these multiple mutants to aminotriazole is not maximal in the presence of αIPM, presumably because only

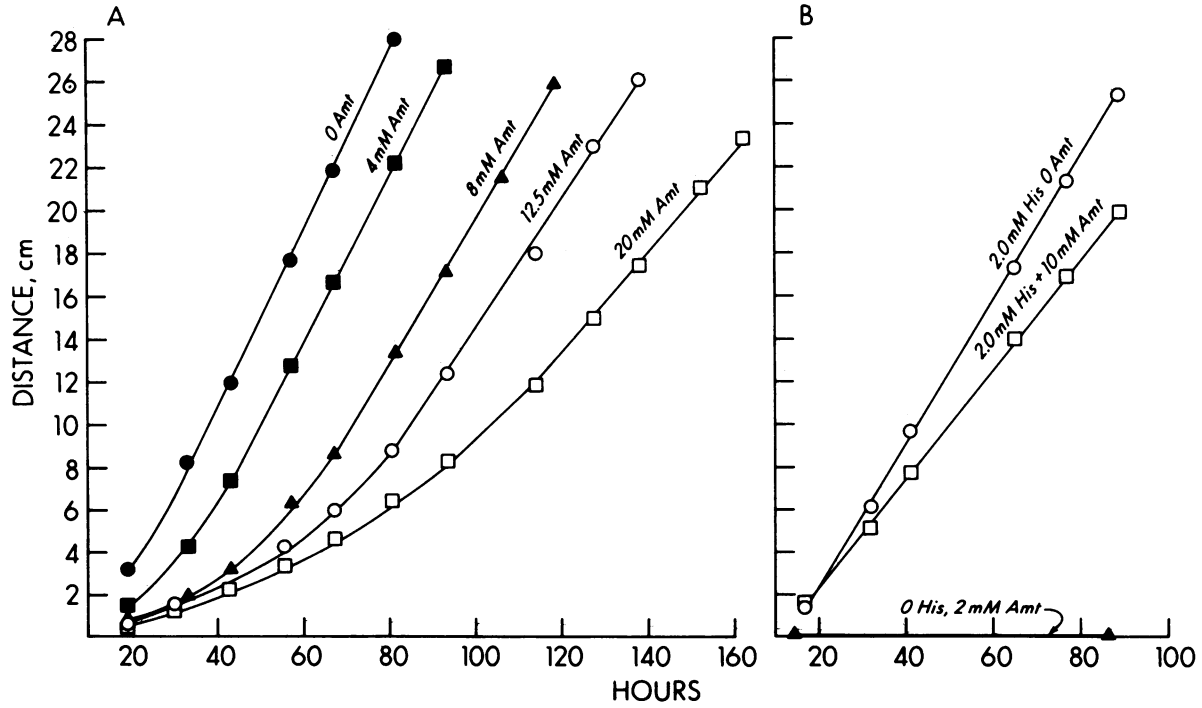

FIG. 1. Inhibition of linear growth by aminotriazole and its reversal by histidine. (A) Inhibition as a function of aminotriazole (Amt) concentration of the resistant strain D3 (*leu-2*); (B) Reversal of aminotriazole inhibition of the sensitive strain R156 (*leu-3*) by histidine. Leucine (2 mM) was present in all tubes.

a small amount of the inducer permeates the cell (26). The endogenous levels of αIPM attained by ut^+ strains have been found sufficient to induce considerable synthesis of the leucine biosynthetic enzymes, but the endogenous αIPM concentration never reaches a high enough level to replace fully the leucine requirement for growth of such strains. Blocking the conversion of αIPM to leucine by interposing a *leu-1* or *leu-2* mutation in *leu-4*, ut^+, however, was found to increase the sensitivity of the pathway to induction (26) and, as indicated in Table 1, also to increase the level of resistance to aminotriazole.

As might have been expected from the many different inhibitory effects claimed for the compound, the effect of aminotriazole on the growth of even the most-resistant strain (Fig. 1A) appears quite complex. The lag in attaining maximum growth rate in the presence of aminotriazole is reasonably proportional to the concentration of the inhibitor but is measured in days rather than hours. As expected, histidine nearly completely reverses aminotriazole inhibition (Fig. 1B). This reversal is rapid (at least within a few hours) and specific; no other amino acid, vitamin, purine, or pyrimidine by itself has been found to significantly reverse the inhibition. Limitation of available leucine to *leu-1* and *leu-2* mutants does have a dose reduction effect, but this effect is noted only by a small shortening of the lag before maximum growth rate is attained. The data, then, conform to the general conclusion that reversal of aminotriazole inhibition by histidine is direct. Reversal in response to the leucine pathway control signals, however, appears metabolically much more complex.

Aminotriazole as a selective probe for regulatory system interconnections. It seemed clear that the specificity of the resistance pattern of the *leu* mutants to aminotriazole not only provided a specific method for selecting for newly arisen *leu-1* and *leu-2* mutations but also provided a general method for probing the metabolic circuit for as yet undetected connections between the regulation of the histidine enzymes and the regulation of other pathways. As a check of the feasibility of using aminotriazole resistance as a probe for other regulatory interconnections, the efficiency of the method was determined by measuring the frequency of *leu* mutants among newly induced aminotriazole-resistant mutants. About 10% of the colonies obtained after UV irradiation and selection for resistance to aminotriazole in the presence of leucine are indeed *leu-1* and *leu-2* auxotrophs (Table 2). For unknown reasons, about half of the colonies isolated as aminotriazole resistant grew poorly on secondary transfer to medium containing the inhibitor. All of the *leu* mutants, however, are highly resistant and grew well. The frequency of *leu* mutants, if calculated on the basis of those that grew well upon secondary transfer, is about twice that indicated in the table. The aminotriazole resistance selection method appears to be much more efficient in yielding *leu-1* mutants than is the inositol-less death method (see pooled mutant hunt data of Table 2); the tightness of the block is probably less crucial for aminotriazole resistance than for surviving inositol starvation.

Searches for other specific auxotrophic mutations leading to aminotriazole resistance have been fruitless so far. Though by no means exhaustive, selection on tryptophan, arginine, and complete media yielded some auxotrophic mutants, but each of them has been found to be complex and, as distinct from *leu-1* and *leu-2* mutants, to contain mutations for resistance which segregate easily from the auxotrophic mutation. It was found further that some of the mutants obtained involved complex chromosomal aberrations. The leaky *leu-4* mutant displayed a tetrad spore pattern characteristic of a complex translocation (23). When backcrossed to wild type, this mutant yielded temperature-sensitive lethal, as well as nonauxotrophic aminotriazole-resistant, segregants, some of which secreted riboflavin or a derivative thereof during growth. (Many of the nonauxotrophic aminotriazole-resistant strains secrete riboflavin; a few secrete a red fluorescent compound tentatively identified as coproporphyrin.) The *leu-1* mutation SLm-1, upon genetic analysis, appeared to involve a translocation of the *leu-1* gene from linkage group III to I. Cytological analysis confirmed the genetic analysis but showed that the breakpoints are quite far from the mutant gene itself (E. Barry, personal communication). It seemed clear, then, that the experiments

TABLE 2. Selective enrichment of auxotrophic mutants by aminotriazole

Strain[a]	Amino-triazole (mM)	Supplement	Isolates	Auxo-trophs	Genotypes (no.)
89601A (*inl*)	10	Leucine	180	21	*leu-2* (16) *leu-1* (5)
	20	Arginine	195	1	complex (*arg meth* B_1)[b]
	15	Tryptophan	180	0	
89601A (*inl*)	20	Arginine	122	0	
	15	Tryptophan	121	0	
89601A(*inl*)	10	Adenine	82	0	
STD8A (wild type)	10	Leucine	85	5	*leu-2* (4) *leu-4* (1)
	10	Complete[c]	45	2	*leu-1* (1) *trp* (1)
89601A (*inl*)	0	Leucine	D series mutant hunts summary[d]		*leu-2* (154) *leu-4* (56) *leu-1* (1)

[a] Conidia were mutagenized by irradiation with UV light to 10 to 20% survival.

[b] The original isolate of this mutant was an unstable thiamine auxotroph which yielded *arg* and *meth* segregants from a cross to wild type.

[c] Synthetic medium supplemented with 0.1 g of peptone (Difco Laboratories) and 0.1 g of yeast extract per liter. This is 2% of the usual complete supplement. Only large, well-defined colonies were isolated.

[d] A series of mutant hunts by the inositol-less death selection procedure (18) after UV irradiation followed by plating on leucine-supplemented medium.

had been carried out to the level of mutational noise, at which point the selective agent, itself a carcinogen (14), might contribute irrelevant mutational and phenotypic complications.

A potentially useful byproduct of the selection procedure described here is a way to isolate *leu-1* and *leu-2* mutants in a *leu-4*, ut$^+$ background. *Leu-4*, ut$^+$ strains, although permeable to αIPM, convert it to leucine and are therefore only slightly more resistant to aminotriazole in the presence of αIPM than in its absence (Table 1). *leu-1*, *leu-4*, and *leu-2*, *leu-4* double mutants are frequent (ca. 10%) among aminotriazole-resistant strains obtained after UV mutagenesis followed by selection as aminotriazole resistant in the presence of αIPM and leucine. It was in this way that the *leu-2*, *leu-4*, ut$^+$ strain α6α, R59 was obtained. Synthesis of such a strain by recombinational means is slow because of the need to identify the *ipm* genotype of segregants by secondary crosses.

Production of histidine biosynthetic enzymes by *leu* auxotrophs. The data obtained from independent measurements of the specific activities of four *his* enzymes in extracts of the four different classes of *leu* mutants grown on limiting or excess leucine and of the wild-type strain grown without added leucine are presented in Table 3. One of the enzymatic activities measured, that of histidinol dehydrogenase, is actually a measure of the *his-3* complex which also includes phosphoribosyl-AMP pyrophosphorylase and phosphoribosyl-AMP cyclohydrolase (collectively called phosphoribosyl-AMP cyclohydrolase [EC 3.5.4.19]), which catalyze the second and third steps of the pathway (1, 2). The results obtained, then, pertain to 6 of the 10 or so enzymatic activities of histidine biosynthesis. Despite some heterogeneity with respect to strain and enzyme, two differences between aminotriazole-resistant *leu* mutants and -sensitive ones are apparent. The difference in production levels of IGP dehydratase appears more likely to be significant with respect to resistance. The activity of this, the target enzyme of aminotriazole inhibition, is high in *leu-1* and *leu-2* grown in low-leucine medium. In fact, IGP dehydratase production by *leu-2* appears to be high even when synthesis of the other enzymes of histidine biosynthesis is extensively repressed by growth in excess leucine. In contrast, IGP dehydratase production by the sensitive strains decreases rather significantly when grown under leucine limitation. Sensitive and resistant mutants also differ with respect to the production of the other three *his* enzymes. In contrast to *leu-1*, in which the three enzyme activities are approximately twofold higher when grown on low leucine, in *leu-4* only the level of production of histidinol dehydrogenase is increased significantly, whereas in *leu-3* only ATP phosphoribosyltransferase production is unresponsive to signals generated by leucine limitation. It should be emphasized, however, that though the responses of sensitive and resistant strains differ markedly with respect to ATP phosphoribosyltransferase, histidinol dehydrogenase, and histidinol phosphatase production, these three enzymes are produced by sensitive strains at levels close to, and in some cases even higher than, resistant strains.

Kinetics of IGP dehydratase production in response to αIPM. Although the data obtained indicate that the response of *his* enzyme production by aminotriazole-sensitive and -resistant strains is quite complicated, they show, more significantly, that the regulation of IGP dehydratase production differs markedly from that of the other enzymes of the pathway. It seemed clear from the outset of the enzymatic analysis, however, that any role of leucine pathway-specific signals in *his* enzyme regulation might be obscured by secondary effects related to general regulatory signals coupled to the state of nutrition of the *leu* strains. Consequently, an experiment was required which allowed an unambiguous distinction between the effect of leucine pathway-specific signals and any others affecting the system that might be perturbed by the growth regimen. To accomplish this, we repeated an experiment that was originally designed to measure the time of onset and rate of IPM isomerase production in response to the inducer, αIPM (26), this time measuring the activity of the histidine biosynthetic enzymes. Use was made of a *leu-1*, *leu-4*, ut$^+$ multiple mutant, which is permeable to αIPM. This strain, lacking αIPM synthetase and βIPM dehydrogenase, can neither produce αIPM nor convert exogenously supplied αIPM to leucine. Such a multiple mutant, when grown in excess leucine will com-

TABLE 3. Histidine enzyme levels produced by *leu* mutants grown on high and low concentrations of leucine

Strain	Leucine (mg/liter)	Sp act (nmol/min per mg of protein)[a]			
		ATP phosphoribosyltransferase	Histidinol phosphatase	Histidinol dehydrogenase	IGP dehydratase
leu-1	50	31.9 ± 2.3 (4)	164.8 ± 15.9 (3)	35.4 ± 3.8 (7)	5.44 ± 1.20 (7)
	500	16.9 ± 1.3 (4)	79.7 ± 7.8 (3)	13.1 ± 2.3 (7)	3.07 ± 0.52 (7)
	50/500 Ratio	1.89	2.07	2.70	1.77
leu-2	50	33.0 ± 4.2 (3)	197.2 ± 13.1 (3)	31.8 ± 6.9 (6)	5.05 ± 0.69 (6)
	500	18.1 ± 2.1 (5)	70.4 ± 6.6 (5)	11.6 ± 2.5 (5)	4.82 ± 0.21 (5)
	50/500 Ratio	1.82	2.80	2.74	1.05
leu-3	50	22.7 ± 3.8 (3)	211.4 ± 4.9 (3)	45.8 ± 7.4 (6)	1.80 ± 0.31 (6)
	500	21.9 ± 1.5 (3)	129.2 ± 19.9 (3)	26.7 ± 1.6 (3)	2.92 ± 0.57 (3)
	50/500 Ratio	1.04	1.64	1.72	0.62
leu-4	50	24.9 ± 0.5 (3)	92.9 ± 9.1 (3)	41.2 ± 4.1 (6)	2.18 ± 0.30 (6)
	500	21.9 ± 1.1 (3)	108.1 ± 7.5 (3)	20.3 ± 1.5 (3)	2.90 ± 0.16 (6)
	50/500 Ratio	1.14	0.86	2.03	0.75
STD8A	0	17.4 ± 3.1 (4)	78.0 ± 5.1 (3)	16.4 ± 2.0 (4)	3.05 ± 0.28 (4)

[a] The numbers within parentheses indicate the number of independent cultures assayed.

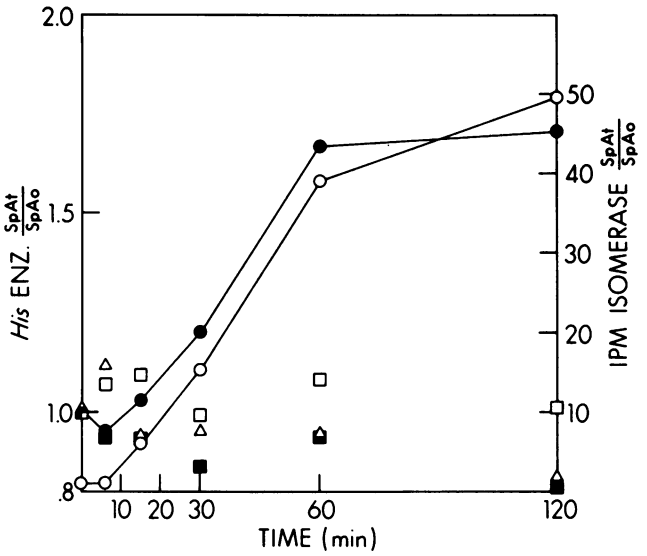

FIG. 2. Kinetics of induction by αIPM of histidine biosynthetic enzymes and IPM isomerase in a *leu-1*, *leu-4*, ut$^+$. Mycelia were grown at a high leucine concentration (500 mg/liter) for 33 h (3 to 4 g of growth). αIPM was added (25 mg/liter), and the cultures were incubated further for the time periods indicated before harvesting. The data plotted are the ratios of the specific activity at time *t* (SpAt) after the addition of αIPM to the specific activity at the time of addition (SpAo). Each point is an average of duplicate determinations. ●, IGP dehydratase; □, ATP phosphoribosyltransferase; △, histidinol phosphatase; ■, histidinol dehydrogenase; ○, IPM isomerase.

mence synthesis of IPM isomerase within 10 min after the addition of the inducer (26). In the experiment illustrated in Fig. 2, in addition to IPM isomerase, the specific activity of each of the histidine biosynthetic enzymes was measured as a function of time after the addition of the inducer. The data show clearly that, of the four histidine enzymes measured, only the aminotriazole target enzyme, IGP dehydratase, increases significantly after the addition of αIPM and that the time and course of induction, within the limits of detection of the experiment, appear to coincide with those of IPM isomerase. Indeed, the rapidity of the increase in activity suggests either that αIPM caused an increase in the rate of transcription or translation or both or that some kind of enzyme activation was involved. We have shown separately that no increase in IGP dehydratase activity follows the addition of αIPM in the presence of 4 μg of cycloheximide per ml. It seems likely, then, that the increase stemmed from some direct effect of the leucine pathway-specific inducer on the *his-1* gene or its transcript.

The histidine content of *leu-1* and *leu-4* mutant strains was measured to determine whether there was a direct correlation among the internal concentration of the amino acid, *his* enzyme activity, and aminotriazole resistance. The two strains grown on 0.2 mM leucine had the same internal histidine concentration (56 nmol per mg of protein). When grown on relatively high concentrations of leucine (2 mM), the internal histidine concentration was actually lower (20 nmol per mg of protein) in the resistant strain, *leu-1*, than in the sensitive one, *leu-4* (31 nmol per mg of protein). The difference between *leu-1* and *leu-4* on high concentrations of leucine may be due to some competition for retention between leucine and histidine, perhaps correlated with amino acid transport, which appears to be quite different in the two strains (22). Clearly, resistance to aminotriazole is not reflected by an increased level of histidine production in the absence of the inhibitor. Indeed, the data indicate that, despite differences in histidine biosynthetic enzyme activities in the two strains when grown on limiting concentrations of leucine, the regulatory system maintains histidine production constant relative to protein synthesis.

DISCUSSION

At the outset of these investigations, it seemed likely that the pattern of resistance to aminotriazole resulted from the potentiation of some general control mechanism which coupled the regulation of the synthesis of enzymes of the leucine and histidine pathways via αIPM and the *leu-3* product. The system thought likely to be involved was cross pathway regulation, which appears to be expressed whenever one of several amino acids becomes growth rate limiting (7). The evaluation of the role of the various regulatory signals that might be involved in aminotriazole resistance seemed difficult because restriction of histidine production by the inhibitor would be expected to perturb histidine pathway-specific signals, as well as a potential galaxy of interacting systems that monitor the nutritional status of the cell. This was especially troublesome because no *his*-specific regulatory mutants of *Neurospora*, feedback negative or otherwise, were available, and as a consequence, there was no simple way to distinguish between the effect of pathway-specific and general control signals on the system (a problem that is especially complicated when regulatory interactions are likely to be interdependent and the amplitude of variation of enzyme production is relatively small [7]). Indeed, only the antithetical response of IGP dehydratase production by *leu-3* and *leu-4* strains grown on limiting concentrations of leucine was of sufficient magnitude to suggest the involvement of some kind of a site-specific response to a leucine pathway-specific signal. It was therefore quite gratifying to be able to show, in an experiment presumably free of extraneous perturbations, that αIPM acts specifically as an inducer of IGP dehydratase production. The kinetics of the increase in IGP dehydratase activity after the addition of the inducer are rather rapid, following closely those of IPM isomerase activity, and require protein synthesis for expression. As a consequence, it seems likely that the effect of αIPM is on the rate of transcription of the *his-1* gene, as has been shown to be the case for *leu-2* (26).

The evidence obtained, then, indicates that it is the target enzyme of aminotriazole inhibition, IGP dehydratase, whose synthesis is controlled, at least in part, by the regulatory elements of the leucine pathway. It is this coincidence that is reflected in the resistance pattern of the *leu* auxotrophs. Thus, it appears likely that binding the *leu* regulatory signals at some nucleotide sequence at or near *his-1*, which is homologous to the corresponding recognition sites of the *leu* genes, is responsible for the increased level of IGP dehydratase synthesis. The changes in enzyme levels observed, however, appear not to have affected the concentration of endogenous histidine in any of the strains in a way that would be expected to have an impact on aminotriazole resistance. The changes do not seem sufficient to lead to the very large differences in sensitivity to the inhibitor characteristic of the different *leu* mutants, the extended time it takes for maximum growth rate to be obtained, or the apparent diversity of avenues by which resistance can be obtained. We pointed out in the introductory section that

aminotriazole has been found to have a rather broad spectrum of inhibitory effects. Some of these, like the inhibition of catalase (20), seem very remote from histidine biosynthesis and may not involve processes essential for growth. Others, like the inhibition of mitochondrial protein synthesis (17), may be related indirectly to histidine synthesis by an effect on respiration and ATP synthesis. The inhibition of purine biosynthesis by aminotriazole has been observed in yeasts and *Salmonella typhimurium* (15) and may be reflected indirectly by the plethora of riboflavin-secreting mutants obtained among the aminotriazole-resistant mutants. Aminotriazole may inhibit histidine synthesis not only via its effect on IGP dehydratase but perhaps also by inhibiting the synthesis of a direct precursor of histidine, ATP. The return to the normal growth rate, then, is likely to require a good deal of metabolic reshuffling before the endogenous level of histidine production becomes high enough to reverse aminotriazole inhibition. This raises the question as to whether the effect on IGP dehydratase production by αIPM is sufficient to account for full resistance or, instead, other synergistic effects of αIPM on the system, in addition to the increase in dehydratase activity, increase the rate of histidine production. In any case, we are led to wonder whether IGP dehydratase, which can be viewed as the first step in histidine biosynthesis that is completely divergent from purine biosynthesis, plays a heretofore unappreciated role in coordinating purine and histidine synthesis.

General application for study of other regulatory interconnections. We expect that the *leu-his* interconnection is not unique and that the methodology used to analyze this small part of the regulatory network might have general applicability in the search for and analysis of other interacting systems. We expect to be able to detect other interconnections involving αIPM and the *leu-3* product by checking resistance patterns of the *leu* auxotrophs to other pathway-specific inhibitors and to extend the investigation to other systems in which the genetics of pathway-specific regulatory signals are manageable. The efficiency and specificity of selection for aminotriazole-resistant mutants also allow use of this system in the analysis of events leading to interference with the fidelity of transcription and translation of the *leu-1* and *leu-2* cistrons. This might be very useful in the recombinant DNA technology of *Neurospora* since this system allows direct selection for insertions, deletions, and translocations.

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LITERATURE CITED

1. **Ahmed, A.** 1968. Organization of the *histidine-3* region of *Neurospora*. Mol. Gen. Genet. **103**:185–193.
2. **Ahmed, A., M. E. Case, and N. H. Giles.** 1964. The nature of complementation among mutants in the *histidine-3* region of *Neurospora crassa*. Brookhaven Symp. Biol. **17**:53–65.
3. **Ames, B. N.** 1957. The biosynthesis of histidine: D-*erythro*-imidazoleglycerol phosphate dehydrase. J. Biol. Chem. **228**:131–143.
4. **Barthelmess, I. B.** 1982. Mutants affecting amino acid cross-pathway control in *Neurospora crassa*. Genet. Res. **39**:169–185.
5. **Barthelmess, I. B., C. F. Curtis, and H. Kacser.** 1974. Control of the flux to arginine in *Neurospora crassa*: derepression of the last three enzymes of the arginine pathway. J. Mol. Biol. **87**:303–316.
6. **Calvo, J. M., and S. R. Gross.** 1970. Isolation and chemical estimation of α-isopropylmalate and β-isopropylmalate. Methods Enzymol. **17**:791–793.
7. **Carsiotis, M., R. F. Jones, and A. C. Wesseling.** 1974. Cross-pathway regulation: histidine-mediated control of histidine, tryptophan, and arginine biosynthetic enzymes in *Neurospora crassa*. J. Bacteriol. **119**:893–898.
8. **Davis, R. H., and F. J. de Serres.** 1970. Genetic and microbiological research techniques for *Neurospora crassa*. Methods Enzymol. **17**:79–143.
9. **Donahue, T. F., R. S. Daves, G. Jucchini, and G. R. Fink.** 1983. A short nucleotide sequence required for regulation of *his-4* by the general control system of yeast. Cell **32**:89–98.
10. **Gressly, R., and H. Aebi.** 1959. Temperaturabhangigkeit der Wirkung Vershiedener Katalase-Inhibitoren. Helv. Chim. Acta **42**:1683–1691.
11. **Gross, S. R.** 1965. The regulation of synthesis of the leucine biosynthetic enzymes of *Neurospora*. Proc. Natl. Acad. Sci. U.S.A. **54**:1538–1546.
12. **Gross, S. R.** 1969. Genetic regulatory mechanisms in the fungi. Annu. Rev. Genet. **3**:395–424.
13. **Hilton, J. L., P. C. Kearney, and B. N. Ames.** 1965. Mode of action of the herbicide 3-amino-1,2,4-triazole (amitrole): inhibition of an enzyme of histidine biosynthesis. Arch. Biochem. Biophys. **112**:544–547.
14. **Hodge, H. C., E. Maynard, W. Downs, J. Ashton, and L. Salerno.** 1966. Tests on mice for evaluating carcinogenicity. Toxicol. Appl. Pharmacol. **9**:583–596.
15. **Hulanicka, D., T. Klopotowski, and G. Bagdasarian.** 1969. Inhibition of aminoimidazole ribotide biosynthesis in *Salmonella typhimurium* by aminotriazole. Acta Biochim. Pol. **16**:127–133.
16. **Klopotowski, T., and A. Wiater.** 1965. Synergism of aminotriazole and phosphate on the inhibition of yeast imidazole glycerol phosphate dehydratase. Arch. Biochem. Biophys. **112**:562–566.
17. **Kumar, C. C., and G. Padmanaban.** 1980. 3-Amino-1,2,4-triazole is an inhibitor of protein synthesis on mitoribosomes in *Neurospora crassa*. Biochim. Biophys. Acta **607**:339–349.
18. **Lester, H. E., and S. R. Gross.** 1959. Efficient method for selection of auxotrophic mutants of *Neurospora*. Science **129**:572.
19. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
20. **Margoliash, E., and A. Novogrodsky.** 1958. A study of the inhibition of catalase by 3-amino-1,2,4-triazole. Biochem. J. **68**:468–475.
21. **Martin, R. G.** 1963. The first enzyme in histidine biosynthesis: the nature of feedback inhibition by histidine. J. Biol. Chem. **238**:257–268.
22. **Olshan, A. R., and S. R. Gross.** 1974. Role of the *leu-3* cistron in the regulation of the synthesis of isoleucine and valine biosynthetic enzymes of *Neurospora*. J. Bacteriol. **118**:374–384.
23. **Perkins, D. D.** 1974. The manifestation of chromosome rearrangements in unordered asci of *Neurospora*. Genetics **77**:459–489.
24. **Perkins, D. D., A. Radford, D. Newmeyer, and M. Björkman.** 1982. Chromosomal loci of *Neurospora crassa*. Microbiol. Rev. **46**:426–570.
25. **Polacco, J., and S. R. Gross.** 1973. The product of the *leu-3* cistron as a regulatory element for the production of the leucine biosynthetic enzymes of *Neurospora*. Genetics **74**:443–459.
26. **Reichenbecher, V. E., Jr., M. Fischer, and S. R. Gross.** 1978. Regulation of isopropylmalate isomerase synthesis in *Neurospora crassa*. J. Bacteriol. **133**:794–801.
27. **Ryan, F. J., G. W. Beadle, and E. L. Tatum.** 1943. The tube method of measuring the growth rate of *Neurospora*. Am. J. Bot. **30**:784–799.