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Regulation of Isopropylmalate Isomerase Synthesis in *Neurospora crassa*

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The capacity to synthesize isopropylmalate isomerase (EC 4.2.1.33) by *Neurospora crassa* increased during induction in the presence of cycloheximide but was inhibited by proflavine and other inhibitors of RNA synthesis. Turnover of the enzyme once formed appeared negligible, but the message (measured as enzyme-forming capacity) had a half-life of 4 to 8 min. A comparison of the kinetics of induction in the wild type and a newly isolated α-isopropylmalate-permeable strain suggested strongly that feedback control by leucine of α-isopropylmalate production can adequately serve as the primary physiological regulator of endogenous inducer concentration. Genetic data are presented which implicate the involvement of two unlinked genes, *ipm-1* and *ipm-2*, in determining permeation of α-isopropylmalate.

The regulation of expression of the three unlinked structural genes of the leucine biosynthetic pathway of *Neurospora crassa* has been shown to involve two distinctly different but interdependent regulatory mechanisms. One of these, repression by leucine of the synthesis of the first enzyme of the pathway, α-isopropylmalate (α-IPM) synthase (EC 4.1.3.12), which is specified by the *leu-4* gene and catalyzes the condensation of α-ketoisovalerate and acetyl coenzyme A in the formation of α-IPM, seems formally analogous to repression mechanisms in procaryotic systems (7, 8, 26). A second, positive regulatory system, involving α-IPM as an effector in conjunction with a regulatory product of the *leu-3* gene, is involved in the regulation of synthesis of the remaining two enzymes of the pathway, isopropylmalate isomerase (the isomerase) and β-IPM dehydrogenase (EC 1.1.1.85), specified by the *leu-2* and *leu-1* genes, respectively (17). Since the production of the isomerase and dehydrogenase in significant quantities is dependent upon the presence of α-IPM and an effective *leu-3* product (*leu-3*$^-$ mutants are leucine auxotrophs), it has been proposed that a complex of the *leu-3* product with either α-IPM or a metabolic derivative serves a primary physiological function in the expression of the *leu-2* and *leu-1* genes (8). In addition, it has been found that in the absence of either α-IPM or an effective *leu-3* product, neither α-IPM synthase nor most of the enzymes of isoleucine and valine synthesis can be synthesized at maximal rate (16). A general coordinating role in the regulation of expression of the genes of branched-chain amino acid biosynthesis has been proposed for the α-IPM-*leu-3* product complex.

The experiments described here were designed with two objectives in mind. First, we wished to obtain some elementary information about the mechanism of α-IPM isomerase induction in vivo. We specifically wanted to settle the question of whether synthesis of α-IPM isomerase is regulated at the level of transcription, and if so, whether the half-life of the message is short enough so that message synthesis can serve adequately as the physiological control point in the production of the isomerase.

The second objective was to obtain information that would confirm or reject our conclusion that α-IPM is the inducer of isomerase synthesis and, if possible, to assess the role of feedback inhibition and repression by leucine of α-IPM synthase in the in vivo regulation of isomerase production. Until recently, induction and repression of synthesis of the isomerase and β-IPM dehydrogenase have had to be studied by varying the endogenous production of α-IPM as a function of the concentration of leucine supplied during growth. This indirect approach was necessitated by the fact that α-IPM is normally impermeable to *Neurospora,* and mutants that allow entry of the compound had not been found. We report here the isolation of an α-IPM-permeable strain and describe the kinetics of induced synthesis of the isomerase and the phenotypic and genotypic complexity of the permeable strain.

MATERIALS AND METHODS

Biological. The strains of *N. crassa* used in this

study originated or were constructed in this laboratory. R59F84, an α-IPM-utilizing strain, was isolated following UV irradiation of R59 (*leu-4*). Irradiated conidia were heavily inoculated (about 10^7 conidia/plate) onto minimal sucrose plates containing α-IPM and overlaid with a thin layer of medium containing sorbose. α-IPM-utilizing mutants were isolated as spidery colonies which appeared after about 3 days of incubation at 34°C.

Crosses were performed at 25°C on synthetic crossing medium (5) supplemented appropriately with growth factors required by the protoperithecial parent. The sorbose plating method of Newmeyer (15) was used for the isolation of segregants. Heterokaryons were formed by the method of Kashmiri and Gross (12).

Growth methods. Mycelia were routinely obtained for biochemical analysis after growth in 2-liter Fernbach flasks containing 1 liter of synthetic medium (5) as described by Polacco and Gross (17).

When mycelia were required for induction studies, a dense suspension of conidia (equivalent to 40 ml of a conidial suspension of 1.0 optical density at 550 nm [OD_{550}]) was inoculated into each Fernbach flask containing 1 liter of minimal medium supplemented with 300 μg of L-leucine per ml (repression medium). After 13 h of growth with aeration at 34°C, the log-phase mycelia were harvested by filtration, washed with fresh medium, and quickly resuspended at a concentration of about 5 g (wet weight) per liter into appropriately supplemented fresh medium. (Minimal medium without L-leucine is referred to as induction medium.) The resuspended mycelia were further incubated at 34°C with vigorous aeration. Periodically, mycelial samples were collected by filtration.

Leucine auxotrophs were grown in medium supplemented with L-leucine at concentrations ranging from 20 μg/ml (growth-limiting conditions) to 300 μg/ml (excess-leucine conditions). Inositol auxotrophs were supplemented with 25 μg of inositol per ml. Cycloheximide and proflavine were used at concentrations of 4 and 100 μg/ml, respectively.

Preparation of extracts. Crude ammonium sulfate precipitates (0 to 80%) were prepared by the method of Gross (7), using 0.1 M potassium phosphate buffer, pH 6.0, containing 0.2 mM L-leucine. A Polytron homogenizer was used instead of a blender to shear the mycelia before sonic treatment. Protamine sulfate (7.5 mg per g [wet weight] of mycelia) was added immediately after sonic treatment rather than after the first centrifugation.

Enzyme assay. Crude ammonium sulfate pellets were dissolved in 0.1 M potassium phosphate buffer, pH 6.0, containing 3 M glycerol, 1 mM dithiothreitol, and 0.2 mM L-leucine. Although the isomerase has been shown to be inactivated by several sulfhydryl-containing reagents (10), dithiothreitol at this pH and concentration has little, if any, effect on activity. Assays were performed after removal of debris by centrifugation for 1 h at 100,000 × *g*. The assay of the isomerase and definition of specific activity are as described by Gross (9). All assays were performed at 34°C in a Gilford spectrophotometer.

Protein was determined by the method of Warburg and Christian (25). When protein was determined in extracts derived from proflavine-treated mycelia, a correction factor, 0.0906 mg/ml per 1.0 OD_{443}, was added to the calculated protein concentration because a small amount of proflavine is unavoidably carried through ammonium sulfate fractionation.

Chemicals. α-IPM and β-IPM were prepared by the method of Calvo and Gross (3). Proflavine dihydrochloride was obtained from Schwarz/Mann. Cycloheximide was obtained from Calbiochem. All other chemicals were obtained from standard commercial sources.

RESULTS

Isomerase induction in wild-type *Neurospora*. When the wild-type strain of *Neurospora* (STD8A) is grown in medium containing excess L-leucine, the endogenous concentration of the inducer of isomerase production, α-IPM, is low because L-leucine feedback inhibits catalysis and represses synthesis of α-IPM synthase (7, 26). In minimal medium, however, feedback inhibition and repression are released, and the consequent increase in α-IPM concentration induces isomerase synthesis (7). The specific activity of isomerase began to increase within 20 min after mycelia were transferred from medium in which production of the inducer was feedback inhibited by L-leucine (repression medium) to L-leucine-free medium in which inhibition of the synthesis of the inducer was released (induction medium) (Fig. 1). The specific activity continued to increase for 30 to 40 more min, at which time it leveled off, having undergone more than a 25-fold increase over the basal level.

Two experiments were performed to test whether enzyme degradation is a significant factor in determining the specific activity of the isomerase. The first involved measuring the decrease in specific activity as a function of growth after transfer of induced mycelia into repression medium. The measured decrease in specific activity for 4 h after establishment of repression

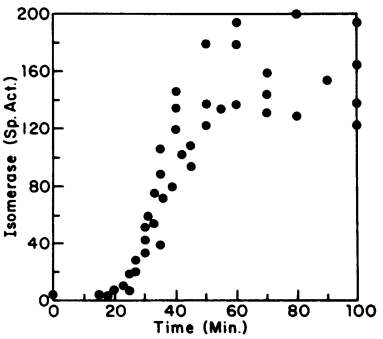

FIG. 1. *Time course of induction of isomerase synthesis after transfer of wild-type (STD8A) mycelia from repression to induction medium. Pooled data from eight independent experiments.*

could be accounted for completely on the basis of dilution during growth. The lack of significant degradation of isomerase activity was confirmed further by measuring the specific activity of the enzyme in the induced wild type and a *leu-4 leu-3*^{cc} double mutant (one that cannot make α-IPM but constitutively synthesizes the isomerase [17]) as a function of time in the presence of cycloheximide. Despite the fact that protein synthesis was negligible, the specific activity remained constant throughout the 2-h duration of the experiment. Cycloheximide has been reported to reduce the rate of turnover of some proteins, presumably by inhibiting the synthesis of specific proteases with short half-lives (20). However, the results of the two different kinds of experiments indicate that the enzyme is stable for several hours after protein synthesis is turned off.

Inhibitors of isomerase synthesis. The effect of excess L-leucine, cycloheximide, and proflavine on induction of isomerase synthesis is shown in Fig. 2. The data indicate clearly that induction does not occur in the presence of cycloheximide or proflavine, suggesting that protein and RNA synthesis are required for an increase in the specific activity of the isomerase.

In addition to proflavine, inhibitors of RNA synthesis which proved effective in preventing isomerase production included acriflavine neutral, Lomofungin (Upjohn) (1, 11), and 8-quinolinol (6). Proflavine was chosen for use in further experiments because its mechanism of action (intercalation) is well established (14) and it is readily available.

Separation of transcription and translation of the isomerase. Preliminary experiments revealed that isomerase mRNA (as enzyme-forming capacity) is synthesized during induction in the presence of cycloheximide and that translation of the message proceeds after the block in protein synthesis is removed and further message synthesis is prevented by incubation in repression medium with proflavine. However, after removal of the cycloheximide, 2 h of incubation was required before maximum isomerase activity was obtained, and the final yield was rather low. In the absence of any method of estimating mRNA other than by its ability to direct the synthesis of the isomerase, it was impossible to determine whether message synthesis was slow or degradation was extensive during cycloheximide inhibition.

The capacity to synthesize the isomerase increased significantly under conditions allowing transcription but preventing translation (Fig. 3). The capacity to synthesize the isomerase increased significantly during the first 2 h of incubation in induction medium containing cycloheximide, while only a slight increase was obtained in the presence of cycloheximide under conditions of repression. However, the maximum yield, after removal of the cycloheximide and translation in the presence of proflavine, was only about 10% of that expected when protein synthesis was unperturbed during induction. The slight increase in isomerase production consistently observed under conditions of repression, while possibly a result of differential synthesis of protein and RNA at the cycloheximide concentration used, is more likely to have resulted from either a small increase in the endogenous concentration of the inducer, α-IPM, or an increase in the relative amount of the

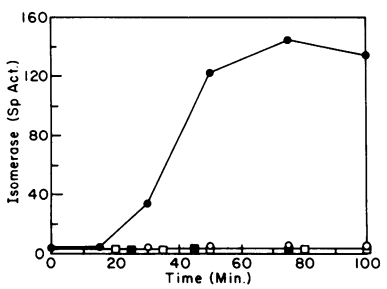

FIG. 2. *Inhibition of induction of isomerase synthesis. At time zero, mycelia grown in repression medium were resuspended into induction medium (●), induction medium supplemented with 300 μg of L-leucine per ml (repression medium) (○), 4 μg of cycloheximide per ml (■), or 100 μg of proflavine per ml (□).*

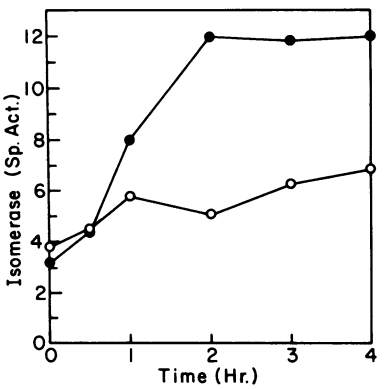

FIG. 3. *Increase in the capacity to synthesize isomerase with increasing duration of the transcription phase. During the transcription phase, mycelia were incubated in either induction medium containing 4 μg of cycloheximide per ml (●) or repression medium containing 4 μg of cycloheximide per ml (○) for the length of the time shown on the abscissa, followed by a translation phase of 2 h in repression medium containing 100 μg of proflavine per ml.*

message for the *leu-3*-positive regulatory element (17).

Results of an experiment designed to estimate the time at which isomerase mRNA first appears after exposure to inducing conditions are plotted in Fig. 4. In this experiment, induction was allowed to proceed as a function of time in the absence of inhibitors. At intervals, mycelia were removed from induction medium and incubated for 1 h in repression medium containing 100 μg of proflavine per ml, allowing translation of preformed messenger but within a few minutes (see Fig. 5B), preventing further message synthesis. The data, uncorrected for residual message synthesis in repression-proflavine medium, indicate that isomerase mRNA begins to appear about 12 min after transfer to induction medium.

Isomerase messenger half-life. Retention of the ability to synthesize the isomerase after relatively long periods of inhibition by cycloheximide could be taken to mean that isomerase mRNA has a relatively long half-life. Cycloheximide inhibition, however, has been found to alter the rate of message turnover (4, 19, 24). As a consequence, it seemed advisable to obtain an estimate of isomerase message half-life without the use of inhibitors. This was accomplished by following the kinetics of isomerase synthesis after pulse induction (4, 13). Mycelia were incubated for 17 min in induction medium and then returned to repression medium to shut off further message synthesis (Fig. 5A). The specific activity of the isomerase was measured as a function of time in repression medium. The remaining synthesizing capacity, *R* (defined as the difference between any point on the induction curve and the maximum point of the curve), represents a measure of the amount of isomerase mRNA remaining at any point in time, on the assumption that a given amount of messenger will eventually be translated into a proportional amount of enzyme. When mRNA synthesis stops, the rate of decrease of *R* is a measure of the rate of decay of functional mRNA. Within 8 min after transfer to repression medium (during which time some messenger was still being synthesized), the kinetics of the decay of *R* became first order (Fig. 5A). Assuming that isomerase mRNA synthesis was completely turned off during the linear portion of the curve, the functional half-life of isomerase mRNA calculated from the slope was found to be 4.0 ± 0.6 min (average of four experiments).

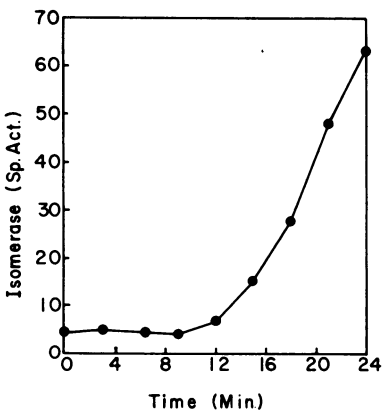

FIG. 4. *Time of first appearance of isomerase mRNA in induced mycelia. Samples of mycelia were incubated in induction medium for the length of time shown on the abscissa and then allowed to undergo translation for 1 h in repression medium containing 100 μg of proflavine per ml.*

The results of a similar experiment in which proflavine was used to turn off further messenger synthesis after 24 min in induction medium are shown in Fig. 5B. The functional half-life of isomerase mRNA, when calculated on the basis of these data, was 7.5 ± 1.3 min (average of four experiments).

Due to the difficulties inherent in such experiments, we feel that the results obtained using these two methods are reasonably consistent. An isomerase mRNA half-life of 4 to 8 min is of the same order of magnitude as the half-lives of many of the messengers of bacteria and simple fungi (4, 13, 19, 22).

Isomerase production in a strain able to utilize exogenous α-IPM. α-IPM is impermeable to *Neurospora* (7) and therefore cannot be used as an inducer in in vivo experiments. Hence the experiments described above were performed by taking advantage of feedback inhibition by leucine of α-IPM synthase to regulate the endogenous production of the inducer. During the course of these studies, a mutant strain was isolated which is able to utilize exogenous α-IPM for growth in place of leucine, and several multiply marked α-IPM-utilizing strains were constructed by recombination. The kinetics of isomerase production by one of these strains after transfer to repression medium to which α-IPM was added is presented in Fig. 6. The permeable strain (R59F84-2-61a) used had the following genotype: *leu-4*$^-$ *leu-1*$^-$ *ipm-1*$^+$ *ipm-2*$^+$. It is blocked both in the synthesis of α-IPM and in its conversion to leucine, and contains the two genes necessary for α-IPM uptake (see below). The isomerase level of the utilizer strain began to increase within 5 to 8 min after α-IPM was supplied and leveled off within 3 h, undergoing a 100-fold increase over the basal level. A *leu-4*$^-$ *leu-1*$^-$ nonpermeable strain showed little or no induction under the same conditions.

The production of the isomerase by the

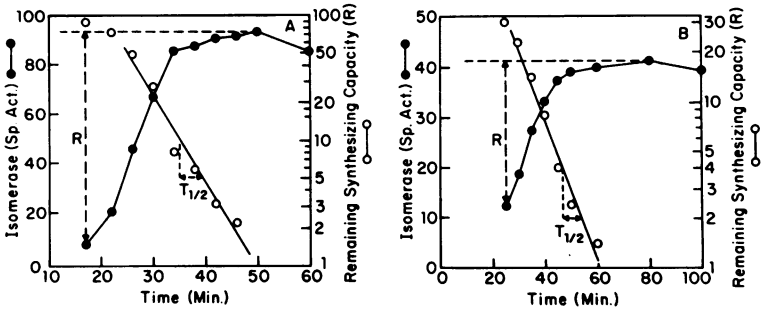

FIG. 5. *(A) Estimation of isomerase messenger half-life by the pulse induction method. At time zero, mycelia were suspended in induction medium and incubated for 17 min and then returned to repression medium. Symbols: Isomerase (●); remaining synthesizing capacity, R (○). (B) Isomerase messenger half-life estimated by the proflavine inhibition method. At time zero, myeclia were resuspended in induction medium and incubated for 25 min, at which time proflavine was added to inhibit further message synthesis. Symbols as for (A).*

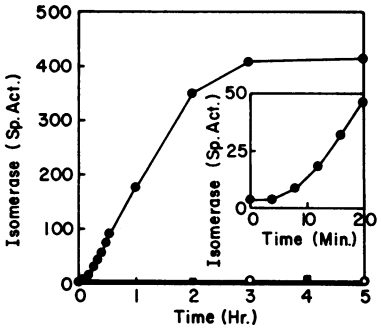

FIG. 6. *Isomerase induction in a strain permeable to exogenous α-IPM. Mycelia from leu-4⁻ leu-1⁻ ipm-1⁺ ipm-2⁺ grown in repression medium (300 μg of L-leucine per ml) were washed, and then resuspended in medium containing either repression medium (○) or repression medium containing 100 μg of α-IPM per ml (●). Mycelia from leu-4⁻ leu-1⁻ ipm-1⁻ ipm-2⁻ were resuspended into medium containing 300 μg of L-leucine per ml + 100 μg of α-IPM per ml (■). Insert: Expansion of the first 20 min of the experiment.*

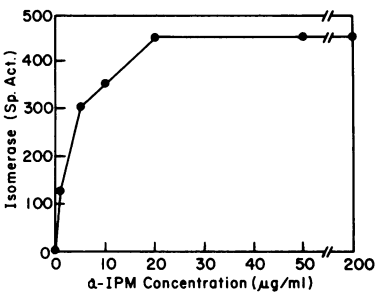

FIG. 7. *Isomerase production as a function of α-IPM concentration. Samples of mycelia from leu-4⁻ leu-1 ipm-1⁺ imp-2⁺ were suspended in medium containing α-IPM and 300 μg of L-leucine per ml and then incubated at 34°C with aeration for 8 h before extraction and assay.*

permeable double leucine auxotroph as a function of α-IPM concentration is presented in Fig. 7. Concentrations of α-IPM as low as 20 μg/ml were sufficient to induce maximum isomerase production. Surprisingly, even though supposedly α-IPM-impermeable *leu-4* mutants would not grow on minimal medium supplemented with α-IPM, both *leu-4* and *leu-4 leu-1* double mutants were induced to produce some isomerase (specific activity <60) when allowed to grow from a small inoculum for longer periods of time (24 h) on leucine in the presence of α-IPM. Apparently, though unable to utilize exogenously supplied α-IPM to satisfy the requirement for leucine, enough α-IPM can get into *ipm⁻* strains for low-level induction of isomerase synthesis.

Genetics of the ability to utilize exogenous α-IPM. The α-IPM-permeable strain (R59F84) was isolated as a spidery colony which grew on plates supplemented with α-IPM following UV irradiation of R59 (*leu-4*) conidia. Analysis of segregation from crosses of the *leu-4⁻* α-IPM-permeable strain to *alcoy*, the multiple-linkage-group tester strain (5), seemed to indicate that a gene allowing α-IPM utilization was linked to *leu-4*, since *leu-4⁻* utilizing progeny were obtained at a much higher frequency than *leu-4⁻* nonutilizing progeny (V. E. Reichenbecher, Jr., M. Fischer, and S. R. Gross, Genetics **80**:s67-s68, 1975). On the other hand, when a direct test of linkage was made by crossing a *leu-4⁻* utilizing strain to an impermeable *leu-3⁻* strain (*leu-3* is 10 to 15 map units from *leu-4*), about half the prototrophic recombinants were inducible by α-IPM, indicating no linkage of the utilizer gene to *leu-4*. It was later discovered that an excess of *leu-4⁻* α-IPM-utilizing segregants was obtained in the *alcoy* cross because, for some unknown reason, nonutilizing *leu-4⁻*

ascospores frequently do not mature properly and fail to germinate.

A tetrad analysis of a cross between R59F84 and a wild-type strain (STD8A) revealed that two genes, *ipm-1* and *ipm-2*, are involved in the ability to utilize α-IPM (Table 1). We designate the genes for the ability to utilize α-IPM as *ipm-1*$^+$ and *ipm-2*$^+$, whereas we designate the lack of this ability as *ipm-1*$^-$ and *ipm-2*$^-$. (In order to avoid misunderstanding, it must be emphasized that in this case the wild-type allele is designated with a minus sign.) Since germination of *leu-4*$^-$ *imp-1*$^-$ *ipm-2*$^-$ ascospores is poor and representatives of each of the four spores pairs in each tetrad were required to establish the pattern of segregation, only 6 of 69 asci dissected proved useful.

The genotype of each spore pair from the six complete tetrads was determined directly by segregation analysis of crosses to tester stocks derived from tetrads V and XXII (whose genotypes were deduced from "brother-sister" spore pair crosses) and matched phenotypically with regard to pattern of induction and, in the case of *leu-4*$^-$ segregants, the growth response to α-IPM on solid medium. (A trace of L-leucine [0.5 to 1.0 μg/ml] must be included in the sorbose-containing plating medium to obtain a consistent response to added α-IPM. Presumably the L-leucine is required for germination of the conidia streaked for test.) The results indicate that a *leu-4*$^-$ strain must contain both *ipm-1*$^+$ and *ipm-2*$^+$ in order to yield confluent growth when incubated on solid medium for 48 h at 34°C in the presence of 50 μg of α-IPM and 0.5 μg of L-leucine per ml. *leu-4*$^-$ strains that were *ipm-1*$^+$ *imp-2*$^-$ yielded growth from discrete conidial foci and, as a consequence, were easily distinguishable from those that were *ipm-1*$^+$ *imp-2*$^+$. Neither *imp-1*$^-$ *imp-2*$^+$ nor *imp-1*$^-$ *ipm-2*$^-$ strains grew more than slightly in response to the trace of L-leucine supplied.

In addition to the above, the data of Table 1 show that both *imp-1*$^+$ *ipm-2*$^+$ and *imp-1*$^+$ *ipm-2*$^-$ segregants are inducible by exogenous α-IPM in liquid culture, while *ipm-1*$^-$ *ipm-2*$^-$ and *ipm-1*$^-$ *ipm-2*$^+$ strains are not. We conclude, then, that the *ipm-1*$^+$ gene is required for isomerase induction by exogenous α-IPM and that the *imp-2*$^+$ gene potentiates the growth response to α-IPM. There is no evidence of linkage of *imp-1* to *ipm-2* or of linkage of either to *leu-4*.

DISCUSSION

The experiments reported here were designed to yield information necessary for the analysis of the molecular events that occur between the time the induction process for isomerase production begins and fully active enzyme is synthesized. The questions asked were of necessity

TABLE 1. *Tetrad analysis of a cross of R59F84-1-93a (leu-4*$^-$ *ipm-1*$^+$ *imp-2*$^+$*) × STD8A (leu-4*$^+$ *ipm-1*$^-$ *ipm-2*$^-$*)*

Tetrad no.	Spore pair			
	1	2	3	4
V	*leu-4*$^+$ *ipm-1*$^-$ *imp-2*$^+$ Ind$^-$	*leu-4*$^+$ *ipm-1*$^+$ *ipm-2*$^-$ Ind$^+$	*leu-4*$^-$ *ipm-1*$^+$ *ipm-2*$^-$ Ind$^+$	*leu-4*$^-$ *ipm-1*$^-$ *ipm-2*$^+$ Ind$^-$
XXII	*leu-4*$^-$ *ipm-1*$^+$ *ipm-2*$^+$ Ind$^+$	*leu-4*$^-$ *ipm-1*$^+$ *ipm-2*$^-$ Ind$^+$	*leu-4*$^+$ *ipm-1*$^-$ *ipm-2*$^-$ Ind$^-$	*leu-4*$^+$ *ipm-1*$^-$ *ipm-2*$^+$ Ind$^-$
LV	*leu-4*$^+$ *ipm-1*$^-$ *ipm-2*$^-$ Ind$^-$	*leu-4*$^+$ *ipm-1*$^+$ *ipm-2*$^+$ Ind$^+$	*leu-4*$^-$ *ipm-1*$^-$ *ipm-2*$^+$ Ind$^-$	*leu-4*$^-$ *ipm-1*$^+$ *ipm-2*$^-$ Ind$^+$
LVI	*leu-4*$^+$ *ipm-1*$^+$ *ipm-2*$^-$ Ind$^+$	*leu-4*$^+$ *ipm-1*$^-$ *ipm-2*$^+$ Ind$^-$	*leu-4*$^-$ *ipm-1*$^+$ *ipm-2*$^-$ Ind$^+$	*leu-4*$^-$ *ipm-1*$^-$ *ipm-2*$^+$ Ind$^-$
LXVI	*leu-4*$^+$ *ipm-1*$^-$ *ipm-2*$^-$ Ind$^-$	*leu-4*$^+$ *ipm-1*$^-$ *ipm-2*$^-$ Ind$^-$	*leu-4*$^-$ *ipm-1*$^+$ *ipm-2*$^+$ Ind$^+$	*leu-4*$^-$ *ipm-1*$^+$ *ipm-2*$^+$ Ind$^+$
LXIX	*leu-4*$^-$ *ipm-1*$^+$ *ipm-2*$^+$ Ind$^+$	*leu-4*$^-$ *ipm-1*$^+$ *ipm-2*$^-$ Ind$^+$	*leu-4*$^+$ *ipm-1*$^-$ *ipm-2*$^-$ Ind$^-$	*leu-4*$^+$ *ipm-1*$^-$ *ipm-2*$^+$ Ind$^-$

[a] The genotype of each segregant was determined by crossing each to tester stocks derived from tetrads V and XXII (whose genotypes were derived from "brother-sister" spore pair crosses) and testing all *leu-4*$^-$ segregants from such crosses for the nature of the growth response to α-IPM on sorbose-containing solid medium (see text). Isomerase inducibility (Ind) by exogenous α-IPM was determined on mycelia pregrown in repression medium at 34°C for 13 h and then transferred to fresh repression medium supplemented with 100 μg of α-IPM per ml and incubated with aeration for 3 h at 34°C.

quite elementary. Is isomerase turnover sufficiently great to be of significance in the regulation of the level of enzyme in the cell? Is synthesis of the isomerase regulated at the level of transcription or translation? What is the half-life of the message? Finally, it was hoped that a careful analysis of the kinetics of induction would permit an estimation of the relevance of endogenous regulation of inducer production to the synthesis of the isomerase.

Enzyme degradation or turnover does not appear to play a significant role in the regulation of the level of isomerase activity in the cell. In confirmation of the observations of Polacco and Gross (17), the decrease in the specific activity observed after transfer of induced mycelia to repression medium corresponds to the decrease expected by dilution during growth. As is shown in the accompanying paper (18), it is difficult to free the isomerase during purification of a contaminating protease. In the absence of demonstrable turnover of the enzyme, however, it seems unlikely that proteolytic cleavage is of physiological consequences to the isomerase under the conditions of the experiments reported here.

Synthesis of the isomerase appears to be regulated at the transcriptional level. The experiments clearly show that the capacity to synthesize isomerase (presumably mRNA) can be induced even when translation is blocked by cycloheximide. Message synthesized in the presence of cycloheximide can be translated after removal of the inhibitor, even when further message synthesis is blocked by proflavine. Although the data do not exclude some secondary translational regulatory step, they do conform to the suggestion of Polacco and Gross (17), derived from genetic arguments, that the α-IPM-*leu-3* product regulatory complex acts at the DNA level to control the rate of transcription of isomerase mRNA. It seems reasonable to assume that the synthesis of the other enzymes under *leu-3* and α-IPM control (i.e., β-IPM dehydrogenase and the enzymes of the isoleucine-valine biosynthetic pathway) is also regulated at the transcriptional level.

The half-life of isomerase message (4 to 8 min) is within the range reported for several other messages studied in *Neurospora* and *Aspergillus* (4, 19, 23) and more comparable to the half-lives of inducible messages in bacteria than the longer half-lives of messages in animal cells (hours or days) (2, 21).

The isolation of a strain of *Neurospora* which is able to utilize exogenous α-IPM allowed, for the first time, a study of the induction process directly by the exogenous addition of α-IPM rather than by inhibition or stimulation of its endogenous synthesis. Because of this, it was possible to show that completed isomerase molecules began to appear within 5 to 8 min after exposure of the permeable strain to α-IPM. This clearly demonstrates that the speed of isomerase induction is not too different from that of β-galactosidase in *Escherichia coli,* in which completed enzyme molecules first appear within 1.5 to 3 min after exposure to inducer (14), despite the fact that in *Neurospora,* in contrast to bacteria, completed messages must first be transported from the nucleus to the cytoplasm before being translated.

The rate of induction in nonpermeable strains of *Neurospora* in response to elevation of the endogenous concentration of α-IPM is also quite rapid. Isomerase mRNA can be detected within 12 min and completed isomerase molecules within 20 min after feedback inhibition of α-IPM production is released. This response is only 12 to 15 min (i.e., less than 0.1 of a generation time) slower than the response seen when α-IPM is directly supplied to the α-IPM-permeable strain. It is clearly rapid enough to add credence to the proposal that the endogenous concentration of α-IPM is the major factor governing the rate of synthesis of the isomerase and, presumably, β-IPM dehydrogenase as well (8). Therefore, during the interval after transfer of the wild-type strain from high-leucine to minimal medium, the following events must occur: reduction of the effective endogenous L-leucine pool to a concentration at which feedback inhibition of α-IPM synthase is released; synthesis of α-IPM in quantities sufficient to induce synthesis of mRNA; completion of the first mRNA molecules; transport of the mRNA to the site of protein synthesis; and completion of the first isomerase molecules. Since isomerase production is induced at low concentrations of α-IPM and the basal level of the isomerase is very low in the wild-type strain grown in excess L-leucine, feedback inhibition of α-IPM synthase must be sufficient to maintain the endogenous α-IPM concentration at a very low level.

The genetics of the α-IPM-permeable strain proved to be somewhat complex. This was not unexpected in view of the difficulty encountered in obtaining a strain capable of growth on α-IPM in place of L-leucine. Tetrad analysis had to be used to discriminate between the several phenotypic responses to α-IPM displayed by segregants from crosses to nonutilizing strains. The segregation patterns obtained in ordered tetrads indicated the involvement of two mutated, independently segregating genes. One of them, *ipm-1*$^+$, permits entry of a sufficient amount of α-IPM to induce isomerase synthesis, but not enough for maximal replacement of L-

leucine for growth. The other, *ipm-2*$^+$, potentiates the growth response to α-IPM in *ipm-1*$^+$ strains, but by itself, in *ipm-1*$^-$ strains, cannot permit sufficient uptake of α-IPM for either growth or isomerase induction. The relationship, if any, between the two genes allowing utilization of exogenous α-IPM and the inducible system for transport of C4-dicarboxylic acids described by Wolfinbarger and Kay (27) is unknown.

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