Differential flux through the quinate and shikimate pathways

Implications for the channelling hypothesis

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The *qutC* gene encoding dehydroshikimate dehydratase has been constitutively overexpressed in *Aspergillus nidulans* from a range of 1–30-fold over the normal wild-type level. This overexpression leads to impaired growth in minimal medium which can be alleviated by the addition of aromatic amino acids to the medium. Overexpression of the *qutC* gene in mutant strains lacking protocatechuic acid (PCA) oxygenase leads to the build up of PCA in the medium, which can be measured by a simple assay. Measuring the rate of production of PCA in strains overproducing dehydroshikimate dehydratase and correlating this with the level of overproduction and impaired ability to grow in minimal medium lacking aromatic amino acids leads to the conclusion that (a) the metabolites 3-dehydroquininate and dehydroshikimate leak from the AROM protein at a rate comparable with the extent of flux catalysed by the AROM protein, (b) the AROM protein has a low-level channelling function probably as a result of the close juxtaposition of five active sites and (c) this channelling function is only physiologically significant under non-optimal conditions of nutrient supply and oxygenation, when the organism is *in situ* in its natural environment.

INTRODUCTION

The quinate and shikimate pathways have in common the two metabolites 3-dehydroquinic acid (DHQ) and dehydroshikimic acid (DHS) see Scheme 1. In fungi the enzymes essential for the catabolism of quinate to protocatechuic acid (PCA) are induced by the presence of quinate, DHQ or DHS, but this induction is subject to carbon catabolite repression; however, steps 2–6, catalysed by enzymes involved in the synthesis of chorismate in the shikimate pathway, are constitutively produced at a low level (Chaleff, 1974; Hawkins *et al.*, 1982; Kinghorn & Hawkins, 1982). Six genes (designated *qut*) have been identified as essential for the catabolism of quinate to protocatechuic acid and its regulation, and they map together in a tight cluster on chromosome VIII (Whittington *et al.*, 1987; Grant *et al.*, 1988). The entire *qut* gene cluster has been isolated by molecular cloning and subjected to DNA sequence determination and Northern-blot analysis. This analysis has shown that regulation of the *qut* gene cluster is at the level of transcriptional control, and eight, not six, genes within the cluster have quinate-inducible mRNAs (Hawkins *et al.*, 1984; 1985, 1988, 1991; Da Silva *et al.*, 1986; Beri *et al.*, 1987; Lamb *et al.*, 1990, 1991a). The six genetically characterized genes are *qutA* (activator), *qutB* (quininate dehydrogenase), *qutC* (dehydroshikimate dehydratase), *qutD* (quininate permease), *qutE* (3-dehydroquinase) *qutR* (repressor), and the two genetically uncharacterized genes are designated *qutG* and *qutH*. The inferred protein sequence of the *qutG* gene shows very strong similarity with bovine *myo*-inositol monophosphatase, whereas the *qutH* inferred protein sequence contains a 'DNA zinc cluster' motif, and shows a low, but significant, similarity to the DNA-directed DNA polymerase of hepatitis B virus (Lamb *et al.*, 1990, 1991a). Steps 2–6 in the shikimate pathway are catalysed by the penta-

functional AROM protein, and the complex *arom* locus which encodes it has been isolated by molecular cloning and shown to consist of a single open reading frame of 4812 nt, which probably evolved by multiple gene fusions (Charles *et al.*, 1985, 1986; Hawkins, 1987). Comparisons of the *qut* gene-inferred protein sequences with that of the *aromA* gene-inferred protein sequence show that they share a long and complex evolutionary history, which has profound implications for the molecular mechanisms by which the *qutR*-encoded repressor protein exerts its effects (Anton *et al.*, 1987; Hawkins *et al.*, 1991).

On a more physiological level it is unclear why the products DHQ and DHS, of the shikimate pathway, do not lead to internal constitutive induction of the *qut*-pathway genes. The two metabolites, DHQ and DHS, are interconverted by catabolic 3-dehydroquinase (cDHQase) encoded by *qutE* and biosynthetic dehydroquinase (bDHQase) encoded within the *aromA* gene, but the two isoenzymes share no similarity at the DNA and protein sequence level, suggesting convergent evolution (Hawkins, 1987). On the basis of the observation in *Neurospora crassa* that mutations abolishing the bDHQase activity of the AROM protein gave rise to constitutive induction of the equivalent *qa* (= *qut*) genes *in vivo*, Giles (1978) proposed that the AROM protein had a channelling function *in vivo* to keep separate the pools of the metabolites DHQ and DHS in the quinate and shikimate pathways. Subsequent biochemical work with purified AROM protein from *N. crassa* lead Gaertner *et al.* (1970) and Welch & Gaertner (1975) to propose in addition the attribute of catalytic facilitation, though Gaertner (1978) later reported that they were working with proteolytically degraded material. Coggins and co-workers (Lambert *et al.*, 1985), demonstrated that the enzyme preparations of Gaertner *et al.* (1970) were also significantly deficient in DHQ synthase activity due to the loss of zinc ions

Abbreviations used: DHQ, 3-dehydroquininate; DHS, dehydroshikimate; *qut*, wild-type allele at a quinic acid-utilization-gene locus; *qut*[−], mutant allele at a quinic acid-utilization-gene locus; *aromA*, the wild-type allele encoding the pentafunctional AROM protein; AROM, the pentafunctional AROM protein; PCA, protocatechuic acid; cDHQase and bDHQase, catabolic and biosynthetic dehydroquinases.

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test for the presence of PCA (Grant *et al.*, 1988). Preliminary experiments indicated that the peak absorbance of the complex was at 565 nm; however, any unbound Fe^{3+} was found to have a significant absorbance at this wavelength; therefore all measurements were taken at 580 nm. After establishing that the PCA and Fe^{3+} form a complex in a 1:1 ratio, in a potassium glycinate buffer, pH 9.2, a linear relationship between the PCA- Fe^{3+} -complex concentration and absorbance at 580 nm showed that the Beer-Lambert Law applied and allowed a molar absorption coefficient of $1.69 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for the complex to be estimated. It must be noted that addition of excess FeCl_3 to a solution of PCA in potassium glycinate buffer leads to the production of a precipitate of iron; however, if this precipitate is removed by centrifugation, accurate absorbance measurements can be taken.

PCA concentration measurements in culture filtrates

Control experiments using *A. nidulans* strains that were wild-type with respect to the quinate- and shikimate-pathway genes showed that these strains did not produce interfering compounds in the culture filtrates that would cause or interfere with PCA- Fe^{3+} -complex formation, even when cultures were grown for 48 h. PCA concentrations were assayed in the following manner. To 750 μl of culture filtrate 250 μl 0.4 M-potassium glycinate buffer, pH 9.2 was added, along with 5 μl of 0.1 M- FeCl_3 ; the solution was vortex-mixed and incubated at room temperature for 2 h; precipitated iron was removed by centrifugation and the absorbance at 580 nm was measured. A further 5 μl of 0.1 M- FeCl_3 was added, and after the solution had been vortex-mixed it was incubated at room temperature for a further 2 h; precipitated iron was removed by centrifugation and the absorbance at 580 nm, which should remain the same as the first measurement, was re-measured.

A semi-quantitative PCA formation test was conducted by inoculating a loopful of conidiospores from chosen transformants into 5 ml of appropriately supplemented minimal medium containing glycerol as carbon source in a boiling tube. After incubation at 37 °C in an orbital incubator for 72 h, culture filtrates were assayed for PCA concentration and mycelial wet weight.

Flux measurements

Flux through the quinate and shikimate pathways was estimated by measuring the rate of production of PCA within the culture filtrate corrected for the extent of fungal mycelial growth. To gain a measure of flux through the quinate pathway, mycelium was initially grown with glucose (20 mM) as carbon source (catabolite-repressing carbon source) and, after harvesting by filtration, shifted to medium containing 20 mM-glycerol (non-catabolite-repressing carbon source) and 1 % quinate as carbon source. To gain a measure of the flux through the shikimate pathway, mycelium was grown with glucose as carbon source and, after harvesting by filtration, subsequently shifted to medium containing 20 mM-glycerol as carbon source.

RESULTS AND DISCUSSION

Strain variation in AROM protein concentrations

In order to assess the variability in AROM protein concentration within multiple samples of the same strain grown under common conditions of nutrient supply and aeration, five cultures of strain R44 were grown in glucose minimal medium and cell free extracts prepared. The five cell free extracts were

assayed for bDHQase and shikimate dehydrogenase activity, and the values obtained showed that there was a 22.5 % variation between the bDHQase assay values and 13 % variation between the shikimate dehydrogenase assay values (results not shown).

To test whether there is any significant difference between strains and whether exogenous supplementation (with a presumed concomitant internal increase) of aromatic amino acids does have an effect on the regulation of the *aromA* gene or activity of the AROM protein, a series of growth tests on strains R153, R44 and R46 were used with various levels of aromatic-amino-acid supplementation (see above). The experimental design was in the form of a 3×3 Latin square, and conidiospores were grown in glucose minimal medium supplemented with (a) no aromatic amino acid supplements, (b) supplements at $0.25 \times$ or (c) supplements at $1 \times$. Cell-free extracts were prepared, the levels of bDHQase and shikimate dehydrogenase measured, and the values subjected to a statistical analysis of variance. The results of the analysis showed that (a) strain R46 has significantly higher AROM protein activity than R44 and R153, being approx. 2-fold higher, and (b) supplementation with aromatic amino acids produced no significant difference in the levels of bDHQase or shikimate dehydrogenase (results not shown).

The data demonstrate that we can accurately detect the 2–3-fold variation in AROM protein concentration seen in transformed strains overexpressing the *gutC* gene (see below), when grown under the same conditions on the same day.

Construction of plasmid pGN1

A 4.5 kb *EcoRI* fragment containing the *gutC* gene was purified from agarose after electrophoresis of an *EcoRI* digest of recombinant cosmid pQ7 (Lamb *et al.*, 1990). The 4.5 kb *EcoRI* fragment was ligated to *EcoRI*-digested plasmid pDJB1 to produce plasmid pGN1. Plasmid pDJB1 is an *A. nidulans* transformation vector that contains the selectable marker, *pyr4*, from *Neurospora crassa* which allows growth of *A. nidulans pyrG* mutants in the absence of uracil (Ballance *et al.*, 1983).

Overproduction of dehydroshikimate dehydratase induces an auxotrophic requirement for aromatic amino acids

Strain 2035 requires uracil for growth and constitutively produces the quinate pathway enzymes, and was used as a host in order to investigate the possibility of obtaining strains constitutively overproducing the quinate pathway enzyme dehydroshikimate dehydratase (encoded by *gutC*), and assessing their ability to grow on minimal medium. These constitutive mutants have previously been shown to have lowered levels of *gut*-pathway enzymes when grown with glucose as carbon source compared with glycerol (Grant *et al.*, 1988). Strain 2035 was transformed with plasmid pGN1 or pDJB1 (control), by selection for growth in the absence of uracil in minimal medium supplemented with aromatic amino acids. The transformants were subsequently inoculated on to glycerol minimal medium lacking aromatic amino acids to see if growth was impaired. A range of growth responses from wild-type to strongly impaired growth were observed, with the impaired-growth phenotype being 'rescued' by the addition of aromatic amino acids. One control and three experimental (nos. 2, 6 and 14) transformants were selected on the basis of impaired growth on glycerol minimal medium, purified by streaking for single colonies on glucose minimal medium and grown with a second control of strain R153, in minimal medium containing glycerol or glucose as sole carbon source. Cell-free extracts were assayed for all three quinate-pathway enzymes, the shikimate-pathway enzyme shikimate dehydrogenase, and the results of the enzyme assays are shown in Table 1. Inspection of Table 1 leads to the following

observations: (1) R153 control strain has the quinate-pathway enzymes at the expected low uninduced level, whereas the control pDJB1 transformant 2038 has the enzymes constitutively expressed; however, this constitutive expression is subject to carbon catabolite repression; (2) all three experimental transformants overproduced the dehydroshikimate dehydratase enzyme, and in each case this overproduction was subject to carbon catabolite repression; (3) transformants 2 and 14 produced levels of quinate dehydrogenase and cDHQase similar to the pDJB1 control, but transformant 6 had substantially reduced levels of these two enzymes; (4) the levels of shikimate dehydrogenase in the control and three experimental transformants was substantially higher (up to 3-fold) in glycerol minimal medium (a non-catabolite-repressing carbon source) lacking aromatic-amino-acid supplementation compared with glycerol minimal medium with supplementation.

We interpret the impaired growth of the experimental strains when grown on glycerol minimal medium as being a consequence of the elevated levels of the dehydroshikimate dehydratase fluxing DHS from the shikimate pathway into the quinate pathway. DNA from the R153 control and experimental strains was purified and, after digestion with restriction endonuclease *EcoRI* and electrophoresis through agar, subjected to Southern-blot analysis using [α - 32 P]dCTP-labelled DNA containing the *qutC* gene or the vector pDJB1 as probes. Transformant number 6 was found to contain multiple (approximately seven) copies of the *qutC* gene inserted into the genome at heterologous sites, whereas transformants 2 and 14 had integration events at the homologous site. These observations are consistent with the view that the heterologous integration events in transformant 6 are effectively 'titrating out' the activator protein encoded by the *qutA* gene causing a reduced induction of the remainder of the *qut* cluster genes.

Chronic drain on the shikimate-pathway intermediates DHQ and DHS leads to the elevation of AROM protein concentration

The apparent increase in AROM protein concentration (as measured by the shikimate dehydrogenase activity) in transformed strains in the *qutR16* background (conferring constitutive expression of the *qut* cluster genes) when they are grown in glycerol minimal medium without aromatic-amino-acid supplementation (see Table 1) is a challenging observation. We believe that this increase in AROM protein concentration is consistent with the view that it represents a response to a chronic drain on the flux through the shikimate pathway. When the elevated levels of the dehydroshikimate dehydratase flux DHS from the shikimate pathway through the quinate pathway over an extended period, we propose that the organism attempts to compensate by elevating the concentration of the AROM protein, thereby fluxing DHS back into the shikimate pathway in accordance with the Law of Mass Action. When cells are grown on glycerol minimal medium in the presence of aromatic amino acids, the physiological consequence of this chronic drain on the flux through the shikimate pathway is alleviated, thereby obviating the necessity for an elevation of the AROM protein concentration. In order to investigate whether this phenomenon was a general response to a reduced ability to produce aromatic amino acids *in vivo* (a starvation response), we investigated whether *aromB*⁻ mutants (lacking functional chorismate synthase) of *A. nidulans* showed the same effect. Strain 1242, which lacks chorismate synthase and consequently cannot synthesize the aromatic amino acids *in vivo*, and strain R44 (the parental strain from which strain 1242 was derived by mutagenesis) were grown in glucose minimal medium, supplemented with 0.25 $\times$ aromatic amino acids. After 18 h growth the mycelium from each strain was harvested by filtration, divided into

Table 1. Quinate- and shikimate-pathway enzyme levels in selected transformants overproducing dehydroshikimate dehydratase

Transformant		Quinic acid-utilization enzyme						Shikimate-pathway enzyme	
		Quinate dehydrogenase		cDHQase		Dehydroshikimate dehydratase		Shikimate dehydrogenase	
		Specific activity (units/mg)	Relative	Specific activity (units/mg)	Relative	Specific activity (units/mg)	Relative	Specific activity (units/mg)	Relative
R153		0.02		0.041		0.010		ND*	
R2038		0.25	1	0.504	1	0.360	1	0.021	1
2	Glycerol-grown	0.21	0.84	0.478	0.95	3.264	9.1	0.037	1.76
6		0.11	0.44	0.100	0.20	3.598	10	0.040	1.90
14		0.37	1.48	0.663	1.32	1.465	4.01	0.024	1.14
R153		0.012		0.013		0.082		ND	
2038		0.085	1	0.193	1	0.205	1	0.008	1
2	Glucose-grown	0.042	0.49	0.100	0.52	0.771	3.76	0.023	2.88
6		0.010	0.12	Not detectable		0.437	2.13	0.016	2.00
14		0.104	1.20	0.160	0.83	0.411	2.00	0.021	2.63
R153		ND		ND		ND		ND	
2038	Glycerol plus	ND		ND		ND		0.013	1
2	aromatic	ND		ND		ND		0.012	0.92
6	amino acids	ND		ND		ND		0.011	0.85
14		ND		ND		ND		0.011	0.85
R153		ND		ND		ND		ND	
2038	Glucose plus	ND		ND		ND		0.014	1
2	aromatic	ND		ND		ND		0.010	0.85
6	amino acids	ND		ND		ND		0.010	0.85
14		ND		ND		ND		0.014	1

* Abbreviation: ND, not determined.

two portions and re-incubated for a further 8 h in glucose minimal medium with and without aromatic-amino-acid supplementation at $0.25 \times$. Cell-free extracts from all four final mycelial pads were assayed for bDHQase and shikimate dehydrogenase activity; the R44 strain showed no significant difference in the enzyme levels between the supplemented and non-supplemented cultures, whereas strain 1242 showed a 2-fold increase in the non-supplemented (starved) culture (results not shown). These observations are consistent with the view that reduction of aromatic-amino-acid synthesis *in vivo* leads to a starvation response in which the concentration of the AROM protein is increased.

Measurement of the rate of PCA production as a function of flux through the quinate pathway

As the measurement of PCA production requires that mycelium be pregrown with glucose as a carbon source and subsequently shifted to inducing media with different carbon sources, a problem arises in that strains of differing genotype show differential growth upon shifting. To circumvent this problem, pyridoxine was left out of the inducing medium because it inhibits mycelial growth, as all strains carry the *pyroA4* mutation which confers a requirement for pyridoxine supplementation.

In order to gain a measure of the flux through the shikimate and quinate pathways, the measurement of the rate of production of PCA in strains with wild-type and mutant (non-functional) PCA oxygenase was attempted. Initial control experiments with shifted mycelium both supplemented and non-supplemented with pyridoxine demonstrated that, after correction of the supplemented culture for continued mycelial growth, the rate of PCA production was essentially identical (6% difference; results not shown) in both cultures. These data showed that the mycelial pad only had to be weighed after the initial growth with glucose as carbon source. Strain 2036 (constitutively expressing the *gut* cluster genes, lacking PCA oxygenase and requiring uracil for growth), strain 2037 (lacking PCA oxygenase) and strain 2038 (strain 2036 transformed with pDJB1 and therefore not requiring uracil for growth), and strain R153 (wild-type with respect to the *gut*, PCA and shikimate-pathway genes), were grown with glucose as carbon source, shifted to inducing medium containing glycerol and 1% quinate and incubated for a further 13 h. Samples of culture filtrate were taken at hourly intervals, and the concentration of PCA determined as described in the Materials and methods section. The results obtained showed that PCA was produced at a constant rate over 8.5 h in all but the R153 strain. The rate of PCA production for strains 2036 and 2038 was approx. $1.9 \text{ mm} \cdot \text{h}^{-1} \cdot \text{g}$ of mycelium $^{-1}$ and for strain 2037 was $0.75 \text{ mm} \cdot \text{h}^{-1} \cdot \text{g}$ of mycelium $^{-1}$. The lower rate for strain 2037 was correlated with an approx. 3-fold relative decrease in dehydroshikimate dehydratase levels; therefore, when corrected for enzyme levels, all three rates of PCA production were essentially identical (< 15% variation). As expected, strain R153, which produces active PCA oxygenase, does not accumulate PCA in the culture filtrate, and these results confirm that it is possible to measure accurate rates of production of PCA in culture filtrates of strains containing the *pcaE200* mutation.

Shikimate-pathway-flux measurements in transformed strains overproducing the dehydroshikimate dehydratase enzyme

Strain 2036 was transformed with plasmids pDJB1 (control) and pGN1 (experimental), and transformants selected in glucose minimal medium, lacking uracil, but supplemented with $1 \times$ concentration of aromatic amino acids. The selective medium was supplemented with aromatic amino acids to obviate the physiological consequences of the predicted drain on the flux

through the shikimate pathway caused by overproduction of dehydroshikimate dehydratase. Five pDJB1 (control) and 42 pGN1 transformants (experimental) were taken at random and subject to growth tests on minimal medium containing (a) glycerol, (b) glycerol plus $0.25 \times$ aromatic-amino-acid supplements, (c) glucose and (d) glucose plus $0.25 \times$ aromatic-amino-acid supplements; 36 of the 42 pGN1 transformants tested showed a range of clearly reduced growth on glycerol non-supplemented compared with glycerol-supplemented medium, a similar but less pronounced growth difference was observed with the supplemented and non-supplemented glucose minimal medium. The five pDJB1 transformed control strains were scored as a relative growth of 5 on supplemented and non-supplemented glycerol medium, and the pGN1 transformants ranged in relative growth value from 1 to 5 on the same medium. Selected pGN1 transformants showing a range of growth values and the control pDJB1 transformant were further screened in a semi-quantitative PCA-formation test as described in the Materials and methods section. The concentration of PCA formed after 72 h growth was closely correlated with the reduced growth rate of particular transformants in both solid and liquid glycerol minimal medium, strongly suggesting that this was a function of the dehydroshikimate dehydratase levels *in vivo*. On the basis of these tests, 11 pGN1 and one pDJB1 transformants were chosen, grown in glucose minimal medium and shifted to glycerol minimal medium containing 0.1% quinate to maximally induce the *gutC* gene(s). After harvesting by filtration, cell-free extracts were prepared and assayed for the level of dehydroshikimate dehydratase and shikimate dehydrogenase. The results of these assays are shown in Table 2, which shows that the dehydroshikimate dehydratase levels in the pGN1 transformants varied from 1 to approx. 30-fold higher than the pDJB1 transformed control and that the shikimate dehydrogenase levels varied by up to a 5-fold increase. The higher levels of dehydroshikimate dehydratase in the transformants correlated exactly with the semi-quantitative growth tests on solid medium and the semi-quantitative PCA test in liquid minimal medium. Although it is not absolute, a higher relative level of shikimate dehydrogenase is correlated with an increase in dehydroshikimate dehydratase levels. We consider that these observations are entirely consistent with our view that shikimate dehydrogenase levels are amplified in response to a chronic drain on the metabolic flux through the shikimate pathway, caused by the constitutive activity of dehydroshikimate dehydratase (see above and Table 2).

Table 2. Dehydroshikimate dehydratase and shikimate dehydrogenase levels in transformants of strain 2036

The values are given in units/mg of protein and as a relative value compared with the control strain 2038.

Transformant	DHS dehydratase		Shikimate dehydrogenase	
	(units/mg)	(Relative)	(units/mg)	(Relative)
1	0.18	7.2	0.016	1.6
2	0.46	18.4	0.028	2.8
4	0.22	8.8	0.043	4.4
5	0.04	1.6	0.023	2.3
7	0.12	4.8	0.012	1.3
13	0.19	7.5	0.014	1.5
14	0.82	33.5	0.053	5.4
18	0.34	13.6	0.022	2.3
19	0.51	20.6	0.036	3.7
20	0.27	10.3	0.016	1.6
21	0.22	8.7	0.011	1.1
2038	0.025	1.0	0.0098	1.0

Fig. 1. Rate of PCA production as a function of dehydroshikimate dehydratase concentration

The rate of PCA concentration in $\mu\text{M} \cdot \text{h}^{-1} \cdot \text{g}$ of mycelium $^{-1}$ is shown as a function of the increase in dehydroshikimate dehydratase levels relative to the wild-type.

In order to gain some measure of the effect on flux through the shikimate pathway caused by overproducing the dehydroshikimate dehydratase, selected transformants were assayed to determine the rate of PCA production. Eight pGN1 transformants (nos. 1, 2, 4, 5, 7, 14, 18, 19) and one pDJB1 control transformant (2038) were grown in glucose minimal medium, subsequently shifted to glycerol minimal medium (lacking pyridoxine, to prevent differential growth), and culture filtrates taken at intervals over a further 24 h period. PCA concentrations for each strain at each time point were determined, allowing the rate of PCA production to be measured. Cell-free extracts were prepared from each transformant at the end of the experiment, and the level of dehydroshikimate dehydratase was determined; the variation in the rate of PCA production as a function of the dehydroshikimate dehydratase level is shown in Fig. 1. Inspection of Fig. 1 shows that the rate of PCA production has a complex relationship with the increase in the concentration of dehydroshikimate dehydratase. Increases in dehydroshikimate dehydratase levels up to approx. 10-fold lead to a linear increase in PCA production, whereas levels over a 15-fold increase have a sharply reduced effect. Previously Lamb *et al.* (1991b) have shown that if the AROM protein has a strict or large associated channelling function then an n -fold change in the concentration of either of the competing enzymes will have a much less than n -fold effect on the relative flux of the common metabolic substrate. Inspection of Fig. 1 shows that at normal physiological concentrations of AROM protein and dehydroshikimate dehydratase, an n -fold increase in dehydroshikimate dehydratase leads to an approx. n -fold increase in the rate of PCA production. This observation suggests that the AROM protein does not have a quantitatively large channelling function and that the rate of leakage of metabolites from the AROM protein is similar to rate of flux through this part of the pathway. However, the fact that doubling the concentration of dehydroshikimate dehydratase from 15- to 30-fold over the normal wild-type level does not significantly further impair growth of such transformants on glycerol minimal medium strongly suggests that, under these conditions, the AROM protein has a low-level channelling function. This still leaves open the possibility that even greater increases in dehydroshikimate dehydratase may cause a greater impairment of growth of transformants on glycerol minimal medium lacking aromatic amino acids. To test this possibility, strain 2036 was retransformed with plasmid pGN1 and plasmid pAL7ΔGE (Berl *et al.*, 1990), which contains the *gutA* activator

gene and therefore in co-transformed strains enhances the potential for high-level overproduction of dehydroshikimate dehydratase. Strain 2036 was transformed with pAL7ΔGE and pGN1 in the ratio of 10:1 and 20:1 (w/w) and transformants selected in glucose minimal medium supplemented with $1 \times$ aromatic amino acids. In general the transformation frequency was lower by about 50 %; the transformants appeared later, and on average had significantly greater impairment of growth on glycerol minimal medium lacking aromatic amino acids. Some strains grew so poorly that they could only be rescued by cutting agar plugs from the selective medium and reincubation on a rich non-selective medium, where some slight increase in growth was observed. Incubation on this rich medium for more than 3 days led to the appearance of fast-growing sectors originating from the initially poor-growing strains. This phenomenon has been described previously (Lamb *et al.*, 1991b) and in this case is probably due to selection for sectors that are now producing dehydroshikimate dehydratase at a low level. We interpret these observations as being entirely consistent with the view that these original co-transformants are producing dehydroshikimate dehydratase at such a high level (> 30 -fold) that the growth of mycelium is almost completely stopped, owing to the extensive drain on shikimate-pathway intermediates.

Implications for the channelling hypothesis

The results presented above strongly suggest that, with normal physiological concentrations of enzymes under steady-state conditions with optimal nutrient and oxygen supply, the channelling function is likely to be physiologically insignificant. Lamb *et al.* (1991b) have previously argued that the ecology of the organism must be considered and that in its natural environment the supply of carbon sources will not be constant. Quinate constitutes an abundant carbon source for *A. nidulans in situ*, but it is likely that quinate will only be available in pulses, for example in heavy dew, rainfall or other transient humid conditions (Lamb *et al.*, 1991b). Fig. 1 demonstrates that, with physiologically normal concentrations of enzymes, the rate of flux of DHS from the shikimate pathway through the quinate pathway in the absence of quinate is approximately proportional to the dehydroshikimate dehydratase concentration. Therefore if the *gutC* gene encoding dehydroshikimate dehydratase is subject to pulsed induction or the enzyme persists *in vivo* when quinate is no longer actively being metabolized, DHS from the shikimate pathway will flux into the quinate pathway. We have tested the ability of dehydroshikimate dehydratase to persist *in vivo* by growing *A. nidulans* strain R153 on glucose minimal medium for 18 h, shifting the washed mycelium to quinate minimal medium for 4 h and finally shifting to glycerol minimal medium for a further 2 h. Cell-free extracts were prepared from the mycelium at various time points after the shift to glycerol minimal medium, and the levels of dehydroshikimate dehydratase and shikimate dehydrogenase measured up to 2 h after the shift. As predicted, the level of shikimate dehydrogenase remained close to the original value (less than 25 % reduction); however, the dehydroshikimate dehydratase level was only reduced by approximately one half. These observations indicate that significant dehydroshikimate dehydratase activity does persist *in vivo* for at least 2 h after quinate is no longer present as a carbon source.

Why are the enzymes for steps 2–6 in the shikimate pathway fused into a single protein?

On the basis of the experiments presented here and previously (Lamb *et al.*, 1991b), we propose that the selective advantage for this fusion is that by the juxtaposition of five closely spaced active sites, a low-level channelling function is associated with the AROM protein. As the AROM protein is so leaky, we believe

that this channelling function is physiologically insignificant under steady-state conditions with optimal nutrient and oxygen supply. However, under conditions of nutrient limitation due, for example, to substrate pulsing in the organism's natural environment, we propose that this low-level channelling function becomes physiologically significant and ensures the minimum flux through the shikimate pathway necessary to prevent mycelial death. On a larger scale this low-level channelling function can be seen as a safeguard to alleviate acute stress caused by short periodic fluxing of shikimate pathway DHS into the quinate pathway. Similarly the ability of the organism to elevate the levels of the AROM protein in response to the long-term fluxing of shikimate-pathway intermediates into the quinate pathway can be seen as a safeguard against chronic depletion of shikimate-pathway intermediates. Further, we suggest that the reason the shikimate pathway DHQ and DHS do not lead to constitutive *in vivo* induction of the quinate pathway genes is simply that the normal physiological concentrations are too low. The genetic model predicts that the quinate-pathway genes, especially the permease gene *qutD*, must be continually expressed at a low level *in vivo* or quinate would only enter the cell at a very low rate in its protonated form when it became available. The DHQ and DHS leaking from the AROM protein may aid in maintaining this low level expression, that is not detectable *in vitro*.

This research was supported by Science and Engineering Research Council grants GR/E1055.5 and GR/F44663 awarded to A.R.H. We are indebted to Dr. H. Nimmo for stimulating discussions that lead to the design of some of the experiments described in this manuscript, and thank Mrs. I. Stobbs for typing the manuscript.

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Received 5 August 1991/7 November 1991; accepted 11 November 1991