Review Article

The pre-chorismate (shikimate) and quinate pathways in filamentous fungi: theoretical and practical aspects

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Purpose of the review

The detailed chemistry and enzymology of the shikimate pathway have been extensively discussed in recent excellent reviews (Pittard, 1987; Bentley, 1990; Braus, 1991). This short review will therefore focus (a) on the web of interconnecting evolutionary and structural relationships within and between the enzymes and regulatory proteins that constitute the pre-chorismate (shikimate) and the related quinate pathways, and (b) the possibility of pathway engineering to divert major new carbon flux from the quinate pathway into the shikimate pathway.

What is the shikimate pathway?

The shikimate pathway includes the production of chorismate, which is the common precursor for the three aromatic amino acids (Trp, Tyr and Phe) and *para*-aminobenzoic acid (see Fig. 1). This pathway is present in those prokaryotes, microbial eukaryotes and plants studied; however, it is absent in those animal species investigated, making it a potential target for specific chemotherapeutic attack. The traditional view of the pathway as solely being of interest because of the production of the aromatic amino acids is inaccurate. The shikimate pathway branches at many points leading to the production of an extensive range of products that include metal chelators, vitamins E and K, folic acid, ubiquinone and plastoquinone. The pre-chorismate shikimate pathway branches at 3-deoxy-D-*arabino*-hep-

tulosonate 7-phosphate and 3-dehydroquinate giving rise to either 3-hydroxy-5-aminobenzoate or 3-amino-benzoate. These compounds are the precursors to a specific moiety that is characteristic of the ansamycins, a complex group of compounds that have a diverse array of biological properties including antibiotic, antiviral and antitumour activities (Bentley, 1990, and references therein). These two branch points identify good examples of pathways that are of medical and biotechnological interest and potential targets for pathway engineering. Other branches of the pathway and references to them are contained within the review of Bentley (1990).

The pre-chorismate shikimate pathway has two metabolites (3-dehydroquinate, DHQ; dehydroshikimate, DHS) in common with the quinate pathway that is responsible for the catabolism of quinate to protocatechuate (PCA) in many fungi and some bacteria (Tresguerres *et al.*, 1970*a, b*; Ingledew *et al.*, 1971; Grund *et al.*, 1990) (see Fig. 1). Quinate is an abundant carbon source for many fungi comprising around 10% by weight of decaying leaf litter. The three enzymes necessary for the catabolism of quinate to PCA are induced by the presence of quinate, but this induction is subject to carbon catabolite repression when quinate is supplied as a mixture with other preferred carbon sources such as glucose. The induction of the production of the quinate pathway enzymes is therefore highly regulated and the molecular mechanisms involved have been intensively studied by genetic and molecular techniques in the filamentous fungi *Neurospora crassa* and *Aspergillus nidulans* (Giles *et al.*, 1985; Grant *et al.*, 1988). The metabolites DHQ and DHS, however, are interconverted by two entirely separate 3-dehydroquinase isoenzymes that have different phylogenetic origins and

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Fig. 1. Genes, enzymes and metabolites comprising the quinate and pre-chorismate (shikimate) pathways in *A. nidulans*. The AROM protein catalyses steps two to six in the pre-chorismate section of the shikimate pathway converting 3-deoxy-D-arabino-heptulosonate 7-phosphate into 5-enolpyruvylshikimate 3-phosphate. The individual enzymes are: 3-dehydroquinase (EC 4.6.1.3; DHQ synthase); 3-dehydroquinase (EC 4.2.1.10); shikimate dehydrogenase (EC 1.1.1.25); shikimate kinase (EC 2.7.1.71); 5-enolpyruvylshikimate-3-phosphate synthase (EC 2.5.1.19; EPSP synthase). The initial step in the shikimate pathway is catalysed by 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (EC 4.1.2.15; DAHP synthase); the final step in the pre-chorismate section is catalysed by chorismate synthase (EC 4.6.1.4).

distinct genetic mechanisms regulating the corresponding gene expression (see below).

Pathway organization

The enzymes catalysing the pre-chorismate section of the shikimate pathway in bacteria are monofunctional with no evidence of *in vivo* association and are encoded by genes that tend to be dispersed throughout the genome (Pittard, 1987). This trend is not absolute, as at least in *Mycobacterium tuberculosis*, *Amycolatopsis methanolica* and *Staphylococcus aureus* the genes encoding DHQ synthase and 3-dehydroquinase are closely linked (Garbe *et al.*, 1991; Euverink *et al.*, 1992; O'Connell *et al.*, 1993). There is evidence that plants in general have the 3-dehydroquinase and shikimate dehydrogenase enzymic activities fused into a single bifunctional protein (Koshiba, 1978; Mousdale *et al.*, 1987). In many microbial eukaryotes, the enzymes catalysing steps two to six are fused into a single polypeptide (the AROM protein) that is active as a dimer of identical subunits (Smith & Coggins, 1983). The genes encoding these proteins in *A. nidulans* and *Saccharomyces cerevisiae* have been isolated by molecular cloning and their nucleotide sequences determined (Hawkins *et al.*, 1985; Charles *et al.*, 1986; Hawkins, 1987; Duncan *et al.*, 1987). Comparison of the

predicted amino acid sequences encoded by these genes with those of the corresponding genes from an array of bacterial species strongly suggests that the genes are related to common ancestors and that the genes encoding the pentafunctional proteins evolved by multiple gene fusions (Hawkins, 1987; Duncan *et al.*, 1987). Amino acid sequence comparisons between the 3-dehydroquinase isoenzymes active in the shikimate (biosynthetic) and quinate (catabolic) pathways of filamentous fungi strongly suggested that these isoenzymes are phylogenetically distinct and have arisen by convergent evolution (Da Silva *et al.*, 1986). Most recent biochemical analyses of the shikimate pathway 3-dehydroquinases from *Escherichia coli* and *Salmonella typhi* and the quinate pathway 3-dehydroquinase from *A. nidulans*, have confirmed that they are representatives of two distinct classes of 3-dehydroquinase (Kleanthous *et al.*, 1992; Moore *et al.*, 1993). Type I 3-dehydroquinases are dimeric enzymes with a subunit M_r of around 27000, whereas type II 3-dehydroquinases are dodecamers with a subunit M_r of 16–18000. Type I enzymes involve Schiff base formation as part of the reaction mechanism and abstract water from DHQ in *cis*, whereas type II enzymes do not form a Schiff base and abstract water from DHQ in *trans*, the opposite stereochemical outcome from type I enzymes, further strengthening the convergent evol-

ution hypothesis (Shneier *et al.*, 1993; Harris *et al.*, 1993). Comparison of 3-dehydroquinases from a range of prokaryotic species has shown that type I and II 3-dehydroquinases show a high degree of sequence conservation within a class. Type I enzymes examined so far are exclusively biosynthetic in nature whereas type II enzymes are active in both the fungal catabolic (quinate) and the biosynthetic (shikimate) pathways (Giles *et al.*, 1967; White *et al.*, 1990; Garbe *et al.*, 1991). Indeed, a type II 3-dehydroquinase in *Amycolatopsis methanolica* has a dual function catalysing the conversion of DHQ to DHS which is then utilized in both the shikimate and quinate pathways (Euverink *et al.*, 1992). Recent experiments in *A. nidulans* have shown that both the type I enzyme of *S. typhi* and the type II enzyme of *Mycobacterium tuberculosis* when produced *in vivo* in *qutE* mutants of *A. nidulans* unable to produce the normal type II (catabolic) enzyme facilitate growth with quinate as sole carbon source (Moore *et al.*, 1992). A full three-dimensional comparison of the two classes of enzyme is now within reach, as the type I enzyme from *S. typhi* and the type II enzyme from *M. tuberculosis* have been purified in bulk and successfully crystallized (Boys *et al.*, 1992; Moore *et al.*, 1992).

The organization and regulation of the genes necessary for the catabolism of quinate and its regulation have been most intensively studied in *N. crassa* (Giles *et al.*, 1985; Geever *et al.*, 1989) and *A. nidulans*. Other species have been characterized (Giles *et al.*, 1991), but the *in vivo* interactions between the quinate and shikimate pathways have been most extensively characterized in *A. nidulans*. A cluster of eight genes (designated *qut*) with quinate inducible mRNAs have been isolated by molecular cloning from *A. nidulans* (although only six of the genes have been characterized by mutational analysis) and their nucleotide sequences determined (Da Silva *et al.*, 1986; Beri *et al.*, 1987; Hawkins *et al.*, 1988, 1992; Lamb *et al.*, 1992a). The eight genes are designated *qutA* (encodes an activator protein), *qutB* (encodes quinate dehydrogenase), *qutC* (encodes a dehydroshikimate dehydratase), *qutD* (encodes a quinate permease), *qutE* (encodes catabolic type II 3-dehydroquinase), *qutG* (encodes a protein homologous with bovine *myo*-inositol monophosphatase), *qutH* (encodes a protein that has a low level similarity with a DNA polymerase from Hepatitis B virus) and *qutR* (encodes a repressor protein). The mutant phenotype of the *qutG* and *qutH* genes is not known as strains mutant in these genes have not been recovered. Transcription of the *qut* gene cluster is positively mediated by the QUTA protein, the product of the *qutA* gene, and negatively mediated by the QUTR protein, the product of the *qutR* gene (Grant *et al.*, 1988). In the context of this review, the most important feature of the regulation is that the QUTR protein is proposed

to repress transcription by binding to the activator protein stoichiometrically and to negate its repressing function by being able to recognize and bind quinate, DHQ and DHS (Hawkins *et al.*, 1992, 1993a).

The shikimate and quinate pathway enzymes and regulatory proteins are based around a small group of conserved structural modules that have evolved pathway specific functions

Amino acid sequence comparisons have revealed a wealth of information showing that the enzymes and regulatory proteins comprising the shikimate and quinate pathways share a long and complex history (see Fig. 2). The bifunctional *qutB* encoded quinate pathway quinate/shikimate dehydrogenase is related to a common ancestor of the prokaryotic shikimate pathway shikimate dehydrogenases and to the fungal shikimate dehydrogenases found in the pentafunctional AROM protein (Hawkins *et al.*, 1988). Unexpectedly however, the amino acid sequences of the *qa-1S* (*N. crassa*) and *qutR* (*A. nidulans*) encoded fungal quinate pathway transcription repressor proteins are similar throughout their entire length to the extreme C-terminus (amino acids 760–863 in *A. nidulans*) of the EPSP synthase domain, and the three complete C-terminal domains shikimate kinase, 3-dehydroquinase and shikimate dehydrogenase of the pentafunctional AROM proteins (amino acids 864–1603 in *A. nidulans*) from fungi (Anton *et al.*, 1987; Hawkins *et al.*, 1992). Indeed, one basic structural motif has formed the basis for the shikimate pathway *aromA* encoded shikimate dehydrogenase, the quinate pathway *qutB* encoded quinate/shikimate dehydrogenase and approximately one-third of the quinate pathway *qutR* encoded repressor protein that controls the expression of the *qutB* gene (see Fig. 2). The domain of the QUTR protein equivalent to the shikimate kinase domain of the AROM protein is of particular interest, as it contains the motif GX₄GKS/T (where X = any amino acid) which is found in purine nucleotide binding proteins (Walker *et al.*, 1982). This observation opens the possibility that regulation of transcription of the *qut* gene cluster may involve phosphorylation events (see below), a view strengthened by the close similarity of the *qutG* encoded QUTG protein to bovine *myo*-inositol monophosphatase (Lamb *et al.*, 1990). In addition, these sequence alignments provide a potential molecular explanation for the genetically based assertion that the quinate pathway *qutR* encoded transcription repressor protein can recognize and bind quinate, DHQ and DHS, as these metabolites or closely related compounds can be metabolized by the three C-terminal AROM protein domains. The simplest explanation is that the repressor proteins have retained the ability to recognize and bind these

Fig. 2. Modular structure of the enzymes and regulatory proteins comprising the quinate and pre-chorismate shikimate pathways in *A. nidulans*. The boxes denoted *aro* designate genes from bacteria encoding monofunctional shikimate pathway enzymes: *aroA*, *E. coli* EPSP synthase (EPSPS); *aroB*, *E. coli* DHQ synthase (DHS); *aroD*, *E. coli* type I 3-dehydroquinase (DQ); *aroE*, *E. coli* shikimate dehydrogenase (SDH); *aroL*, *E. coli* shikimate kinase (SK); and *aroQ*, *M. tuberculosis* type II 3-dehydroquinase (DQII). AROM designates the modular structure of the AROM protein of *A. nidulans* which is specified by the *aromA* gene that arose by the fusion of the bacterial *aroA*, *B*, *D*, *E* and *L* genes. The boxes denoting the genes of the *qut* pathway are: *qutA*, activator; *qutB*, quinate/shikimate dehydrogenase (QDH); *qutD*, permease; *qutE*, type II 3-dehydroquinase (DQII); *qutG*, possibly a phosphatase; and *qutR*, repressor. The genes encoding the quinate pathway activator and repressor proteins are proposed to have arisen by duplication of the *aromA* gene followed by cleavage in the DNA sequence specifying the C-terminus of the EPSP synthase domain (*aroA* equivalent). The *aromA* sequence encoding the DHQ synthase domain in the duplicated copy is proposed to have undergone an event in which the DNA sequence encoding a pre-formed zinc binuclear cluster motif (designated Zn) has become inserted into its N-terminus. The box denoted *aroQ* designates the type II 3-dehydroquinase from *M. tuberculosis*. The arrows between boxes are there to indicate that genes denoted by the boxes are related to one another. The use of *A. nidulans*, *E. coli* and *M. tuberculosis* is to give specific examples as the homologies between the various genes are evident across a wide range of species. Refer to the text for references to these and other examples in the literature.

metabolites, but have lost enzymic activity with respect to them. These observations about the QUTR and equivalent *N. crassa* QA-1S proteins, whilst providing a potential basis to explain the phenotypes of *qutR* and *qa-1S* mutants, also raise the fundamental question of how a protein that is related to three shikimate pathway enzymes can recognize and negate the activity of a eukaryotic transcriptional activator protein by binding to it. Most remarkably, it has recently been reported that the *qutA* encoded quinate pathway activator protein is homologous with the two amino terminal domains of the pentafunctional AROM protein, DHQ synthase and EPSP synthase (Hawkins *et al.*, 1993a). The fundamental change is the insertion into the DHQ synthase like domain of an amino acid sequence motif that is able to co-ordinate zinc atoms and form a putative zinc binuclear cluster domain, thereby facilitating DNA binding (see Fig. 2). Immediately adjacent to the C-terminus of the zinc binuclear cluster domain is a specificity region which acts to direct binding to defined upstream activator sequences in the promoters of quinate pathway specific genes (Hawkins *et al.*, 1988). This hypothesis requires that the two N-terminal domains of

the AROM polypeptide fold to give a stable protein, and it has recently been demonstrated that the two N-terminal domains (DHQ synthase and EPSP synthase) of the AROM polypeptide are able to fold and function independently of the three C-terminal domains (Hawkins & Smith, 1991; Moore & Hawkins, 1993). This observation therefore gives added credence to the proposal that the activator protein is homologous to this functionally independent part of the AROM polypeptide. The overall sequence similarity of the *A. nidulans* QUTA protein compared with the AROM protein is 23%, with localized regions as high as 35%; this situation is similar to the sequence similarity of the repressor protein with the three C-terminal domains of the AROM protein.

A speculative model for the molecular control of transcription of the qut gene cluster

This sequence similarity suggests a possible mechanism for how the repressor protein is able to recognize and bind to the activator. Early pioneering genetic studies demonstrated that the native AROM protein in *N. crassa* is a dimer of identical subunits, and that the dimerization

interface involved contacts between the two DHQ synthase domains and between the two shikimate dehydrogenase domains (Case *et al.*, 1969; Case & Giles, 1971). Later work confirmed that the native AROM protein in *N. crassa* is a dimer and demonstrated through the use of limited proteolysis peptide mapping and suberimidate cross-linking studies that proteolytically cleaved AROM protein retains its native quaternary structure (Lumsden & Coggins, 1978; Smith & Coggins, 1983). The cross-linking studies further strongly suggest that the two individual polypeptides comprising the native AROM protein adopt a compact globular shape with the N- and C-terminal domains (DHQ synthase and shikimate dehydrogenase respectively) closely juxtaposed (Smith & Coggins, 1983). These observations suggest a mechanism for how the repressor protein is able to recognize and bind to the activator. As the activator and repressor appear to have evolved by splitting a duplicated copy of the gene encoding the pentafunctional AROM polypeptide in half, the two proteins may come into close juxtaposition with the shikimate dehydrogenase domain of the repressor in close proximity with the N-terminus (the DHQ synthase like domain) of the activator in a manner similar to the AROM protein. This arrangement would give the potential for the repressor 3-dehydroquinase like and/or shikimate kinase like domains to occlude the surface acidic residues located near the C-terminus of the activator that are proposed to be important for its interaction with the accessory transcription complex (Ptashne, 1988). These observations also reinforce the link between the fact that the N-terminal domain of the repressor protein is homologous to a metabolic kinase (shikimate kinase) and that the quinate inducible *qutG* gene encodes a protein that is homologous with bovine *myo*-inositol monophosphatase. It is hard to believe (though not impossible) that the metabolic kinase like domain of the QUTR repressor protein could have evolved into a protein kinase; similarly, it is unlikely that the *qutG* encoded *myo*-inositol phosphatase like protein is a protein phosphatase. The discovery that the QUTA activator protein is homologous with the two N-terminal domains of the AROM protein (DHQ synthase and EPSP synthase), however, suggests a speculative model for how transcriptional regulation may be influenced by a reversible phosphorylation event. The C-terminal half of the QUTA activator is homologous with the EPSP synthase domain of the AROM protein, and this domain is able to recognize shikimate phosphate as a substrate. It is therefore possible that the C-terminal half of the QUTA protein could bind a similar metabolite that is subject to reversible phosphorylation by the products of *qutG* encoded protein and the shikimate kinase like domain of the QUTR repressor protein. These events may generate

a phosphorylated isoform of the activator that is transcriptionally hyperactive in a manner analogous to the GAL4 activator in *S. cerevisiae* (Mylin *et al.*, 1990).

The quinate pathway may be the first unequivocally documented case where it is shown that virtually an entire catabolic pathway (the only exception is the *qutC* gene which shows no extensive homology to any protein in known databases) including regulatory proteins has been constructed by co-opting preformed domains from related pathways and evolving pathway specific functions (see Fig. 2). Four genes, *qutA*, *R*, *E* and *B* have been co-opted from the shikimate pathway, the *qutD* encoded permease is almost certainly a modified sugar transporter (Whittington *et al.*, 1987; Geever *et al.*, 1989), *qutG* is a member of a large group of phosphatases involved in dephosphorylating sugars (Neuwald *et al.*, 1991) and *qutH* shows a low level homology with the Hepatitis B DNA polymerase and has a conserved zinc binuclear cluster motif (Geever *et al.*, 1989; Lamb *et al.*, 1990, 1992).

Domain structure within the AROM protein

Domain structure and interaction within the *A. nidulans* pentafunctional protein have been investigated using *in vivo* and *in vitro* techniques. Sub-fragments of the *aromA* gene (encoding the AROM protein), have been expressed in appropriate *E. coli* mutant strains lacking particular shikimate pathway enzymes using prokaryotic expression vectors to complement the *aro*⁻ mutations and allow growth in minimal medium (Hawkins & Smith, 1991). These initial studies defined the approximate limits of the functional domains within the AROM protein, and subsequent work using polymerase chain reaction (PCR) amplified DNA was able to more precisely delineate the limits of these domains (van den Hombergh *et al.*, 1992; Moore & Hawkins, 1993). The complete AROM protein has been overproduced in *A. nidulans* by around 150-fold, constituting around 6% of the soluble cell protein (Moore *et al.*, 1992). The two N-terminal domains DHQ synthase and EPSP synthase have been substantially overproduced in *E. coli* as monofunctional domains (DHQ synthase), bifunctional domains (DHQ synthase and EPSP synthase) or as fusion proteins with glutathione *S*-transferase (EPSP synthase) (van den Hombergh *et al.*, 1992; Moore & Hawkins, 1993). An *in vivo* protein based complementation test in *E. coli* has also identified an interaction between the N-terminal domains DHQ synthase and EPSP synthase that stabilizes the activity of the latter enzyme (Moore & Hawkins, 1993). The type I 3-dehydroquinase domain of the *A. nidulans* AROM protein has also been overproduced in and purified from *E. coli*, and has substantially different kinetic parameters compared to the same domain of the

native *N. crassa* AROM protein (Hawkins *et al.*, 1993*b*). The two C-terminal domains (3-dehydroquinase and shikimate dehydrogenase) have also been shown to be capable of independent folding and retain enzymic activity. *In vitro* studies with the *N. crassa* AROM protein demonstrated that a proteolytic fragment specifying 3-dehydroquinase and shikimate dehydrogenase can be renatured in a polyacrylamide gel and bind the respective substrates (Smith & Coggins, 1983). *In vivo* studies with the *A. nidulans* AROM protein have demonstrated that a C-terminal bifunctional fragment of the protein can be overproduced in *A. nidulans* to around 50-fold over the normal wild-type native AROM protein level. These overexpressing strains show an apparent change in the specific activity ratios of the 3-dehydroquinase and shikimate dehydrogenase functions when compared to the native AROM protein; however, this observation must be considered with caution as the measurements were not performed on purified material (Moore & Hawkins, 1993). Taken as a whole with the data from *N. crassa* discussed earlier in this section, these experiments strongly support an emerging view that the domains that make up the AROM protein do not all act independently of one another, but that there is substantial interaction at a gross tertiary level to stabilize enzyme activity.

The physiology of the AROM protein

The contemporary functional significance of why the enzymes catalysing steps two to six in the shikimate pathway are fused into a single multifunctional protein in fungi is the subject of a long running controversy. Early work based on genetic studies and *in vitro* analysis of purified AROM protein proposed that the fused protein acted as a channelling mechanism to keep the quinate and shikimate pathway derived metabolites DHQ and DHS separate (Giles, 1978). Further work additionally ascribed the function of catalytic facilitation to the AROM protein, but these *in vitro* studies were subsequently reported to have been carried out with protein that was deficient in zinc (DHQ synthase requires zinc) and had suffered significant proteolysis (Gaertner *et al.*, 1970; Welch & Gaertner, 1975, 1976; Lambert *et al.*, 1985). The channelling hypothesis was challenged and an alternate explanation of ensuring the co-ordinate production of all five enzymes was proposed to explain the functional significance of having a pentafunctional protein (Duncan *et al.*, 1988).

Most recently, this controversy has been addressed in *A. nidulans* by taking an *in vivo* approach in contrast to earlier *in vitro* experiments. Recombinant DNA technology has been used to produce strains of *A. nidulans* that have particular quinate or shikimate pathway

enzyme concentrations up-modulated. Strains of *A. nidulans* lacking the *qutE* encoded catabolic type II 3-dehydroquinase are unable to utilize quinate as carbon source (see Fig. 1). If the AROM protein concentration in these strains is increased 5-fold, they are able to utilize quinate as carbon source demonstrating that the AROM protein type I 3-dehydroquinase is able to synthesize DHS from quinate pathway DHQ and subsequently release it, thereby showing that the AROM protein is leaky *in vivo* (Lamb *et al.*, 1991).

If the AROM protein concentration is raised over 5-fold in *A. nidulans* strains with wild-type *qut* gene cluster genes, then these strains are unable to grow in the presence of quinate (even on mixed carbon sources with glycerol) and have the *qut* pathway enzymes induced to a much lower level than in wild-type strains. The proposed cause of this effect is that the overproduced AROM protein is diverting flux of quinate pathway derived DHQ and DHS into the shikimate pathway at an increased rate, leading to one of the shikimate pathway metabolite concentrations reaching toxic levels (Lamb *et al.*, 1991). Reciprocal experiments in which the *qutC* encoded dehydroshikimate dehydratase concentration is manipulated demonstrate that substantial flux of DHS from the shikimate pathway can be diverted into the quinate pathway. Dehydroshikimate dehydratase catalyses the conversion of DHS to protocatechuate and is therefore active at the branch point between the quinate and shikimate pathways (see Fig. 1). With increases of dehydroshikimate dehydratase up to 10-fold over the normal wild-type level, an *N*-fold increase in enzyme concentration gives an *N*-fold increase in pathway flux (Lamb *et al.*, 1992*b*). This is equivalent to saying that the dehydroshikimate dehydratase enzyme has a shikimate pathway deviation control index (D) of -1 , where D^i is defined as $(\Delta J_i/J_i)/(\Delta E_i/E_i)$, where Δ stands for a large change, J stands for a pathway flux and E stands for the enzyme concentration under consideration (Niederberger *et al.*, 1992).

The *A. nidulans* strains overproducing the dehydroshikimate dehydratase display a varying auxotrophic requirement for aromatic amino acids which becomes more pronounced as the enzyme concentration increases (Lamb *et al.*, 1992*b*). This increasing auxotrophic requirement for aromatic amino acids is most simply interpreted as a consequence of diverting substantial flux from the shikimate pathway into the quinate pathway with an *N*-fold increase in enzyme (dehydroshikimate dehydratase) concentration giving an approximate *N*-fold change in the flux ratio of the two branches of the pathway (Lamb *et al.*, 1991; Hawkins *et al.*, 1993*c*). Additionally, strains overproducing the dehydroshikimate dehydratase enzyme show levels of the AROM protein elevated up to 5-fold over the wild-type level

(Lamb *et al.*, 1992*b*). These observations are consistent with the proposal that the AROM protein and dehydroshikimate dehydratase enzyme are displaying a mass action effect on a substrate (DHS) that approximates pool behaviour, without any need to impute a channelling function for the AROM protein (Lamb *et al.*, 1991; Hawkins *et al.*, 1993*c*).

These experiments whilst unequivocally demonstrating that the AROM protein is extremely leaky *in vivo* are dealing with the fungus growing under ideal laboratory conditions and do not rule out the possibility that there is a low level channelling property associated with the AROM protein. In this regard, analysis of strains overproducing the dehydroshikimate dehydratase up to 30-fold over the wild-type level have given evidence that the AROM protein does have a very low selective flux (channelling) effect due to the close juxtaposition of five active sites, but that this effect is only of physiological significance when the organism is growing in its natural environment where nutrient supply, aeration and water supply will not be optimal (Lamb *et al.*, 1992*b*).

Pathway manipulation

In *A. nidulans*, when quinate is supplied as the sole carbon source, there is an approximately 30-fold difference in the level of flux through the quinate pathway compared to the shikimate pathway as judged by the experiments up-modulating the dehydroshikimate dehydratase enzyme (Lamb *et al.*, 1992*b*). The quinate pathway is an entirely dispensable high flux pathway as *A. nidulans* and related fungi have a wide array of alternate carbon sources that they can utilize for energy and growth. It should therefore be possible to divert *de novo* carbon flux in the form of the metabolites DHQ and DHS from the quinate pathway into the lower flux shikimate pathway thereby circumventing the end-product inhibition on the DAHP synthases (Doy, 1968; Nimmo & Coggins, 1981; Braus, 1991), converting the latter into a high flux pathway possibly allowing the increased production of metabolites that are of commercial interest. Experiments with *A. nidulans* overproducing a mutant form of the AROM protein lacking the 3-dehydroquinase synthase enzyme activity have shown that substantial flux of DHQ and DHS can be diverted from the quinate pathway into the shikimate pathway (Lamb *et al.*, 1991; Moore & Hawkins, 1993). These experiments showed that (a) it is possible to circumvent the end-product inhibition on the DAHP synthases, the first step in the pathway and so increase pathway flux, and (b) that this increase in flux is limited to the pre-chorismate section of the shikimate pathway leading to one or more pathway intermediates reaching toxic levels. This inability to increase overall pathway

flux by up-modulating a single block of pathway enzymes is to be expected from a consideration of the principles of metabolic control analysis (MCA). MCA theoretically deals with infinitesimal changes in enzyme and metabolite concentrations, and in fluxes, but in practice small but finite changes are introduced into experimental systems. The changes in enzyme concentration and fluxes reported for *in vivo* quinate and shikimate pathway manipulations in *A. nidulans* clearly cannot be considered as small (Lamb *et al.*, 1991, 1992*b*); however, recent theoretical developments in MCA based on experiments with the shikimate pathway in *S. cerevisiae* are pointing the way forward (Niederberger *et al.*, 1992). Essentially these new observations show that in order to increase substantial overall pathway flux then ideally all of the enzymes in the pathway should be up-modulated simultaneously (Niederberger *et al.*, 1992). This requirement presents technically difficult obstacles unless the genes encoding all the enzymes in a chosen pathway have been isolated by molecular cloning. This is a greater or lesser problem depending on the tractability of the organism under study, but a second way of circumventing this problem is to express genes that have previously been isolated from other species encoding the required enzyme activities in the organism under study. This approach has been successfully employed in *A. nidulans* where a 158 bp truncated promoter from the *qutE* gene containing a putative upstream activator sequence recognized by the QUTA protein has been engineered into an *A. nidulans* expression vector containing the *qutE* transcription termination signals (Lamb *et al.*, 1991; Moore *et al.*, 1992; Moore & Hawkins, 1993). This plasmid successfully places any gene appropriately fused to the promoter under the control of the positively acting QUTA transcription regulator protein allowing quinate inducible production of heterologous proteins in *A. nidulans* (Lamb *et al.*, 1991; Moore *et al.*, 1992; Moore & Hawkins, 1993). Later versions of this expression plasmid containing the constitutive high level *A. nidulans* phosphoglycerate kinase promoter (Clements & Roberts, 1986; H. K. Lamb & A. R. Hawkins, unpublished data), should allow up-modulation of all the steps in the shikimate pathway by expression of appropriate heterologous genes in *A. nidulans*. This approach may allow the diversion of major new carbon flux from the dispensable quinate pathway into the shikimate pathway turning the latter into a high flux pathway.

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