Control of metabolic flux through the quinate pathway in *Aspergillus nidulans*

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The quinic acid utilization (*qut*) pathway in *Aspergillus nidulans* is a dispensable carbon utilization pathway that catabolizes quinate to protocatechuate via dehydroquinone and dehydroshikimate (DHS). At the usual *in vitro* growth pH of 6.5, quinate enters the mycelium by means of a specific permease and is converted into PCA by the sequential action of the enzymes quinate dehydrogenase, 3-dehydroquinase and DHS dehydratase. The extent of control on metabolic flux exerted by the permease and the three pathway enzymes was investigated by applying the techniques of Metabolic Control Analysis. The flux control coefficients for each of the three quinate pathway enzymes were determined empirically, and the flux control coefficient of the quinate permease was inferred by use of the summation theorem. These measurements implied that, under the standard growth conditions used, the values for the flux control coefficients of the components of the quinate pathway were: quinate permease, 0.43; quinate dehydrogenase, 0.36; dehydroquinase, 0.18; DHS dehydratase, < 0.03. Attempts to partially decouple quinate permease from the control over flux by measuring flux at pH 3.5 (when a significant percentage of the soluble quinate is protonated and able to enter the mycelium without the aid of a permease) led to an increase of approx. 50% in the flux control coefficient for dehydroquinase. Taken together with the fact that *A. nidulans* has a very efficient pH homeostasis mechanism, these experiments are consistent with the view that quinate permease exerts a high degree of control over pathway flux under the standard laboratory growth conditions at pH 6.5. The enzymes quinate dehydrogenase and 3-dehydroquinase have previously been overproduced in *Escherichia coli*, and protocols for their purification published. The remaining *qut* pathway enzyme DHS dehydratase was overproduced in *E. coli* and a purification protocol established. The purified DHS dehydratase was shown to have a K_m of 530 μ M for its substrate DHS and a

requirement for bivalent metal cations that could be fulfilled by Mg^{2+} , Mn^{2+} or Zn^{2+} . All three *qut* pathway enzymes were purified in bulk and their elasticity coefficients with respect to the three quinate pathway intermediates were derived over a range of concentrations in a core tricine/NaOH buffer, augmented with necessary cofactors and bivalent cations as appropriate. Using these empirically determined relative values, in conjunction with the connectivity theorem, the relative ratios of the flux control coefficients for the various quinate pathway enzymes, and how this control shifts between them, was determined over a range of possible metabolite concentrations. These calculations, although clearly subject to caveats about the relationship between kinetic measurements *in vitro* and the situation *in vivo*, were able to successfully predict the hierarchy of control observed under the standard laboratory growth conditions. The calculations imply that the hierarchy of control exerted by the quinate pathway enzymes is stable and relatively insensitive to changing metabolite concentrations in the ranges most likely to correspond to those found *in vivo*. The effects of substituting the type I 3-dehydroquinases from *Salmonella typhi* and the *A. nidulans* AROM protein (a pentadomain protein catalysing the conversion of 3-deoxy-D-arabinoheptulosonic acid 7-phosphate into 5-enolpyruvylshikimate 3-phosphate), and the *Mycobacterium tuberculosis* type II 3-dehydroquinase, in the quinate pathway were investigated and found to have an effect. In the case of *S. typhi* and *A. nidulans*, overproduction of heterologous dehydroquinase led to a diminution of pathway flux caused by a lowering of *in vivo* quinate dehydrogenase levels. With *M. tuberculosis*, however, quinate dehydrogenase levels increased above those of the wild type. We speculate that these changes in quinate pathway enzyme activities may be due to changes in the pool sizes of quinate and dehydroquinone.

INTRODUCTION

The quinic acid utilization (*qut*) pathway in *Aspergillus nidulans* is a dispensable pathway for the use of quinate as an alternative carbon source. The production of three enzymes quinate/shikimate dehydrogenase (EC 1.1.1.24/25; encoded by *qutB*), 3-dehydroquinase (EC 4.2.1.10; encoded by *qutE*) and dehydroshikimate (DHS) dehydratase (encoded by *qutC*) is induced by the presence of quinate; however, this induction is subject to carbon catabolite repression [1,2]. These three enzymes catalyse the conversion of quinate into protocatechuic acid (PCA) via the intermediates 3-dehydroquinone (DHQ) and DHS. DHQ and DHS are also metabolites in the constitutively expressed shikimate pathway which leads to the production of the aromatic

amino acids (for a review, see [3]). These common metabolites are interconverted in the shikimate pathway by the pentadomain AROM protein (a pentadomain protein catalysing the conversion of 3-deoxy-D-arabinoheptulosonic acid 7-phosphate into 5-enolpyruvylshikimate 3-phosphate), which is encoded by the *aromA* gene.

The genes *qutB*, *qutC* and *qutE* are three of eight genes comprising the *qut* gene cluster, which is controlled at the level of transcription by the *qutA*-encoded QUTA activator protein and the *qutR*-encoded QUTR repressor protein [2]. Induction is initiated by the uptake of quinate into the mycelium by a quinic acid permease encoded by the *qutD* gene; this induction, however, is greatly decreased in the presence of glucose. This repressive effect is due to carbon catabolite repression rather than substrate

Abbreviations used: AROM, a pentadomain protein catalysing the conversion of 3-deoxy-D-arabinoheptulosonic acid 7-phosphate into 5-enolpyruvylshikimate 3-phosphate; DHS, dehydroshikimate; DHQ, dehydroquinone; IPTG, isopropylthiogalactoside; MCA, Metabolic Control Analysis; PCA, protocatechuic acid.

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exclusion [4]. The *aromA* gene encoding the pentafunctional AROM protein apparently evolved by multiple gene fusions to form a gene with a single open reading frame [5,6].

The genes encoding the enzymes and regulatory proteins comprising the quinate and shikimate pathways share a long and complex evolutionary history based around the shuffling of a small group of protein modules that have evolved pathway-specific functions [3]. Perhaps the most remarkable finding is that the QUTA activator and QUTR repressor proteins are homologous with the pentafunctional AROM protein: the QUTA protein is homologous with the two N-terminal domains, and the QUTR protein with the three C-terminal domains [3,7].

Flux of the common metabolites DHQ and DHS within and between the quinate and shikimate pathways has been studied previously by *in vivo* techniques based upon the principles of Metabolic Control Analysis (MCA) [8,9]. The AROM protein has been overproduced in *A. nidulans* by recombinant DNA techniques, and at relatively low levels (up to 5-fold over the wild-type level) it can substitute for the lack of quinate pathway dehydroquinase in *qutE*[−] mutant strains of *A. nidulans*. This demonstrates that the AROM protein is highly leaky *in vivo*, as *qut*-pathway-derived DHQ can be converted into DHS and released again, allowing *qutE*[−] mutant strains (lacking the *qut* pathway 3-dehydroquinase) to utilize quinate as a carbon source. When higher levels (up to 30-fold over the wild type) of the AROM protein are produced *in vivo* the presence of quinic acid is toxic, presumably because the increased levels of AROM protein lead to an increased flux of quinate-derived DHQ and DHS into the shikimate pathway, causing one of the shikimate pathway intermediates to reach toxic levels [8,9]. In a series of essentially reciprocal experiments, DHS derived from the shikimate pathway was fluxed into the quinate pathway to form PCA by increasing the *in vivo* concentration of DHS dehydratase in the absence of exogenously supplied quinate. It was found that, at levels up to 10-fold over the wild-type level of enzyme, an *n*-fold increase in the concentration of DHS dehydratase gave rise to an *n*-fold increase in pathway flux from the shikimate pathway into the quinate pathway [9]. Expressed in terms of MCA this means that, under the conditions of the experiment, the DHS dehydratase enzyme had a shikimate pathway flux control coefficient/deviation control index of -1 [9–11].

The quinate pathway has been shown to have an approx. 30-fold higher level of flux than the shikimate pathway, and when the quinate pathway 3-dehydroquinase is selectively amplified by recombinant technology the growth medium turns purple due to the accumulation of PCA, which complexes with iron (a component of the essential salts) to form a purple-coloured compound in minimal medium [12]. This observation strongly suggests that the 3-dehydroquinase exerts a high level of control on flux through the quinate pathway. In order to gain an understanding of how the control of flux is apportioned amongst the various components of the quinate pathway and how this control varies with changing metabolite concentrations, we report here the results of experiments based on the principles of MCA [13–15].

MATERIALS AND METHODS

Strains and plasmids

The genotypes of the *A. nidulans* and *Escherichia coli* strains, other than those constructed as part of this research, have been detailed previously, as have methods for their growth and propagation [2,8,16–18]. All recombinant plasmids and λ , other than those constructed as part of this research, have been described previously [19].

Materials

Chemicals and solvents other than DHQ were of AnalaR or greater purity and were purchased from local suppliers. Quinate and NAD⁺ were from Sigma, general molecular biology reagents were from Pharmacia or Gibco/BRL, *Taq* polymerase was from BCL, and specific oligonucleotides were purchased from the University of Newcastle upon Tyne Central Facility for Molecular Biology.

Standard molecular biology and biochemical techniques

Recombinant DNA techniques were as described by Maniatis et al. [20] or followed manufacturers' recommendations. Specific DNA fragments were recovered from agarose by the 'freeze-squeeze' method of Tautz and Renz [21], and quinate and shikimate pathway enzymes were assayed as previously described [2,22]. DHQ was prepared from quinate by the method of Grewe and Haendler [23], and DHS was prepared enzymically from DHQ using either purified type I or type II 3-dehydroquinase from *Salmonella typhi* or *Mycobacterium tuberculosis* respectively [24]. Small- and large-scale isopropylthiogalactoside (IPTG)-induced synthesis of heterologous proteins in *E. coli* [25–27], and quinate induction of mycelium grown with glucose as the carbon source [2,8], were as previously described. SDS/PAGE was performed by the method of Laemmli [28] using a 5% acrylamide stacking gel and a 10% separating gel, and proteins were made visible by staining with Coomassie Brilliant Blue. Conditions for the amplification of specific DNA sequences by PCR were as described by van den Hombergh et al. [26]. DNA-mediated transformation of *A. nidulans* was essentially done by the method of Ballance et al. [29], with the modification that protoplasts were prepared from an overnight mycelial culture. Flux through the quinate pathway in *A. nidulans* was measured by monitoring the rate of PCA accumulation in the medium of strains lacking PCA oxygenase, as previously described [9]. Detection of the *qutE*-encoded type II 3-dehydroquinase with rabbit antibodies was as previously described [30]. Quinate dehydrogenase and 3-dehydroquinase were purified as described in [24,27]. *K_m* determinations for the DHS dehydratase activity were carried out in 100 mM Tris, pH 7.5, 2.5 mM MgCl₂ at 37 °C with DHS concentrations ranging from 0.012 to 3.0 mM. The kinetic constants were obtained by computer analysis from Hanes plots ($[S]/v$ against $[S]$) using linear regression. Measurements were taken in triplicate and all replicates were within 5%.

Strain construction

In order to determine the flux control coefficients (*C*) of the *qutB*-encoded quinate dehydrogenase, the *qutE*-encoded 3-dehydroquinase and the *qutC*-encoded DHS dehydratase, appropriate *A. nidulans* strains had to be constructed. The strains were required to carry the *qutB*, the *qutE208* or the *qutC* mutation so that we could manipulate the strain by transformation to produce levels of the quinate dehydrogenase, 3-dehydroquinase and DHS dehydratase enzymes substantially below or above the normal wild-type level. Additionally the strains were required to carry the *pyrG189* mutation, to allow selection of transformants by complementation with the *Neurospora crassa pyr-4* gene [29], and the *pcaE200* mutation [31], resulting in quinate pathway PCA accumulating in the growth medium due to the lack of PCA oxygenase. *A. nidulans* strain 2041 (*qutE208*; *pcaE200*; *pyrG189*; *pabaA1*; *qutR16*) was constructed for dehydroquinase flux control coefficient determination. *A. nidulans* strain 2042 (*qutB42*; *pcaE200*; *pyrG189*; *qutR16*) was constructed for quinate dehydrogenase flux control

coefficient determination, and strain 2053 (*qutC374*; *pcaE200*; *pyrG189*; *pyroA4*; *wA3*; *qutR16*) was constructed for DHS dehydratase flux control coefficient determination. The *qutR16* mutation is a recessive mutation that leads to the constitutive production of the *qut* pathway enzymes, and was included in an attempt to reduce the period required for maximal induction of these enzymes.

Construction of vectors containing wild-type and mutant versions of the *qutE* promoter

The 338 nt upstream of the *qutE* coding region were amplified by means of PCR, using oligonucleotides introducing a *SacI* site at the 5' end and an *NcoI* site incorporating the ATG translational start codon at the 3' end. Following digestion with *SacI* and *NcoI*, the *qutE* promoter was subcloned into appropriately digested pNUFC 102 DNA to produce plasmid pNUFC 209. Plasmid pNUFC 102 contains a unique *XbaI* site allowing the subcloning of the selectable marker *pyr-4* on an *XbaI* fragment (K. A. Wheeler, H. K. Lamb and A. R. Hawkins, unpublished work). The oligonucleotide for the 5' end was 5' AGTGGAG-ATCAGAGCTCAGGCCGCTGATA 3' and that for the 3' end was 5' ATTTTCCATGGTGCAGAGTA 3'.

The *qutE* promoter in plasmid pNUFC 209 was subjected to PCR-mediated mutagenesis by the 'back to back' method of Hemsley et al. [32]. Mutagenic oligonucleotides designed to make three separate alterations (in plasmids pNUFC 210–212) to a 16 nt sequence that is the presumed binding site for the QUTA transcriptional activator [33] were used in conjunction with a non-mutagenic oligonucleotide of sequence 5' TCCCTTA-TCCGTTTCCCCGCA 3'. The mutagenic oligonucleotide used to generate plasmid pNUFC 210 was 5' AAGTGGTGGCGG-ATCCCTCTGCCGGC 3'; that for plasmid pNUFC 211 was 5' AAGTGGTGGCAGAATTCTCTGCCGGC 3'; and that for pNUFC 212 was 5' AAGTGGTGGCAGAGCGCTCTGCCG 3'. The appropriate regions of plasmids pNUFC 210, 211 and 212 were subcloned into M13 sequencing vectors and the nucleotide sequences of the promoters determined. The relevant sequences of the promoters were as follows: wild-type sequence in pNUFC 102, GGGAAGTGGTGGCAGAACGCTCTGCCGG; sequence in pNUFC 210, GGGAAGTGGTGGCGGATCCCTCTGCCGG; sequence in pNUFC 211, GGGTC-CCTTATCCGTTTCGTGGTGGCAGAATTCTCTGCCGG; sequence in pNUFC 212, GGGAAGTGGTGCAGAGCGC-TCTGCCGG.

Construction of recombinant plasmids to transcribe the wild-type *qutB*, *qutC* and *qutE* genes in *A. nidulans*

The plasmid pNUFC 77 contains truncated *qutE* promoter and *qutE* terminator sequences, and has previously been shown to drive efficient expression of any gene placed under their control by subcloning into a short polylinker containing sites for the restriction endonucleases *NcoI*, *PstI* and *HindIII* [34]. The coding sequence of the *qutE* gene was amplified by PCR using 30-mer oligonucleotides that introduce an *NcoI* site incorporating the AUG start codon and a *HindIII* site at the 3' end of the gene. Following digestion with *NcoI* and *HindIII*, the amplified DNA was subcloned into suitably digested pNUFC 77 DNA to form plasmid pNUFC 231. As plasmid pNUFC 231 has no selectable marker for *A. nidulans* transformation, the *qutE* gene with the truncated promoter and terminator were amplified by PCR using 30-mer oligonucleotides that introduce *BamHI* sites at the ends of amplified DNA. Following digestion with *BamHI* the amplified DNA was subcloned into appropriately digested plasmid pCAP2 DNA. Plasmid pCAP2 is an *A. nidulans* cloning vector that has

a unique *BamHI* site, and carries the *N. crassa pyr-4* gene which will complement the *A. nidulans pyrG* gene allowing growth of transformants in minimal medium lacking uracil supplementation. The recombinant pCAP2 plasmid generated by this procedure was designated pCAP10. The coding sequence of the *qutB* gene was amplified by means of PCR and subcloned into *NcoI/HindIII*-digested pNUFC 209, 210, 211 and 212 plasmid DNA (producing plasmids pNUFC 240–243 respectively), putting it under the control of the three mutagenized *qutE* promoters. Similarly, the coding region of the *qutC* gene was amplified by means of PCR and, after digestion with *BspHI* and *HindIII*, subcloned into *NcoI/HindIII*-digested plasmids pNUFC 209, 210, 211 and 212 DNA to yield plasmids pNUFC 236, 237, 238 and 239 respectively.

Purification of DHS dehydratase

DHS dehydratase was purified to homogeneity using the following protocol. (i) The cell pellet from 2 litres of IPTG-induced cells grown at 30 °C was sonicated in 100 ml of 50 mM potassium phosphate, pH 7.2, 1 mM dithiothreitol (buffer 1) and the resulting protein solution clarified by centrifugation. (ii) The 104 ml of clarified sonicate was made 50% saturated with solid ammonium sulphate (at 4 °C) and the resulting precipitate was recovered by centrifugation. The precipitate was redissolved in 40 ml of buffer 1, made 1.0 M with solid ammonium sulphate and clarified by centrifugation. (iii) The clarified solution was loaded on to a phenyl-Sepharose column (1.5 cm × 16 cm) that had previously been equilibrated by passing 1 litre of 1.0 M (NH₄)₂SO₄, 50 mM potassium phosphate, 1 mM dithiothreitol, 2.5 mM MgCl₂, pH 7.2 (buffer 2) through it. All of the DHS dehydratase enzyme bound to the column, which was then washed with 700 ml of buffer 2. The column was eluted with a 1 litre gradient from 1.0 to 0 M (NH₄)₂SO₄ in 50 mM potassium phosphate, 2.5 mM MgCl₂, pH 7.2. Fractions of 10 ml were collected at a rate of 50 ml/h. The enzyme was eluted in fractions 45–55 [0.35–0.25 M (NH₄)₂SO₄], and active fractions containing 90% of the total activity were pooled and dialysed against 2 × 5 litre changes of 50 mM Tris, pH 8.0, 2.5 mM MgCl₂ for 18 h at 4 °C. (iv) Samples of 100 ml of the dialysed enzyme were loaded on to a 4 ml NeoBar AQ⁴ strong anion-exchange FPLC column that had previously been equilibrated with 20 mM Tris, pH 7.5 (buffer 3). Following a wash with 16 ml of buffer 3, the bound proteins were eluted with an 80 ml linear 0–0.5 M NaCl gradient in buffer 3. Fractions of 1 ml were collected at a rate of 4 ml/min. DHS dehydratase eluted as a single peak of activity at around 0.1 M NaCl, and SDS/PAGE analysis showed that this peak contained a single protein of the predicted molecular mass (39.1 kDa).

An overview of the methodology

The purpose of the investigations presented in this paper is to investigate how the control of flux through the quinate pathway is distributed between its various component parts, and how control of the system responds to changes in metabolite concentrations caused by varying the enzyme activity. A full quantitative description therefore requires an estimate of how the contributions from each of the components (the three quinate pathway enzymes and the quinate permease) to the control of flux (expressed as flux control coefficients) vary over a range of *in vivo* metabolite concentrations. This ideal, however, is extremely difficult to achieve in this or any other experimental system.

It is, however, possible to empirically measure the flux control coefficients of the three pathway enzymes under standard laboratory conditions and then use the connectivity theorem to

estimate the flux control coefficient of the quinate permease. In this way it is possible to gain some understanding of how the control of flux is apportioned between the various components of the system under one set of defined conditions. It is also possible to gain a measure of the relative contributions of each of the three enzymes in the pathway to the control of flux, and to make some predictions about how this control varies with changing metabolite concentrations. This can be achieved by using *in vitro* methods to estimate the elasticity coefficients for each of the enzymes with respect to their substrates and products, and using the connectivity theorem to estimate the relative ratios of the flux control coefficients corresponding to each of the enzyme pairs. In this manner it is possible to make predictions about possible changes in the hierarchy of control for the enzymes over a range of metabolite concentrations. However, for this analysis supplies of purified enzymes are required. By the application of recombinant DNA technology, it is a relatively straightforward procedure to simplify the purification of desired enzymes by overproduction in *E. coli*.

This *in vitro* experimental procedure is clearly open to questions about the applicability of such empirically determined values for the elasticity coefficients *in vitro* to the situation *in vivo*. The type of questions that can be fairly asked are: are the measurements of cellular metabolite concentrations representative of the actual concentrations experienced by the enzyme in the cell, and have all the significant effectors of the enzyme's activity been accounted for? [15]. Such arguments, however, are not limited to the application of MCA. Fell [15] has discussed in detail the conditions and caveats pertaining to the use of *in vitro* data in MCA, pointing out that the reductionist ethos of much of biochemistry (to seek an understanding of the functioning of biochemical systems through the study of the molecular properties of its components) is undermined if there is no confidence in the applicability of such results. Where checked, however, values for elasticity-derived flux control coefficients determined *in vitro* have been found to be in broad agreement with the values determined *in vivo* and to be valuable aids in predicting how enzymes in a pathway are likely to respond to changing metabolite concentrations [35].

RESULTS AND DISCUSSION

Overproduction, purification and characterization of the *qutC*-encoded DHS dehydratase

Subcloning the *qutC* gene and overproduction of DHS dehydratase in *E. coli*

In order to determine the elasticity coefficients of DHS dehydratase, the *A. nidulans qutC* gene was overexpressed in *E. coli*. The coding region of the *A. nidulans qutC* gene was amplified by PCR using specific 30-mer oligonucleotides containing *NdeI* and *HindIII* sites at the 5' and 3' ends respectively of the gene, using the previously described cosmid pQ7 [19] as a template. Following

Figure 1 Purification of DHS dehydratase

Approx. 5 µg of protein from each stage in the purification of DHS dehydratase from an overproducing strain of *E. coli* BL21(DE3) harbouring plasmid pRF18 was analysed by SDS/PAGE. Lane 1, boiled cells containing unmodified pRSETB; lane 2, boiled cells containing pRF18; lane 3, crude extract; lane 4, (NH₄)₂SO₄ precipitate (0–50% saturation); lane 5, after phenyl-Sepharose chromatography; lane 6, after strong anion (NeoBar AQ⁴) chromatography. SDS/PAGE was standardized by electrophoresing protein molecular-mass markers (shown in the unmarked lanes) comprising phosphorylase B (97 kDa), BSA (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and β-lactoglobulin (18 kDa).

digestion with *NdeI*/*HindIII*, the PCR-amplified DNA was purified from low-melting-point agarose and subcloned into the appropriately digested pRSETB. This procedure placed the coding region of *qutC* under the control of the powerful T7 promoter contained within the *E. coli* expression vector pRSETB, giving the potential to overproduce a native (i.e. non-fusion) DHS dehydratase enzyme in *E. coli*. Recombinant pRSETB plasmids were transformed into *E. coli* strain BL21(DE3), which contains T7 polymerase under an IPTG-inducible promoter, and screened for DHS dehydratase production. All of the recombinant plasmids directed overproduction of soluble DHS dehydratase to around 15% of the total cell protein, and one such plasmid was designated pRF18 and used for the bulk purification of the enzyme. Two 1 litre cultures of *E. coli* BL21(DE3) were grown to mid-exponential phase at 30 °C, and the production of DHS dehydratase was induced by the addition of 200 µg·ml⁻¹ IPTG. After a further incubation for 5 h, the *E. coli* cells were harvested by centrifugation and used as the starting material for bulk preparation of DHS dehydratase.

Characterization of the purified enzyme

DHS dehydratase was purified according to the protocol described in the Materials and methods section. Table 1 summarizes the enzyme purification, and Figure 1 shows SDS/PAGE analysis of each of the purification stages. *N. crassa qa-4*-encoded DHS

Table 1 Purification of DHS dehydratase

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units·mg ⁻¹)	Purification (fold)	Recovery (%)
Crude extract	104	927	3395	3.66	1.0	100
Ammonium sulphate precipitate	40	42.4	3147	74.2	20.3	92.7
Phenyl-Sepharose chromatography	132	6.6	1697	257	70.2	50.0
NeoBar AQ ⁴ strong anion chromatography	10	6.1	1594	261	71.3	46.9

Figure 2 Elasticity coefficients of the three quinate pathway enzymes as a function of metabolite concentration

(a) Elasticity coefficient of quinate dehydrogenase as a function of the concentration of its product DHQ; (b) elasticity coefficient of DHS dehydratase as a function of the concentration of its substrate DHS; (c) elasticity coefficient of 3-dehydroquinase as a function of the concentration of its substrate DHQ; (d) elasticity coefficient of 3-dehydroquinase as a function of the concentration of its product DHS. For the activation profiles, substrate concentrations were varied in the range 0–4.0 mM for dehydroquinase and 0–3.0 mM for DHS dehydratase. For the inhibition profile of quinate dehydrogenase, the substrate concentrations were fixed at 0.45 mM quinate and 0.75 mM NAD⁺ and the product concentration varied over the range 0–1.25 mM. For the product inhibition profile of dehydroquinase, the substrate concentration was fixed at 0.17 mM DHQ and the product concentration varied over the range 0–0.08 mM. All assays were carried out in a core buffer consisting of 20 mM Tricine/NaOH, pH 7.5, that was augmented with specific cofactors and essential bivalent cations as appropriate [2,22]. The elasticity coefficients were determined over the desired metabolite range by taking tangents ($\partial v/\partial [S]$) to the metabolite activation or inhibition curves (results not shown) multiplied by the ratio of the metabolite concentration to the velocity (expressed as μmol of product $\cdot \text{min}^{-1}$ at 37 °C) at each individual point ($[S]/v$). Because of the nature of the calculation, elasticity coefficients are dimensionless numbers.

dehydratase has been purified previously [36], and shown to require Mg²⁺ for activity. In order to confirm that the *A. nidulans* enzyme had the same characteristics, aliquots of enzyme were dialysed against 2 × 5 litre changes of 20 mM Tris, pH 7.5, in the presence or absence of 1 mM EDTA. Taking the value for the sample dialysed in the absence of EDTA as 100 %, the sample dialysed in the presence of EDTA lost all its activity. The ability of various cations to restore catalytic activity was tested by assaying 30 pmol of enzyme in 100 mM Tris, pH 7.5, at 37 °C in the presence of various concentrations of specific cations. In all cases the buffers for these assays were stored over a chelating resin prior to use to obviate adventitious enzyme activation. The addition of Mg²⁺ restored activity with a linear dose response up to 1.3 mM, where the activation formed a plateau. The addition of Mn²⁺ or Zn²⁺ gave similar activation profiles with a plateau at 2.0 mM in each case. The cations K⁺, Na⁺, NH₄⁺ and Ca²⁺ all showed no ability to substitute for Mg²⁺ or restore enzyme activity. In the case of Mg²⁺, Mn²⁺ and Zn²⁺, the final maximum activation gave an enzyme specific activity equal to that of the control sample dialysed in the absence of EDTA. These observations confirm that the *A. nidulans* DHS dehydratase has a requirement for bivalent metal cations that can be met by Mg²⁺, Mn²⁺ or Zn²⁺. The K_m value for DHS dehydratase with respect to its substrate DHS at 37 °C was determined as described in the Materials and methods section. A value of 530 μM was obtained, which is comparable with the value obtained by Stroman et al. [36] for the equivalent *N. crassa* enzyme.

Determination of the relative flux control ratios for the three quinate pathway enzymes over a range of possible *in vivo* metabolite concentrations

The substrate-activation and product-inhibition curves for each of the three quinate pathway enzymes were determined empirically *in vitro*. The elasticity coefficients for each enzyme were determined as a function of metabolite concentration by taking tangents to the curves at intervals corresponding to 0.05 mM over the concentration range 0.05–0.5 mM. The various elasticity profiles derived from these data are shown in Figure 2. These elasticities were then used in conjunction with the connectivity theorem to predict the relative ratios of the flux control coefficients of the appropriate enzyme pairs. This particular limit to the range of substrate concentrations was chosen because in the range 0.6–1.0 mM all three quinate pathway enzymes become inactivated by end-product inhibition. This makes it highly unlikely that these higher concentrations of metabolites are produced *in vivo*, as they would inhibit enzyme activity thereby halting metabolic flux. Additionally, the sensitivity of each of the enzymes to inhibition by PCA was determined (Figure 3).

Calculation of the actual *in vivo* metabolite pools is difficult, as the internal volume of the mycelium that is accessible to each metabolite is not known. Actively growing mycelium also presents particular difficulties because of its coenocytic nature, as the outer actively growing halo is more metabolically active than the older parts of the mycelium. Concentrations can be expressed

Figure 3 Sensitivity of quinate pathway enzyme activities to various concentrations of PCA

(a) Quinate dehydrogenase activity as a function of PCA concentration. The quinate concentration was 0.45 mM ($1 \times K_m$) and the NAD^+ concentration was 0.75 mM ($5 \times K_m$). (b) 3-Dehydroquinase activity as a function of PCA concentration. The DHQ concentration was 0.16 mM ($1 \times K_m$). (c) DHS dehydratase activity as a function of PCA concentration. The DHS concentration was 0.53 mM ($1 \times K_m$). In all cases a core buffer of 20 mM Tricine/NaOH, pH 7.5 (supplemented with various substrates and cofactors as necessary), was used. The assays were carried out in triplicate at 37 °C and were within 5% of each another. The units of velocity were μmol of product $\cdot \text{min}^{-1}$ at 37 °C.

in terms of $\text{mM} \cdot \text{g}$ of freeze dried mycelium $^{-1}$ [37], but how these values relate to the concentrations in the actively growing parts of the mycelium remains unclear.

By plotting the relative ratios of the flux control coefficients of sequential pairs of enzymes as a function of the concentration of their shared metabolites, the relative contribution to the control of flux by each individual of each pair of enzymes was estimated. By pooling the relative sets of values for the two pairs of enzymes (quinate dehydrogenase/dehydroquinase; dehydroquinase/DHS dehydratase), a relative hierarchy was determined. This relative hierarchy and its variation as a function of the DHQ and DHS concentrations is shown in Figure 4. The essential feature of Figure 4 is that, for DHQ concentrations up to 0.5 mM and DHS concentrations up to 0.3 mM, the majority of the control exerted by the three quinate pathway enzymes

Figure 4 Predicted relative hierarchy of control exerted by the three quinate pathway enzymes as a function of DHQ and DHS concentrations

The three bar diagrams indicate how the hierarchy of the distribution of control by the three quinate pathway enzymes is predicted to shift due to changes in the DHQ and DHS concentrations. The boundaries on each bar diagram indicate the metabolite concentration at which there is a cross-over in the hierarchy of control. For simplicity, the actual ratios of flux control coefficients of the enzymes are omitted. The top diagram indicates which enzyme is predicted to exert the most control over flux at any combination of the concentrations of DHQ and DHS between 0.05 and 0.50 mM. Similarly, the middle and bottom diagrams show the enzymes that have the medium and least predicted control over flux for particular combinations of DHQ and DHS concentrations. Open boxes, quinate dehydrogenase; black boxes, 3-dehydroquinase; hatched boxes, DHS dehydratase.

resides in quinate dehydrogenase and dehydroquinase. Between values of 0.3 and 0.5 mM DHS there is a predicted sharp transition, with most of the control over flux then residing in DHS dehydratase.

The overall picture that arises from these predictions is that, in the range of metabolite concentrations most likely to correspond to the actual values *in vivo* (up to 0.5 mM), there is a relatively stable hierarchy of enzyme flux control coefficients in which the value for DHS dehydratase is considerably lower than those for quinate dehydrogenase and 3-dehydroquinase.

It must be stressed, however, that: (i) any values determined in this manner are relative and do not predict anything about the absolute values of the flux control coefficients at any of the

Figure 5 Western blot analysis of the *qutE*-encoded 3-dehydroquinase

Approximately 10 μ g of cell-free extracts derived from *A. nidulans* wild-type strain R21 and mutant strain 2041 (lacking the type II 3-dehydroquinase activity) were subjected to SDS/PAGE. The proteins were immobilized by electroblotting to a nitrocellulose sheet which was subsequently blocked by overnight incubation in 1% (v/v) BSA rinse buffer [137 mM NaCl, 1.5 mM KH_2PO_4 , 8 mM Na_2HPO_4 , 2.7 mM KCl, 0.5% (w/v) Tween 20] at 37 °C and processed for antibody detection as previously described [30] using rabbit antibody at a dilution of 1:50. Lanes 1 and 2, strain *qutE208*, non-induced, neat (N) and N/2; lanes 3–5, strain *qutE208*, quinate-induced, neat (N), N/2 and N/4 respectively; lanes 6–8, strain R21 induced with quinate; neat (N), N/2 and N/4 respectively; lanes 9–11 wild-type strain R21, non-induced, neat (N), N/2 and N/4 respectively. The SDS/PAGE was standardized by electrophoresing protein molecular-mass markers (shown in the outside lanes) comprising phosphorylase B (97 kDa), BSA (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), β -lactoglobulin (18 kDa) and lysozyme (14.3 kDa). The position of the dehydroquinase subunits is marked by a dot.

metabolite concentrations used, and (ii) these predictions must be interpreted in the light of the caveats pertaining to the use of *in vitro* data discussed above and in more detail by others [15]. Biological precedent [38] suggests that quinate permease will have a relatively large flux control coefficient, and this can be estimated by subtraction using the summation theorem if the values for the flux control coefficients of the other three enzymes are known.

Determination of the flux control coefficients for *qutE*-encoded type II 3-dehydroquinase, *qutB*-encoded quinate dehydrogenase and *qutC*-encoded DHS dehydratase

Western blot analysis of *A. nidulans* strain 2041

Strain 2041 lacks *qutE*-encoded type II 3-dehydroquinase activity; however, the nature of the *qutE208* mutation is not known. Type II 3-dehydroquinases are dodecameric, and therefore if the *qutE208* mutation is a mis-sense mutation it is possible that hetero-oligomeric proteins could be formed between the resident mutant subunits and wild-type subunits encoded by wild-type *qutE* genes introduced by transformation. This possible formation of heterododecamers could adversely affect enzyme activity by changing the elasticity coefficients. In order to ascertain if strains carrying the *qutE208* mutation produce non-functional type II 3-dehydroquinase subunits, cell-free protein extracts of wild-type strain R21 and strain 2041, both uninduced and induced by quinate, were immobilized on nitrocellulose by Western blotting. The resulting nitrocellulose filter was then probed with rabbit anti-*(A. nidulans* type II 3-dehydroquinase) polyclonal antibodies and the conjugated protein made visible as previously described [30]. The results of this experiment are shown in Figure 5, inspection of which shows that type II 3-dehydroquinase subunits (identified by a dot) could only be detected in the cell-free extracts derived from the quinate-induced wild-type culture, implying that they were not being produced in the mutant strain. This makes it unlikely that mutant type

II 3-dehydroquinase subunits interfere with 3-dehydroquinase activity by forming heterododecamers with wild-type subunits.

Empirical determination of the 3-dehydroquinase flux control coefficient.

It has previously been shown that flux through the quinate pathway can be estimated by measuring the rate of PCA accumulation in the medium of strains carrying the *pcaE200* mutation and thereby lacking PCA oxygenase [9]. These initial studies showed that the rate of PCA formation from exogenously supplied quinate was 1.9 mM $\cdot$ h $^{-1}$ $\cdot$ g of mycelium $^{-1}$ (strains 2036 and 2038) in liquid culture. This mycelium (which required pyridoxine and *p*-aminobenzoic acid for growth) was pre-grown with glucose as carbon source and subsequently inhibited from further growth by omitting pyridoxine and *p*-aminobenzoic acid when shifted to quinic acid-containing medium. These flux measurements were repeated with strains 2036 and 2037 [9], and additionally the parental strain 2039 (*pyrG189*; *pcaE200*; *qutR16*; *pabaA1*), which was used to generate the recombinant strain 2041, was included in the experiment. The results obtained were very similar to the previously published results [9], with strain 2039 producing PCA at a rate of 1.2 mM $\cdot$ h $^{-1}$ $\cdot$ g of mycelium $^{-1}$. Strain 2039 carries the *pabaA1* mutation but not the *pyrA* mutation present in the doubly mutant (*pyrA*; *pabaA1*) strains previously reported [9]. Strain 2039 continued to grow in quinate medium lacking *p*-aminobenzoic acid after shifting from glucose, presenting problems for standardizing the conditions for flux measurements. Continued growth of these strains is possible since, although quinate cannot be used as a carbon source (due to the presence of the *pcaE200* mutation), the mycelium can utilize glycerol which is present in the medium and traces of *p*-aminobenzoic acid left over after washing the mycelium prior to shifting to the quinate medium. Flux measurements were taken again using these strains and the effects of different concentrations of glycerol and quinate in the quinate medium were estimated. The results of these experiments (not shown) established that (i) the rate of PCA production from exogenously supplied quinate over an 11 h period was not adversely affected by omitting glycerol from the quinate medium, (ii) increasing the quinate concentration above 52 mM [9] did not increase the rate of production of PCA over an 11 h period, and (iii) the absence of glycerol did not adversely affect the levels of *qut* pathway enzymes over an 11 h period. These conditions resulted in no further growth of the mycelium.

A. nidulans strain 2041 was transformed with plasmid pCAP10 and transformants were selected in minimal medium using glucose as carbon source but lacking uracil supplementation. Eight transformed strains selected because they displayed a range of type II 3-dehydroquinase activities below and above the wild-type level present in the original parental strain 2039 were used to determine the flux control coefficient of the type II 3-dehydroquinase, as previously described [9]. In all the measurements of the flux control coefficients, the variation in the relative activity ratios of the three quinate pathway enzymes did not exceed the natural variation seen when replicate cultures of wild-type strains were grown under the same conditions. The rates of PCA production for all strains were estimated by linear regression (Minitab 8.2) and the experiment was repeated three times. The data from one of the flux control determinations are shown in Figure 6(a), and the overall estimate for the flux control coefficient from all three determinations is 0.18 ± 0.03 .

Empirical determination of the quinate dehydrogenase flux control coefficient

Transformants of *A. nidulans* strain 2042 were isolated on the

Figure 6 Flux control coefficients of quinate pathway enzymes

(a) Flux control coefficient of 3-dehydroquinase in growth medium at pH 6.5. The rate of PCA accumulation in $\text{mM} \cdot \text{h}^{-1} \cdot \text{g}$ of mycelium $^{-1}$ is shown as a function of the increase in *qutE*-encoded type II 3-dehydroquinase levels relative to wild type. (b) Flux control coefficient of quinate dehydrogenase. The rate of PCA accumulation is shown as a function of *qutB*-encoded quinate dehydrogenase levels relative to wild type. (c) Flux control coefficient of DHS dehydratase. The rate of PCA production is shown as a function of *qutC*-encoded DHS dehydratase levels relative to wild type. (d) Flux control coefficient of 3-dehydroquinase in growth medium at pH 3.5. The rate of PCA accumulation is shown as a function of *qutE*-encoded type II 3-dehydroquinase levels relative to wild type. All lines were plotted using non-linear least-squares regression [41].

basis of their ability to grow in minimal medium in the absence of uracil following co-transformation of plasmid pCAP2 and pNUFC 241, 242 or 243 at a ratio of 25:1. The reason for the co-transformation strategy was that the *qutB* gene contains *Bam*HI, *Bgl*II and *Xba*I sites, thereby preventing subcloning into plasmid pCAP2 or insertion of the *pyr-4* gene on an *Xba*I fragment into plasmids pNUFC 241, 242 or 243.

Cell-free extracts from 120 individual quinate-induced cultures of the *qutB* transformants were screened for quinate dehydrogenase activity, and nine were selected on the basis of showing a range of enzyme activities above and below the wild-type level. The flux control coefficient of the quinate dehydrogenase enzyme was then determined using these transformants by the methods described above, and was found to be 0.36 (Figure 6b). For this estimate to be of value, we are making the assumption that there is a sufficient pool of NAD^+ to fully accommodate the increased levels of quinate dehydrogenase present in some of the transformants. If *in vivo* levels of NAD^+ are insufficient to service the increased levels (relative to wild type) of quinate dehydrogenase, this will have the effect of lowering the estimate for the value of the flux control coefficient made under these standard laboratory conditions. Taken together, however, these data indicate that around 54% of the control of flux resides in the first two enzymes of the pathway and that around 46% is apportioned between DHS dehydratase and quinate permease.

Empirical determination of the DHS dehydratase flux control coefficient

Transformants of *A. nidulans* strain 2042 were isolated on the basis of their ability to grow in minimal medium in the absence of uracil following co-transformation of plasmid pCAP2 and pNUFC 236, 237, 238 or 239 at a ratio of 25:1. The reason for the co-transformation strategy was that the recombinant plasmids containing the *qutC* gene contain *Bam*HI, *Bgl*II and *Xba*I sites, thereby preventing subcloning into plasmid pCAP2 or insertion of the *pyr-4* gene on an *Xba*I fragment. Ten transformants identified in a screen as having a range of DHS dehydratase activities above and below the wild-type level were used to determine the flux control coefficient of DHS dehydratase by the methods described above. The value for the flux control coefficient of DHS dehydratase was determined as 0.005 (see Figure 6c). This value is lower than the experimental error (± 0.03) in determining the flux control coefficient of 3-dehydroquinase. Thus all that can be reasonably inferred from this empirical measurement is that the flux control coefficient of DHS dehydratase is less than 0.03 under these standard laboratory growth conditions.

This measurement is in agreement with the prediction arising from the use of *in vitro* data on elasticity coefficients that DHS dehydratase exerts the least control over flux. Clearly this agreement between the predicted and empirically determined values does not confirm that the pattern of control over flux predicted in Figure 3 is a completely accurate description of what will happen *in vivo*. However, it does lend support to the idea of

a relatively stable hierarchy of control that buffers against fluctuations in intramycelial metabolite concentrations.

Flux control coefficient of quinate permease

The combined control exerted by the three quinate pathway enzymes under standard laboratory conditions is 0.57; therefore application of the summation theorem implies that the flux control coefficient of quinate permease under these conditions is 0.43. This value is approximately equal to the value for quinate dehydrogenase, showing that under these standard conditions the majority of control over flux resides with quinate permease and quinate dehydrogenase. The relatively high value for the flux control coefficient of quinate permease is consistent with biological precedent [38].

The QUTR repressor protein is proposed to mediate its repressor activity on transcription activation by a direct stoichiometric interaction with the QUTA transcription activator protein; however, this repressor activity is proposed to be negated by binding one or more of the *gut* pathway metabolites [39]. As the repressor protein helps to determine the extent of transcription initiation, this will ultimately affect the rate of production of and/or steady-state levels of the *gut* pathway enzymes, and thereby alter the flux through the pathway. Any flux control coefficients associated with the repressor protein may therefore change rapidly from a high to a low value as the mycelium induces production of the *gut* pathway enzymes in response to the presence of quinate and steady-state levels are approached. In the experiments reported here, however, the repressor protein in the strains used was inactive due to the presence of the recessive *gutR16* mutation. The value of the flux control coefficient calculated for the permease is therefore unlikely to have a component associated with the repressor protein, but initial considerations suggest that it is possible that it may have a term associated with the efflux of the PCA produced by the *gut* pathway.

It is possible that the experiments described here may by their very nature give a distorted view of the control of flux in the quinate pathway. For example, it can be argued that the presence of the *pcaE200* mutation (which eliminates PCA oxygenase activity), essential for us to carry out the flux measurements, may lead to a build-up of intramycelial PCA which could have a negative effect on quinate pathway enzyme activities. Inspection of Figure 2 shows that these enzyme activities are indeed sensitive to the presence of PCA, with DHS dehydratase around 80% inhibited at a PCA concentration of 25 μ M. Quinate dehydrogenase is the least sensitive, requiring the presence of around 1.4 mM PCA for 80% inhibition. The empirical studies described above demonstrate that, under standard laboratory growth conditions, DHS dehydratase has the lowest flux control coefficient of any of the quinate pathway components. DHS dehydratase activity is, however, exquisitely sensitive to the PCA concentration; therefore it is hard to see how these two facts can be reconciled unless the *in vivo* concentrations of PCA in the experiments we describe are very low. We believe, therefore, that there is unlikely to be a large component for PCA efflux embedded within the value for the flux control coefficient of the permease, and that the values we report are probably an accurate reflection of the values that would be obtained in a strain with normal levels of PCA oxygenase.

Our interpretation has some biological precedent, as Flint et al. [37], investigating the control of arginine flux in *N. crassa*, measured urea efflux into the media. Only a small increase in intramycelial concentration was detected for large changes in urea efflux, and this was suggested to be consistent with a

relatively unsaturated enzyme mechanism or with a diffusion-dependent mechanism [37].

The role of quinate permease

If quinate permease does have a large flux control coefficient, then decoupling the permease should lead to an increase in the flux control coefficients for quinate dehydrogenase and dehydroquinase. Quinic acid is completely deprotonated at the usual growth pH of 6.5, and requires quinate permease for transport into the mycelium. At pH 3.5, however, significant amounts (around 50%) of quinate remain protonated and can enter the mycelium by a concentration-driven diffusion gradient in the absence of the permease [4]. In order to attempt to at least partially decouple the control exerted by the permease, the flux control coefficient of dehydroquinase was determined at an external growth medium pH of 3.5 using quinate at a concentration of 104 mM. The experiment was repeated twice, giving a value of 0.28 ± 0.03 for the flux control coefficient of dehydroquinase (see Figure 6d).

Interpretation of these results is problematic, as growth at such a low pH may affect the internal pH of the mycelium leading to differential effects on the activities of the various enzymes. *A. nidulans* is unusual in that it is able to grow in media as acidic as pH 3.5 or as basic as pH 9.0, and this ability to grow over such a wide pH range has been studied genetically and physiologically [40]. It has been shown that *A. nidulans* has an efficient pH homeostatic mechanism so that non-secreted enzymes are required to act only over a narrow pH range [40]. Growth at pH 3.5 (instead of the usual growth pH of 6.5) has been shown to decrease the internal pH of the mycelium only to approx. pH 5.7 [39]. We have previously shown that growth of *A. nidulans* in a range of pH values from 6.5 to 3.5 does not increase the variation in the levels, or relative ratios, of the three quinate pathway enzymes over that observed when replicate samples of mycelium are grown at pH 6.5 [2,4]. These considerations suggest that flux through the quinate pathway at pH 3.5 is unlikely to be subject to major changes attributable to altered levels, or altered relative activity ratios, of the quinate pathway enzymes.

The 56% increase in the value of the flux control coefficient for dehydroquinase in medium incubated at pH 3.5 is therefore consistent with an at least partial decoupling of the control over flux exerted by quinate permease.

It is extremely likely that *Aspergillus* in its natural environment will usually have access to quinate under acidic conditions, suggesting that the control over flux exerted by the permease may be lower than under laboratory conditions, as the permease is apparently only required for the uptake of the dissociated quinate molecule [4]. Alternatively, however, it is possible that, because the fungus in its natural environment is unlikely to be submerged in a quinic acid solution, the permease may take on an important role in scavenging.

Replacement of the *gutE*-encoded type II 3-dehydroquinase with heterologous type I and II 3-dehydroquinases: a cautionary tale

One of the goals of biotechnology is to attempt to increase flux through commercially important pathways. An ever-increasing number of genes encoding equivalent enzymes from different species are becoming available, giving the potential to substitute equivalent enzymes which have different elasticity coefficients in chosen pathways. This strategy therefore offers the possibility of pathway engineering by substituting enzymes with elasticity coefficients 'tailored' to help circumvent a particular problem.

Having estimated the flux control coefficients for the various

Figure 7 *A. nidulans* quinate pathway flux as a function of the levels of the heterologous type I and II dehydroquinases encoded by *S. typhi* and *M. tuberculosis*

Top: quinate pathway flux is shown as a function of the *S. typhi* type I dehydroquinase (left; $r^2 = 0.86$; $P < 0.0001$) and quinate dehydrogenase (right; $r^2 = 0.76$; $P < 0.001$) activities relative to the respective wild-type controls. Bottom: quinate pathway flux is shown as a function of the *M. tuberculosis* type II dehydroquinase (left; $r^2 = 0.83$; $P < 0.003$) and quinate dehydrogenase (right; $r^2 = 0.43$; $P < 0.05$) activities relative to the respective wild-type controls. Pathway flux was measured in $\text{mM} \cdot \text{h}^{-1} \cdot \text{g}$ of mycelium $^{-1}$ and is expressed as the ratio of transformant flux to control wild-type flux. The lines were plotted using linear least-squares regression.

components comprising the quinate pathway, the effect of substituting heterologous 3-dehydroquinases into this pathway was investigated. The enzymes used were the type I enzyme from *Salmonella typhi* (K_m for DHQ = $18 \mu\text{M}$), the domain of the *A. nidulans* shikimate pathway pentafunctional AROM protein specifying a type I enzyme (K_m for DHQ = $250 \mu\text{M}$), and the shikimate pathway type II enzyme from *Mycobacterium tuberculosis* (K_m for DHQ = $64 \mu\text{M}$) [24,27]. The nucleotide sequences encoding these three dehydroquinase enzymes were subcloned in the *A. nidulans* expression vector pNUFC 102 as described in the Materials and methods section to produce recombinant plasmids pNUFC 244 (*S. typhi*), 245 (*M. tuberculosis*) and 246 (*A. nidulans* AROM), placing them under the control of the truncated *qutE* promoter. These three plasmids were then used to transform *A. nidulans* strain 2041, selecting transformants by their ability to grow in minimal medium lacking uracil. Transformants were subsequently screened for the production of the appropriate dehydroquinase and selected transformants were used to determine the flux as a function of dehydroquinase activity. The results are shown in Figure 7. Inspection of Figure 7 reveals the following points. (1) *S. typhi* type I dehydroquinase was able to substitute efficiently for the *qutE*-encoded type II dehydroquinase leading to a pathway flux that was up to 92% of the wild-type value. *A. nidulans* AROM-derived type I dehydroquinase similarly was able to substitute for the *qutE*-encoded type II dehydroquinase, giving a pathway flux that was up to 84% of the wild-type value (results not shown). The *M. tuberculosis* type II enzyme was only able to produce maximum

levels of flux which were 35% of the wild-type value. (2) Increasing levels of the *M. tuberculosis* dehydroquinase were correlated with an increase in pathway flux; however, the maximum level of enzyme activity was only 15% of the control activity (Figure 7, bottom panels). The levels of *S. typhi* (Figure 7, top panels) and *A. nidulans* (results not shown) dehydroquinases in the transformants, however, showed a much greater range and gave substantially higher activities than the control. Surprisingly, in these two latter cases increasing dehydroquinase activity was inversely correlated with pathway flux when the enzyme activities rose above 2-fold greater than the wild-type value. This seemingly anomalous behaviour is explainable by looking at the levels of quinate dehydrogenase in these transformants (Figure 7, top). It is apparent that the levels of quinate dehydrogenase are inversely correlated with increasing levels of heterologous dehydroquinase production. As quinate dehydrogenase is the enzyme with the greatest control over pathway flux under these conditions ($C = 0.36$), the flux observed is a direct consequence of this enzyme activity.

Why, then, is increasing heterologous dehydroquinase activity associated with diminishing quinate dehydrogenase activity? *A. nidulans* strain 2041, which is the strain with which these experiments were done, carries the *qutR16* mutation resulting in constitutive production of the quinate pathway enzymes and the heterologous dehydroquinases. It is therefore highly unlikely that heterologous dehydroquinase overproduction is having any significant effect on transcriptional regulation, which could be proposed as an explanation for these observations. Instead, we

speculate (i) that the substitution of heterologous dehydroquinases having elasticity coefficients different from that of the wild-type dehydroquinase is leading to new steady-state or quasi-steady-state conditions with significantly lower pool sizes for quinate and/or DHQ (the substrate and product of quinate dehydrogenase), (b) that these putative changed pool sizes are negatively affecting the stability of the endogenous quinate dehydrogenase enzyme *in vivo*, leading to a lowering of the enzyme concentration *in vivo*, and (c) that overproduction of the *S. typhi* and *A. nidulans* AROM dehydroquinases accentuates the putative lowering of pool sizes, thereby increasing the effect on quinate dehydrogenase.

Figure 7 (bottom panels), which shows the effect of the *M. tuberculosis* dehydroquinase on quinate pathway flux, is consistent with this speculation. The maximum pathway flux is 35 % of the wild-type value and the highest level of heterologous dehydroquinase production is only 15 % of the wild-type value. Under these conditions, increasing pathway flux is correlated with increasing heterologous dehydroquinase production. Figure 7 shows that, under these conditions of very low flux, the levels of quinate dehydrogenase are elevated, in this case up to approx. 4-fold over the wild-type value. It is clear, therefore, that production of heterologous dehydroquinases does not have some general negative effect inevitably leading to diminution of quinate dehydrogenase activity. Under these conditions summarized in Figure 7 we speculate that a possible increase in quinate and DHQ pool sizes increases the stability of quinate dehydrogenase, leading to an increased active enzyme concentration *in vivo*.

Conclusions

In this paper we have attempted to provide a description of how the control of flux within the quinate pathway is apportioned between the various components (permease and three enzymes) that comprise it, and how this control may vary as a result of changes in metabolite concentrations. The main themes that arise from these studies are as follows.

(1) The use of *in vitro* values for the elasticity coefficients of the three quinate pathway enzymes in conjunction with the connectivity theorem predicts a hierarchy of control amongst the enzymes that is relatively insensitive to changes in metabolite concentrations. These calculations predict that, over the range of metabolite concentrations most likely to correspond to values *in vivo*, most of the control of flux attributable to the enzymes will reside with quinate dehydrogenase and 3-dehydroquinase, with DHS dehydratase exerting the lowest amount of control.

(2) When the flux control coefficients of the three quinate pathway enzymes were determined empirically under standard laboratory growth conditions, the values obtained were consistent with the hierarchy predicted by the use of *in vitro* values for the elasticity coefficients of the enzymes.

(3) The flux control coefficients of the three enzymes (quinate dehydrogenase, 0.36; 3-dehydroquinase, 0.18; DHS dehydratase, 0.03), in conjunction with the summation theorem, allowed an estimate of 0.43 to be calculated for the flux control coefficient of quinate permease. Flux control determinations for 3-dehydroquinase carried out at low pH are consistent with the prediction that quinate permease exerts a high degree of control over pathway flux.

(4) The substitution of the normal quinate pathway 3-dehydroquinase by heterologous enzymes with different elasticity coefficients led to somewhat unexpected results. Flux through the quinate pathway was affected by significant changes in the levels

of the other quinate pathway enzymes. We speculate that these changes in enzyme levels may be due to altered metabolite pool sizes, caused by the use of heterologous enzymes with different elasticity coefficients.

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