71. K. Walsh and D. E. Koshland, Jr., *J. Biol. Chem.* **259**, 9646 (1984).
72. D. G. Gadian, *Nuclear Magnetic Resonance and Its Applications to Living Systems* (Oxford Univ. Press, Oxford, 1982); E. J. Fernandez and D. S. Clark, *Enzyme Microb. Technol.* **9**, 256 (1987).
73. J. V. Shanks and J. E. Bailey, *Biotechnol. Bioeng.* **32**, 1138 (1988).
74. T. D. Brock, Ed., *Thermophiles: General, Molecular and Applied Microbiology* (Wiley-Interscience, New York, 1986).
75. H. Kacser and J. A. Burns, *Symp. Soc. Exp. Biol.* **27**, 65 (1973); R. Heinrich, S. M. Rapoport, T. A. Rapoport, *Prog. Biophys. Mol. Biol.* **32**, 1 (1977).
76. J. L. Galazzo and J. E. Bailey, *Enzyme Microb. Technol.* **12**, 162 (1990); *ibid.* **13**, 363 (1991).
77. C. Reder, *J. Theor. Biol.* **135**, 175 (1988).
78. P. M. Schlosser and J. E. Bailey, *Math. Biosci.* **100**, 87 (1990).
79. J. P. Delgado and J. Liao, *Biotechnol. Prog.* **7**, 15 (1991).
80. M. M. Domach *et al.*, *ibid.* **26**, 203 (1984).
81. S. B. Lee and J. E. Bailey, *Plasmid* **11**, 151 (1984); *ibid.*, p. 166; *Biotechnol. Bioeng.* **26**, 66 (1984); *ibid.*, p. 1372; *ibid.*, p. 1283.
82. J. E. Bailey, N. Da Silva, S. M. Peretti, J.-H. Seo, F. Srienc, *Ann. N.Y. Acad. Sci.* **469**, 194 (1986).
83. N. Danhash, D. C. J. Gardner, S. G. Oliver, *Biotechnology* **9**, 179 (1991).
84. K. Walsh, M. Schena, A. J. Flint, D. E. Koshland, Jr., *Biochem. Soc. Symp.* **54**, 183 (1987).
85. M. Kosinski, U. Rinas, J. E. Bailey, unpublished data.
86. Supported by NSF and by the Catalysis and Biocatalysis Program of the Advanced Industrial Concepts Division of the U.S. Department of Energy. Advice and assistance in the preparation of this paper were provided by D. S. Clark, B. D. Ensley, T. Hashimoto, R. J. Kaufman, C. Khosla, R. A. Lazarus, A. J. Sinskey, K. N. Timmis, and colleagues at the California Institute of Technology.

Network Rigidity and Metabolic Engineering in Metabolite Overproduction

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In order to enhance the yield and productivity of metabolite production, researchers have focused almost exclusively on enzyme amplification or other modifications of the product pathway. However, overproduction of many metabolites requires significant redirection of flux distributions in the primary metabolism, which may not readily occur following product deregulation because metabolic pathways have evolved to exhibit control architectures that resist flux alterations at branch points. This problem can be addressed through the use of some general concepts of metabolic rigidity, which include a means for identifying and removing rigid branch points within an experimental framework.

ALL ORGANISMS USE PRIMARY METABOLIC PATHWAYS TO supply precursor metabolites and energy to anabolic pathways that synthesize cellular constituents that are necessary for growth and maintenance. In many industrial strains of microorganisms (as well as tissue and plant cultures), these anabolic pathways have been exploited for the overproduction of compounds (such as amino and nucleic acids, antibiotics, vitamins, enzymes, and proteins) that cannot be synthetically produced or for which it is not economical to do so. In general, a particular metabolite is overproduced by deregulating the pathway directly associated with the synthesis of that metabolite, or, more recently, by transforming a robust host organism (typically *Escherichia coli*) with the genes that encode for the synthesis of the desired product (*1, 2*). This approach, however, does not necessarily result in high product yields (defined as the moles of product formed per mole of substrate consumed) since carbon flux distributions at key branch points (nodes) in the primary metabolism [such as glycolysis, tricarboxylic acid (TCA) cycle, and pentose phosphate pathway] must often be radically redirected from the flux distributions that are normally associated with balanced growth. Such metabolic flux alterations are often directly opposed by mechanisms for controlling enzyme activity that have evolved to maintain flux distributions that are optimal for growth. We refer to this inherent resistance to flux alterations as metabolic or network rigidity and to the genetic modifications of specific nodes in the primary metabolism for the purpose of enhancing yield and productivity as metabolic engineering (*3*). Although genetic manipulations can now be readily performed, there are relatively few accounts of successful metabolic flux alterations because of the complex, nonlinear nature of the metabolic control architectures.

The nature and types of metabolic rigidity are reviewed in this article along with methods to identify and possibly circumvent such undesirable nodal controls. The overproduction of lysine by *Corynebacterium glutamicum* [and related strains (*4*)] is used as a vehicle to illustrate key points because of: (i) the lack of compartmentalization in bacteria; (ii) the need for significant flux alterations to optimize lysine biosynthesis; and (iii) the apparent marginal success of mutation-selection (*5, 6*) or genetic engineering (7) techniques used to that end. The concepts, however, are of general value, and the methods are applicable to other metabolic products as well.

Basis of Metabolic Rigidity

Although intracellular metabolite concentrations can fluctuate during growth, on average, the distributions of the major cellular groups (proteins, RNA, DNA, lipids, and so forth) remain relatively proportional to one another throughout balanced growth (*8*). In fact, metabolites and energy required to synthesize an *E. coli* cell have been calculated on the basis of its known composition (*9*). In order to preserve this regularity in cellular composition, the primary metabolism has evolved coordination of pathway control, such that building-block metabolites, energy [such as adenosine triphosphate (ATP)], and biosynthetic reducing power [such as nicotinamide adenine dinucleotide phosphate (NADPH)] are synthesized in approximate stoichiometric ratios during balanced growth. Al-

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though cellular metabolism can support radically different flux distributions in response to different environmental stimuli (*10*), such flux alterations are a consequence of the metabolic feedbacks that control the optimum synthesis of the major cellular groups required for the survival of the cell.

Since primary metabolism is coordinated to synthesize a relatively uniform distribution of building block metabolites and energy, it is not well suited for the overproduction of any single metabolite. Consequently, if overproduction of a particular metabolite demands significant flux alterations in the primary metabolism compared to the flux distributions observed under balanced growth, then simple deregulation of product feedback inhibition frequently results in product yields that are significantly less than theoretical. An example of the effects that overproduction of a metabolite can elicit on metabolic flux distributions is shown in the flux diagrams in Fig. 1, which represent lysine overproduction by *C. glutamicum* ATCC 21253 under varying metabolic burdens. The flux distributions that support lysine production at typically observed yields (30 to 40% molar yield) (*11*) (Fig. 1B) do not deviate significantly from the flux distributions that support balanced growth (Fig. 1A). However, the flux distributions required to support maximum lysine yield (Fig. 1C) represent a significant divergence from the flux distributions that occur during balanced growth or typical lysine production conditions.

If primary metabolism could readily shift to match lysine synthesis demands, then significant yield improvements could be obtained by simply blocking pathways leading to by-products, such as the pyruvate dehydrogenase complex (PDC) that leads into the TCA cycle. However, such attempts have not resulted in significant yield improvements (*5, 6*), which indicates that the metabolic network does not respond readily to the demands of such perturbations. Since modification of the primary metabolism in order to redirect fluxes in support of maximum product yield is the objective of metabolic engineering, the sources of metabolic rigidity must be identified.

Principal Nodes

It is often assumed that poor product yield results from an enzyme or enzymes in a reaction sequence that limit the throughput to the product. Although overall enzymatic activity along the product branch certainly governs product synthesis rate, product yield is ultimately a function of flux partitioning that occurs at intermediate branch points. The latter, also referred to as nodes, are points in a metabolic network where a reaction sequence bifurcates among two or more different pathways. Since the pathways that synthesize primary metabolites can typically support substantial fluxes, metabolic engineering efforts should be directed toward altering flux partitioning at selected metabolic branch points to enhance product yield.

An important realization in the analysis of metabolic networks is that product yield typically depends on the flux partitioning that occurs at only a small subset of all possible nodes that comprise the metabolic network. Identification of these nodes, referred to as the principal nodes, is accomplished by searching for those nodes in the metabolic network where significant changes in flux partitioning occur as a function of product yield. Flux distributions required for this analysis can be determined from metabolite balance constraints with or without (as in Fig. 1) the use of experimental data (see below). The lysine example illustrates the point. Of ~30 branch points in the lysine biosynthetic network, significant changes in flux partition occur at only three nodes, glucose-6-phosphate (G6P), phosphoenolpyruvate (PEP), and pyruvate (Pyr), as lysine yield increases from 35% (Fig. 1B) to 75% (Fig. 1C). Close inspection of the oxaloacetate (OaA) branch point reveals that it is a trivial principal node (*12*) since all of the OaA synthesized from PEP condenses with Pyr to form lysine. The OaA synthesized through malate dehydrogenase is consumed by citrate synthase, so that net OaA production occurs only through the anaplerotic PEP carboxylase reaction (*10*). The fructose-6-phosphate (F6P) node can also be ruled out as a principal node. For a lysine yield of less than 60%, the F6P node is simply a condensation point; however, for yields greater than 60%, F6P becomes a principal node and G6P becomes a condensation point (Fig. 1C). This change is due to the pentose phosphate pathway, which must operate in a cyclic manner to fulfill NADPH requirements when lysine yield exceeds 60%. Since typically observed lysine yields are 30 to 40%, metabolic modifications need only target the G6P, PEP, and Pyr principal nodes. Hence metabolic engineering efforts need only focus on these three nodes and not on the entire network, which dramatically reduces problem complexity. The duality of the G6P and F6P nodes also illustrates the point that the location of the principal nodes depends on the product under investigation, the substrate utilized, and the by-products that are typically associated with the product. The connectivity arrangement of the principal nodes in the network is also an important consideration.

In general, network architectures fall into two basic classes: dependent and independent. If each principal node of a network, or subnetwork, contributes a stoichiometrically consumed component

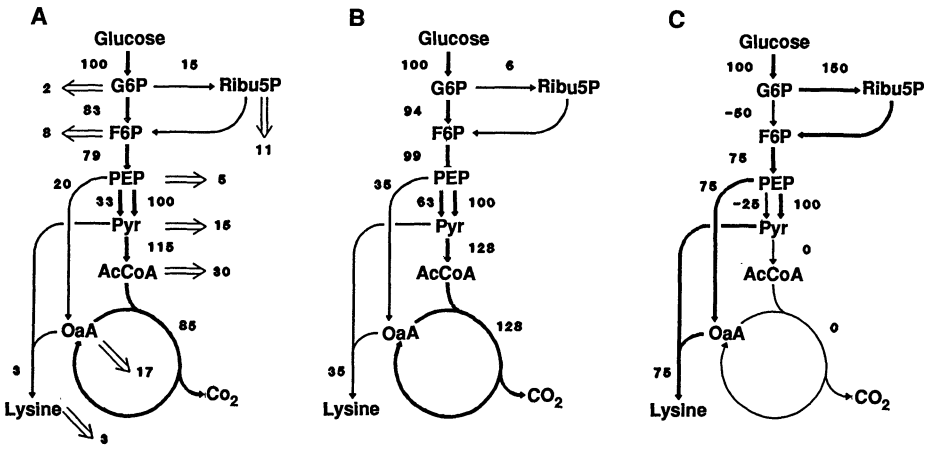

Fig. 1. Flux distributions, normalized by glucose uptake in the metabolic network of *C. glutamicum* (illustrated in condensed form) during: (**A**) balanced growth (at a typically observed yield of 0.5 grams of biomass per gram glucose) without lysine overproduction; (**B**) lysine overproduction at 35% molar yield without growth; and (**C**) lysine overproduction at maximum molar yield of 75%. Fluxes at the indicated yields were determined solely from metabolic balance constraints (that is, experimental data were not required) (*36, 37*) for a network consisting of 34 reactions, including a lumped reaction to account for biomass synthesis (*9, 36, 37, 39*) indicated by the double arrows (⇒). The two fluxes between PEP and Pyr account for reactions catalyzed by pyruvate kinase (or PEP synthetase) (left) and the glucose:PEP phosphotransferase system (*43*) (right). A negative flux indicates that flow is in the opposite direction to that indicated by the arrow. Abbreviations: G6P, glucose-6-phosphate; Ribu5P, ribulose-5-phosphate; F6P, fructose-6-phosphate; PEP, phosphoenolpyruvate; Pyr, pyruvate; AcCoA, acetyl coenzyme A; and OaA, oxaloacetate.

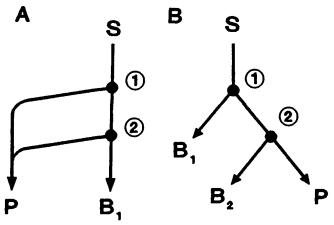

Fig. 2. Examples of (**A**) dependent and (**B**) independent two-node networks, where S is the substrate, B_1 and B_2 are by-products, and P is the desired product.

of the final product, then the network or subnetwork is considered dependent (Fig. 2A), such as in the lysine network (NADPH, OaA, and Pyr are all lysine precursors). In such networks, the flux in all condensing branches must be stoichiometrically balanced to prevent accumulation or excretion of intracellular components (for example, flux partitioning at nodes 1 and 2 of Fig. 2A must be equal). Under this restriction, flux partitioning at all principal nodes that comprise a dependent network must be coordinated, and all principal nodes must be considered of equal importance, regardless of their "remoteness" from the product of interest. Conversely, if the metabolites produced from the principal nodes do not condense (Fig. 2B), then the network is considered independent, and product yield may be improved by altering the flux partitioning that occurs at a single principal node. In the simple two-node network (Fig. 2B), the yield of the desired product P can be enhanced if the flux partitioning at either node 1 or 2 is altered to favor the synthesis of P, while in the dependent network, flux partitioning at both nodes must be altered.

Although network architecture affects metabolic response to enzyme modifications, in both dependent and independent networks the degree of metabolic rigidity ultimately resides with the ease by which flux partitioning at the principal nodes may be altered to obtain a flux distribution that is optimal for product formation. Since the type of nodal rigidity dictates the types of metabolic modifications required to render the rigid node flexible, factors that influence nodal flux partitioning must be examined.

Branch Point Classifications

The metabolic control structures used by different organisms to regulate pathway fluxes vary markedly (*13*). It is, nevertheless, possible to classify these control structures into three general categories on the basis of their branch point rigidity. In the discussion that follows, branch kinetics refers to the lumped response of all of the enzymes that constitute the branch, and the branch split ratio refers to the carbon flux channeled through that branch normalized by the flux into the node.

Nodes where flux partitioning into each branch readily changes to meet metabolic demands are referred to as flexible nodes. In a flexible node, enzymes participating in each branch show similar affinity for the node metabolite, and reaction velocities of each branch are of similar magnitude. The flux through each branch is controlled by feedback inhibition by the corresponding terminal metabolite (Fig. 3A). As a result, the branch split ratio can change from zero to one to meet the demands placed on the terminal metabolites. For example, if the feedback inhibition by metabolite P (Fig. 3A) is removed, or if P (but not by-product B) is a precursor to a desired product, the split-ratio of the P branch would approach unity as the product yield is maximized. Many terminal branch points in amino acid biosynthesis exhibit such flexibility (*14*). For example, the flux into the aspartate semialdehyde branch point can be completely redirected toward lysine synthesis by deregulating the concerted feedback inhibition of aspartokinase by lysine plus threonine (*15*). Flexible nodes are the most amenable to alterations of branch flux distributions and seldom require modification. Perhaps because of this, many metabolic modifications intended to enhance product yield assume the principal nodes (hence the network) to be flexible, something that cannot be expected to be generally true.

A node is said to be weakly rigid if the flux partitioning at the node is dominated by the kinetics of one of its branches. This can be the result of high enzymatic activity or high enzymatic affinity for the node metabolite and lack of feedback inhibition in the dominant branch (Fig. 3B). Even if the subordinate (product) branch is deregulated (or a high demand is placed on P), a significant fraction of the flux still enters the dominant branch and limits product yield. Product yield may still be enhanced, however, by attenuating the activity of the dominant branch. This may have to be accompanied by activity amplification of the subordinate branch or end product deregulation in order to prevent accumulation of the branch point intermediate (N). Nevertheless, weakly rigid nodes are typically amenable to mutation-selection techniques.

Weakly rigid nodes are often found in catabolic pathways leading to CO_2 formation. One example is the isocitrate branch point in *E. coli* between the glyoxylate shunt and the TCA cycle. When glucose is added to an *E. coli* culture grown on acetate, the flux supported by isocitrate lyase (ICL), the first enzyme of the glyoxylate shunt, drops to zero even though the glyoxylate shunt is completely active (*16*). Under these conditions, TCA cycle isocitrate dehydrogenase (ICDH) exhibits complete dominance over ICL since the Michaelis constant K_m of ICDH for isocitrate is 1/75 that of ICL, and both enzymes exhibit similar activity (*16–18*). However, if the activity of ICDH is attenuated by 60% or more, a significant fraction of the isocitrate synthesized is diverted into the glyoxylate shunt (*16–19*). In *E. coli*, ICDH activity is attenuated by phosphorylation, so that growth on acetate can be sustained.

A node is said to be strongly rigid if the split ratio of one or more of its branches is tightly controlled. This is commonly achieved by a combination of feedback control and enzyme trans-activation by a metabolite in an opposite branch (Fig. 3C). In this particular case, besides inhibiting their own synthesis, metabolites P and B also act as positive effectors of the opposite branch, a property exhibited by many allosteric enzymes (*20*). This architecture effectively stabilizes the flux partitioning at the node, provided the steady-state concentrations of P and B are sufficiently high to inhibit their own synthesis in the absence of their corresponding branch activators, B and P, respectively (*21*). For example, if the flux through the B branch is attenuated as means of increasing the split ratio of the P branch, the synthesis rate of P would also be attenuated because of the loss of activation by B and dominance of the remaining end product inhibition by P, leaving the split ratios of each branch relatively unaffected. Interestingly, simulations indicate that other nodal modifications, such as increasing the demand for P (by either affinity or activity modulation), removing the feedback inhibition of P, or blocking the demand for B, have little effect on the split ratio of the P branch as well. Because of the hypothetical nature of this example, the details of these modifications are not discussed. However, several examples of rigid nodes have been previously documented.

In *Bacillus subtilis*, histidine activates anthranilate synthetase, an enzyme required for tryptophan synthesis (*22*). This activation by histidine allows *B. subtilis* to grow in the presence of 5-methyltryptophan, an anti-metabolite of tryptophan. Furthermore, the addition of histidine increases tryptophan excretion by a factor of 4 in mutant strains that express anthranilate synthetase constitutively. It is hypothesized that this trans-activation, which the investigators referred to as "metabolic interlock," has evolved to control the level of 5-phosphoribosyl-1-pyrophosphate, a metabolite that is common to the synthesis of both amino acids. This type of pathway interaction has also been referred to as compensatory control or activation (*14, 23, 24*). Remote effectors also influence phenylalanine biosyn-

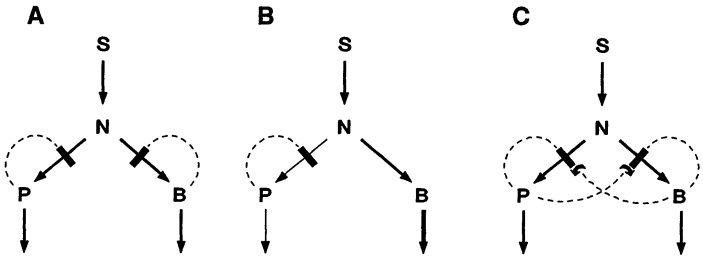

Fig. 3. Control architectures that would render the illustrated branch point (**A**) flexible, (**B**) weakly rigid, or (**C**) strongly rigid to modifications in flux partitioning (see text for details). Dashed lines indicate negative (‖) or positive (↘) feedback from the corresponding metabolite.

thesis by modulating prephenate dehydratase in both coryneform bacteria and *B. subtilis* (*25*). In regard to primary metabolism, significant coupling interactions have been identified between anaplerotic pathways—PEP and Pyr carboxylases—and the catabolic pathways of glycolysis and the TCA cycle (*24, 26*). In general, flux partitioning at strongly rigid nodes cannot be easily modified since the kinetics of the enzymes associated with the branch point may require specific modifications, such as alterations in effector and substrate affinities.

Network Responses

The presence of a rigid node (either strong or weak) in a metabolic network can have profound implications with regard to the outcome of perturbations intended to enhance product yield. Consider again the simple independent network (Fig. 2B), where increasing the yield of P is the desired goal. It is further assumed that node 1 is flexible, node 2 is strongly rigid, and that B_1 does not inhibit its own synthesis. If the B_1 branch is blocked, the yield of P would be increased; however, if the B_2 branch is blocked, the yield of P would actually decrease because of the rigidity of node 2. This occurs because attenuation of the flux through the B_2 branch causes a decrease in the flux to the product branch (due to node 2 rigidity). Since node 1 is flexible, the flux to node 2 would be diverted to B_1, thereby decreasing the yield of P. If the architecture is reversed, so that node 1 is rigid but node 2 is flexible, then blocking B_2 synthesis would improve the yield of P. However, if B_1 synthesis is blocked, the yield of P would not be affected while the rate of P synthesis would be severely attenuated.

Nodal rigidity in dependent networks presents even greater problems in metabolic engineering. For example, if either node 1 or node 2 in the simple dependent network (Fig. 2A) is rigid, then attenuation of B_1 synthesis would not affect the yield of P, but would cause its synthesis rate to be diminished by the same extent as B_1 synthesis. Hence, modifications intended to improve flux distributions in dependent networks often result in overall metabolic collapse (or excretion of intermediate metabolites). In a dependent network, flux distribution response is independent of the location of the rigidity since alterations in flux partitioning at all principal nodes must be coordinated if product yield is to be enhanced.

Metabolic networks may not always exhibit the binary response to flux perturbations implied by the above classifications because of the diverse control architectures and enzyme kinetics that are associated with branch points. Thus, attenuation of one branch of a strongly rigid node may only result in partial attenuation of the competing branch, so that some improvement in product yield may be attained in a dependent network that harbors a rigid node. Also, attenuating nondesirable pathways may cause excretion of intermediate metabolites instead of metabolic flux collapse or flux redirection. Finally, as with the location of the principal nodes, the status of a branch point (that is, whether flexible or rigid) may depend on the product under investigation. For instance, if a node consists of the two branches, A → B + C and A → D, where B and D (but not C) inhibit their own synthesis, then the node would appear to be flexible to products that consume B or D, but weakly rigid to products that require only C. In any event, the extent of rigidity of principal nodes in a network must first be assessed before any metabolic modifications can be suggested.

Assessment of Principal Node Rigidity

Once the principal nodes for the product of interest have been identified in the metabolic network, their degree of rigidity must be assessed. Several techniques are available to analyze metabolic networks, all with their own advantages and disadvantages but none completely comprehensive. If detailed kinetic expressions are available for all of the enzymes included in the network representation of the organism's biochemistry, then a mathematical model of the metabolism can be constructed, such as those developed for glycolysis and respiration (*27, 28*) and for erythrocytes (*29*). With the aid of such models, simulations of the metabolic network, or subnetwork, are conducted to assess the rigidity of the principal nodes by comparing the steady-state flux distributions for the nominal case to those generated from the same network following a perturbation intended to enhance product yield (that is, alteration of a principal node's split-ratio), such as enzyme deletion or deregulation. Suggestions for metabolic alterations are then based on the type of principal nodes identified (Fig. 3). Although these perturbation techniques are governed by trial and error, they are quite informative since the models incorporate the full nonlinear nature of metabolic networks. The obvious disadvantage of simulation-based assessment techniques is that detailed kinetic information is limited and, when available, its applicability to in vivo situations not readily established.

Other related approaches, such as metabolic control theory (MCT) (*30*) or biochemical systems theory (BST) (*31*), have received extensive attention (*2, 32*); however, their value in directing metabolic engineering efforts remains questionable (*33*). Both techniques rely on approximating the actual metabolic system (assuming enzyme kinetics are available) around a particular operating point, so that the sensitivity of steady-state fluxes can be evaluated with respect to perturbations in enzyme velocities. The

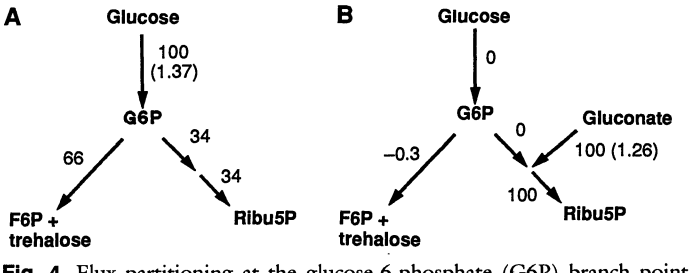

Fig. 4. Flux partitioning at the glucose-6-phosphate (G6P) branch point during lysine fermentations of *C. glutamicum* ATCC 21253 with (**A**) glucose (control) or (**B**) gluconate at the primary carbon source. Fermentations were conducted aerobically in a 10-liter fermentor in which the consumption of glucose (or gluconate), oxygen, and ammonium and the production of lysine, biomass, carbon dioxide, and by-products (trehalose, acetate, pyruvate, alanine, valine, and lactate) were frequently monitored (*37*). Accumulation or consumption rates of extracellular metabolites were determined following the termination of exponential growth and the commencement of lysine overproduction. These rates were then used with the metabolite balance constraints to estimate the metabolic flux distributions. Absolute fluxes (in parentheses) are in units of millimoles per hour per gram of cells.

results of this sensitivity analysis in turn provide the basis for suggesting various metabolic modifications. A major drawback of these methods is that they are valid only in the local neighborhood of the operating point evaluated. Yet, the objective in metabolic engineering is to modify primary metabolism such that the resulting flux distributions (as in Fig. 1C) differ radically from those observed during growth (as in Fig. 1A). Furthermore, enzymes with large flux control coefficients (*30*) are not always the ones to be modified, especially if they are involved in feedback control loops. For example, in a sequence of reactions with a simple feedback loop, the enzyme that consumes the feedback metabolite strongly controls the flux. However, it is usually the removal of the feedback loop that should be considered, not activity amplification of the enzymes that consume the feedback metabolite. Nevertheless, MCT, BST, and related analysis techniques (*34*) are still useful tools in the analysis of metabolic networks.

When enzyme kinetics are unavailable or incomplete, metabolic flux distributions can be obtained by applying stoichiometrically derived mass balance constraints to experimental data on the rates of accumulation or depletion of extracellular metabolites. We (*35–37*), as well as others (*19, 38, 39*), have used this method to determine metabolic fluxes under different conditions and during different phases of culture. There is obviously a limit to the extent that the structure of a biochemical network can be delineated by using extracellular measurements only (*40*), and the use of radioactive (*16, 41*) or stable (*42*) isotope tracers can enhance the power of this approach. However, such tracer measurements should be made in addition to the usual extracellular measurements taken in the course of a fermentation if the metabolic network and its flux distributions are to be further elucidated. Application of mass balances can also yield flux distributions for a specified metabolic burden (such as in Fig. 1, A and B) as well as estimate the maximum theoretical yield for a product (such as in Fig. 1C) but cannot be used to predict flux responses to metabolic perturbations. However, in conjunction with experimental perturbations, flux distribution estimates can often be used to identify the source or sources of metabolic rigidity, as described below.

The degree by which flux partitioning at a principal node limits product yield can be assessed by experimentally perturbing a branch that is proximal to the principal node under investigation and comparing the resulting local flux distributions to those observed under nominal conditions. If the flux partitioning at the node can be clearly redirected in a manner consistent with the perturbation, then the node is either flexible or weakly rigid; otherwise, the node may be strongly rigid. It is not possible to provide a comprehensive map relating metabolic response to nodal type since metabolic responses are case-specific due to the variability of network structure (dependent versus independent), control architecture, and perturbation used. For this same reason, the types of perturbations used to assess nodal rigidity are also case specific, but can be classified into four general categories: (i) attenuation of enzyme activity through the addition of a specific inhibitor; (ii) amplification or attenuation of enzyme activity through genetic modifications; (iii) environmental perturbation, such as a change in carbon source; and (iv) deregulation of a different metabolite to increase metabolic burden. These types of perturbations were applied to investigate the rigidity of the G6P, Pyr, and PEP principal nodes in the lysine biosynthetic network (Fig. 1) (*37*).

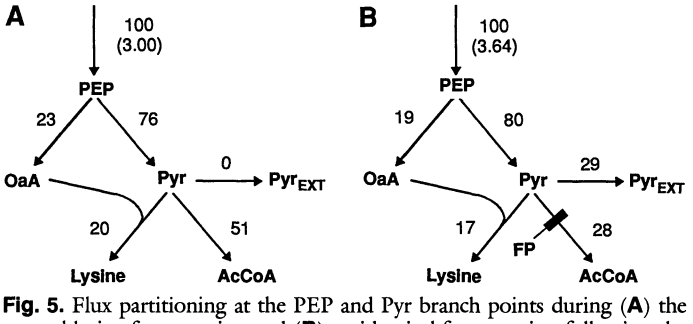

Fig. 5. Flux partitioning at the PEP and Pyr branch points during (**A**) the control lysine fermentation and (**B**) an identical fermentation following the addition of 2 mM fluoropyruvate (FP) at the termination of exponential growth and at the start of lysine overproduction (*37*). The addition of FP resulted in the extracellular accumulation of pyruvate (Pyr_{EXT}) and diminished the flux into the TCA cycle; however, lysine yield remained unaffected at 30% molar. Absolute fluxes (in parentheses) are in units of millimoles per hour per gram of cells, and fluxes to biosynthesis are not shown. See caption of Fig. 4 for measurements taken to construct flux diagrams.

In order to examine the possibility that lysine synthesis is limited by NADPH availability (that is, the rigidity of the G6P node), *C. glutamicum* was cultivated on gluconate instead of glucose since the former is known to bypass the G6P node by directly entering the pentose phosphate pathway (*43*). Flux partitioning at the G6P node (Fig. 4) calculated from the fermentation data reveals that 50% more NADP was reduced per mole of substrate consumed in the gluconate fermentation than in the glucose fermentation (a result of the higher flux supported by the second dehydrogenase in the oxidative branch of the pentose phosphate pathway). Lysine yield in both fermentations, however, was ~30% molar, which indicates that lysine yield is not limited by flux partitioning at the G6P node. In support of these findings, a second perturbation of the G6P node was conducted in which a strain of *C. glutamicum* was isolated that expressed G6P isomerase at only 10% of its native activity. Interestingly, the mutation did not affect lysine yield or flux partitioning but did result in a 75% attenuation of all metabolic fluxes (*37*), a response consistent with a dependent network that harbors a rigid node. These results, along with the efficiency by which flux distributions at the G6P node shifted in response to an increased demand for NADPH during lysine synthesis in the control fermentation (*37*), established the G6P node as flexible and imply that either the Pyr or PEP nodes must be rigid.

The possibility that lysine yield is limited by the flux partitioning at the Pyr principal node was examined by perturbing this node (*37*) by adding fluoropyruvate [a competitive inhibitor of PDC (*6*)] during normal lysine fermentation. Flux distributions estimated from the fermentation data (Fig. 5) demonstrate that pyruvate was diverted from the TCA cycle, but the increase in pyruvate availability did not enhance lysine yield. In a second perturbation experiment (*37*), flux distributions were determined for a strain of *C. glutamicum* that expressed only 1% of nominal PDC activity. This mutation did not effect instantaneous lysine yield or flux partitioning but did result in reducing metabolic fluxes by 75% in an analogous manner as the G6P isomerase mutation. These two perturbation experiments indicate that lysine yield is not limited by pyruvate availability (that is, Pyr node is flexible), which implies, in conjunction with the previous results, that the PEP node must be the source of the network rigidity. However, the control architecture underlying the cause of the PEP node rigidity cannot be determined from the perturbation flux analysis technique; therefore, the kinetics of the enzymes associated with the PEP node were examined in detail.

From inspection, the PEP node control architecture in *C. glutamicum* exhibits characteristics of a strongly rigid node since PEP carboxylase (PPC) is inhibited strongly by aspartate, but this inhibition is alleviated by activation by acetyl coenzyme A (AcCoA) (*44*). To further examine the rigidity of the PEP branch point, a kinetic model of the node was constructed utilizing enzyme kinetics available from glutamic acid bacteria (*37*). The model consists of kinetic expressions for enolase (ENO), PPC, Pyr kinase (PK) (*45*), aspartate aminotransferase, PDC, aspartate kinase (AK), and citrate

synthase (CS). The flux supported by the glucose:PEP phosphotransferase system (*43*) was assumed to be one-half the enolase flux, and all of the aspartyl phosphate synthesized was assumed to condense with pyruvate to produce lysine (kinetics of dihydrodipicolinate synthase were not included in the model).

The rigidity of the PEP node is illustrated by the steady-state flux distributions obtained from the model following attenuation of PK activity (Fig. 6). For the nominal system (Fig 6, solid lines), attenuation of PK activity results in a decrease of PPC flux and a general collapse of flux through the PEP node. If the effects of Asp and AcCoA are removed from the kinetics of PPC, the PEP node reverts to a weakly rigid node since attenuation of PK activity results in a diversion of flux into the PPC branch (Fig. 6, dashed lines). The strong rigidity of the PEP node is enhanced by (i) the high activity of PK and its strong affinity for PEP and (ii) the low activity of AK and its poor affinity for Asp, which maintains the high intracellular pool of Asp. On the basis of these simulations, it appears that flux partitioning at the PEP node can be made favorable for lysine synthesis by transforming *C. glutamicum* with a deregulated PPC enzyme at approximately tenfold its native activity (amplification of the native PPC activity did not result in significant flux partitioning enhancement in simulations).

The analysis of the PEP node brings up an important point regarding mitigation of strong rigidity. Although genetic engineering techniques can be used to modify a rigid node and render it flexible (such as changing substrate or inhibitor affinity), at this time such modifications are still quite difficult to achieve. Another procedure that may prove more advantageous is to use enzymes from organisms that have different control architectures for the same biochemical pathway. A good example is the regulation of the PPC flux in photoautotrophs. Since C_4 plants and some cyanobacteria use PPC in CO_2 fixation (*46*), the control architecture of the PEP node in many photoautotrophs differs from heterotrophs in that PPC is not inhibited (or not inhibited as strongly) by Asp nor does it require activation by AcCoA (*47, 48*). Consequently, flux partition at the PEP node in *C. glutamicum* may be improved by transforming this strain with PPC from a cyanobacterium, such as *Anacystis nidulans* (*48*), although other possibilities exist (*49*). It is important to realize that flux distributions that are optimal for product synthesis may not support growth. Consequently, such modifications should be constructed so that they may be "turned on" after sufficient biomass has accumulated.

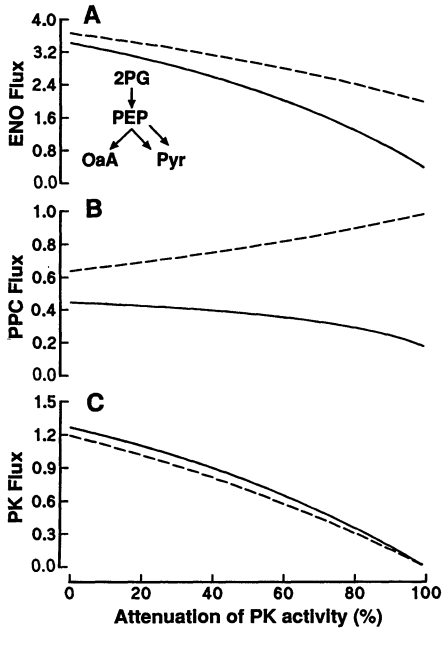

Fig. 6. Simulated steady-state fluxes (in millimoles per hour per gram of cells) supported by (**A**) enolase (ENO), (**B**) PEP carboxylase (PPC), and (**C**) pyruvate kinase (PK) following attenuation of PK activity in the PEP branch point model with either native PPC kinetics (solid lines) or deregulated PPC kinetics (dashed lines) (*37*). Concentrations of 2-phosphoglycerate (2PG), ATP, and AMP were held constant at 4, 7, and 1 mM, respectively. These parameters were adjusted such that the steady-state fluxes matched those observed in a typical lysine fermentation.

Conclusions

Relatively few examples have been offered in strain improvement through alterations of primary metabolism. This lack of significant progress in the field is partly attributed to the complex interactions of metabolic networks but also to the lack of a general, practical approach to metabolic engineering. To address this problem, we have proposed a working hypothesis regarding metabolic rigidity and have developed an approach to identify the source or sources of rigidity that should foster attempts to optimize metabolic flux distributions for metabolite overproduction. We have proposed that the control architecture of primary metabolism, which is optimized for the synthesis of cellular components, prevents radical alterations of metabolic flux distributions that would allow certain metabolites to be overproduced near maximum yield. Analysis of metabolic flux distributions that are optimal for product synthesis reveals that modifications in flux partitioning need occur only at the principal nodes of the network. Control architecture or the kinetics of a principal node may limit the extent to which flux partitioning would occur following product deregulation. Flux mapping coupled with metabolic perturbations can be used to assess the flexibility of the principal nodes. Since the alteration or bypass of a strongly rigid node may require significant alterations of enzyme kinetics, we propose that nodal rigidity may be alleviated by tapping into the diversity of control architectures that are found in other organisms.

REFERENCES AND NOTES

1. S. E. Lindow, N. J. Panopoulos, B. L. McFarland, *Science* **244**, 1300 (1989).
2. D. B. Kell and H. V. Westerhoff, *Trends Biotechnol.* **4**, 137 (1986).
3. J. E. Bailey, S. Birnbaum, J. L. Galazzo, C. Khosla, J. V. Shanks, *Ann. N.Y. Acad. Sci.* **589**, 1 (1990); J. W. Cary, D. J. Petersen, G. N. Bennett, E. T. Papoutsakis, *ibid.*, p. 69.
4. S. Kinoshita, in *Biology of Industrial Microorganisms*, A. L. Demain and N. A. Solomon, Eds. (Benjamin/Cummings, Menlo Park, CA, 1985), pp. 115–142.
5. Although high lysine–producing strains have been isolated, the genetic modifications that lead to such strains remain uncertain due to the isolation techniques used: I. Shiio, A. Yokata, S.-i. Sugimoto, *Agric. Biol. Chem.* **51**, 2485 (1987); A. Yokata and I. Shiio, *ibid.* **52**, 455 (1988); I. Shiio, Y. Toride, S.-i. Sugimoto, *ibid.* **48**, 3091 (1984).
6. O. Tosaka, Y. Yoshihara, S. Ikeda, K. Takinami, *Agric. Biol. Chem.* **49**, 1305 (1985).
7. Unfortunately, emphasis on transforming *C. glutamicum* (and related strains) has taken precedence over documenting the metabolic effects of the genetic alterations: J. Cremer, C. Treptow, L. Eggeling, H. Sahm, *J. Gen. Microbiol.* **134**, 3221 (1988); E. Menkel, G. Thierbach, L. Eggeling, H. Sahm, *Appl. Environ. Microbiol.* **55**, 684 (1989); M. O'Regan *et al.*, *Gene* **77**, 237 (1989).
8. D. Herbert, *Symp. Soc. Gen. Microbiol.* **11**, 391 (1961).
9. J. L. Ingraham, O. Maaløe, F. C. Neidhardt, *Growth of the Bacterial Cell* (Sinauer, Sunderland, MA, 1983), pp. 87–173; A. H. Stouthamer, *Antonie van Leeuwenhoek* **39**, 545 (1973).
10. H. L. Kornberg, *Essays Biochem.* **2**, 1 (1966).
11. S. Kinoshita and K. Nakayama, in *Economic Microbiology*, A. H. Rose, Ed. (Academic Press, New York, 1978), vol. 2, pp. 209–261.
12. The OaA branch point would be considered a principal node if glutamate (or some other TCA-derived metabolite) was produced as a by-product during lysine overproduction. For example, a significant fraction of the net OaA synthesized during balanced growth is consumed by the glutamate amino acid family (Fig. 1A).
13. B. D. Sanwal, *Bacteriol. Rev.* **34**, 20 (1970).
14. H. E. Umbarger, *Annu. Rev. Biochem.* **38**, 323 (1969).
15. K. Nakayama, in *Microbial Production of Amino Acids*, K. Yamada, S. Kinoshita, T. Tsunoda, K. Aida, Eds. (Kodansha, Tokyo, 1972), pp. 369–397; O. Tosaka, M. Ishihara, Y. Morinaga, K. Takinami, *Agric. Biol. Chem.* **43**, 265 (1979).
16. K. Walsh and D. E. Koshland, Jr., *J. Biol. Chem.* **260**, 8430 (1985).
17. D. C. LaPorte, K. Walsh, D. E. Koshland, Jr., *ibid.* **259**, 14068 (1984).
18. D. E. Koshland, Jr., K. Walsh, D. C. LaPorte, *Curr. Top. Cell. Regul.* **27**, 13 (1985).
19. W. H. Holms, *Biochem. Soc. Symp.* **54**, 17 (1987).
20. B. I. Kurganov, *Allosteric Enzymes: Kinetic Behavior* (Wiley, New York, 1982).
21. This is easily achieved if the enzymes that consume P and B exhibit poor affinity for these metabolites and are at or near saturation under nominal conditions.
22. Interestingly, histidine also represses anthranilate synthetase: J. F. Kane and R. A.

Jensen, *J. Biol. Chem.* **245**, 2384 (1970); R. A. Jensen, *ibid.* **244**, 2816 (1969).
23. B. D. Sanwal, M. Kapoor, H. W. Duckworth, *Curr. Top. Cell. Regul.* **3**, 1 (1971).
24. B. D. Sanwal and P. Maeba, *J. Biol. Chem.* **241**, 4557 (1966); E. R. Stadtman, *Enzymes* **1**, 397 (1970).
25. A. M. Faxel and R. A. Jensen, *Arch. Biochem. Biophys.* **200**, 165 (1980).
26. A. L. Miller and D. E. Atkinson, *ibid.* **152**, 531 (1972); C.-L. Liao and D. E. Atkinson, *J. Bacteriol.* **106**, 37 (1971); D. E. Atkinson, *Cellular Energy Metabolism and Its Regulation* (Academic Press, New York, 1977), pp. 193–200.
27. J. L. Galazzo and J. E. Bailey, *Enzyme Microb. Technol.* **12**, 162 (1990).
28. B. Chance, D. Garfinkel, J. Higgins, B. Hess, *J. Biol. Chem.* **235**, 2426 (1960); D. Garfinkel and B. Hess, *ibid.* **239**, 971 (1964); D. Garfinkel, *Trends Biochem. Sci.* 6, 69 (1981).
29. A. Joshi and B. O. Palsson, *J. Theor. Biol.* **141**, 515 (1989); *ibid.* **142**, 41 (1990).
30. H. Kacser and J. A. Burns, *Symp. Soc. Exp. Biol.* **27**, 65 (1973); R. Heinrich and T. A. Rapoport, *Eur. J. Biochem.* **42**, 89 (1974); R. Heinrich, S. M. Rapoport, T. A. Rapoport, *Prog. Biophys. Mol. Biol.* **32**, 1 (1977).
31. M. A. Savageau, *J. Theor. Biol.* **25**, 365 (1969); *ibid.* **26**, 215 (1970).
32. Discussion Forum, *Trends Biochem. Sci.* **12**, 4 (1987); *ibid.*, p. 216; A. K. Groen and J. M. Tager, *Biochem. J.* **253**, 619 (1988).
33. D. E. Atkinson *et al.*, in *Control of Metabolic Processes*, A. Cornish-Bowden and M. L. Cárdenas, Eds. (Plenum, New York, 1989), pp. 3–27; *ibid.*, pp. 413–427. Several other papers on MCT and related topics can also be found in this publication.
34. B. O. Palsson and E. N. Lightfoot, *J. Theor. Biol.* **113**, 261 (1985); R. A. Majewski and M. M. Domach, *BioSystems* **18**, 15 (1985); J. C. Liao and E. N. Lightfoot, *Biotechnol. Bioeng.* **31**, 869 (1988); R. A. Majewski and M. M. Domach, *ibid.* **36**, 166 (1990).
35. J. J. Vallino and G. Stephanopoulos, *Ann. N.Y. Acad. Sci.* **506**, 415 (1987).
36. _____, in *Frontiers in Bioprocessing*, S. K. Sikdar, M. Bier, P. Todd, Eds. (CRC Press, Boca Raton, FL, 1990), pp. 205–219.
37. J. J. Vallino, thesis, Massachusetts Institute of Technology, Cambridge (1991).
38. S. Aiba and M. Matsuoka, *Biotechnol. Bioeng.* **21**, 1373 (1979); E. T. Papoutsakis, *ibid.* **26**, 174 (1984); K. F. Reardon, T.-H. Scheper, J. E. Bailey, *Biotechnol. Prog.* **3**, 153 (1987); S. P. Tsai and Y. H. Lee, *Biotechnol. Bioeng.* **32**, 713 (1988); S. C. Niranjan and K.-Y. San, *ibid.* **34**, 496 (1989).
39. W. H. Holms, *Curr. Top. Cell. Regul.* **28**, 69 (1986).
40. S. P. Tsai and Y. H. Lee, *Biotechnol. Prog.* **4**, 82 (1988).
41. J. J. Blum and R. B. Stein, in *Biological Regulation and Development*, R. F. Goldberger and K. R. Yamamoto, Eds. (Plenum, New York, 1982), vol. 3A, pp. 99–125; R. B. Stein and J. J. Blum, *J. Biol. Chem.* **254**, 10385 (1979).
42. L. Inbar and A. Lapidot, *Eur. J. Biochem.* **162**, 621 (1987); J. A. den Hollander, K. Ugurbil, R. G. Shulman, *Biochemistry* **25**, 212 (1986); K. Yamaguchi, S. Ishino, K. Araki, K. Shirahata, *Agric. Biol. Chem.* **50**, 2453 (1986).
43. M. Mori and I. Shiio, *Agric. Biol. Chem.* **51**, 129 (1987).
44. I Shiio and K. Ujigawa-Takeda, *ibid.* **43**, 2479 (1979); M. Mori and I. Shiio, *J. Biochem.* **97**, 1119 (1985); *ibid.* **98**, 1621 (1985); *Agric. Biol. Chem.* **50**, 2605 (1986).
45. The Monod allosteric model [J. Monod, J. Wyman, J.-P. Changeux, *J. Mol. Biol.* **12**, 88 (1969)] was used to represent the kinetics of PPC and PK, where the kinetic parameters were obtained from nonlinear least-square fits to kinetic data published for PPC and PK [H. Ozaki and I. Shiio, *J. Biochem.* 66, 297 (1969)].
46. M. D. Hatch and C. R. Slack, *Annu. Rev. Plant Physiol.* **21**, 141 (1970); B. Colman, K. H. Cheng, R. K. Ingle, *Plant Sci. Lett.* 6, 123 (1976); G. W. Owttrim and B. Colman, *Biochem. Cell Biol.* 66, 93 (1988).
47. M. F. Utter and H. M. Kolenbrander, *Enzymes* 6, 117 (1972).
48. T. Kodaki, F. Katagiri, M. Asano, K. Iqui, H. Katsuki, *J. Biochem.* **97**, 533 (1985).
49. Another possibility is to use an effectorless pyruvate carboxylase, such as that found in *Pseudomonas fluorescens*: A. I. Higa, S. R. Milrad de Forchetti, J. J. Cazzulo, *J. Gen. Microbiol.* **93**, 69 (1976).
50. We acknowledge the contribution of A. J. Sinskey and M. Follettie of the MIT Department of Biology in our work on the analysis of *C. glutamicum* metabolism. Financial assistance was provided by the National Science Foundation in part through a PYI grant (CBT-8514729) and in part through the MIT Biotechnology Process Engineering Center. Lysine fermentations were conducted in equipment donated by MBR Bioreactors.