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Surprising insights that aren't so surprising in the modeling of sulfur amino acid metabolism

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Summary. The modeling of whole organism sulfur amino acid flux control has been aided in recent years by advancements in proteomics and mass spectroscopy-based metabolite analysis. The convergence of these two fields and their respective techniques, as demonstrated by a new study using yeast by Lafaye et al., has shown that researchers seeking to model whole cell/organism metabolism should give careful consideration to the relationships connecting enzyme concentration, enzyme activity, substrate concentration, and metabolic flux. In this paper, we outline some of the fundamental concepts for modeling sulfur amino acid metabolism and how they relate to our current understanding of mammalian sulfur amino acid metabolism.

Keywords: Sulfur – Metabolism – Amino acid

Introduction

In a recent paper Lafaye et al. (2005a) revisited the issue of flux control in the sulfur assimilation pathway of yeast. Yeast use this pathway to incorporate inorganic sulfur into the free amino acid pool (see Fig. 1 for a schematic illustration of the sulfur assimilation pathway in yeast). Like all metabolic pathways, flux through the sulfur assimilation pathway is tightly regulated. Indeed, many investigations over the course of the past four decades have uncovered multiple levels of transcriptional, translational, and post-translational control that assiduously sculpt flux through the sulfur assimilation's constituent pathways. Whereas these previous studies provided details of how flux is controlled within limited segments of the sulfur assimilation pathway, a comprehensive picture of how well proteomic changes correlated with changes in sulfur metabolite pools and the direction of assimilation flux remained missing. Lafaye et al. attempted to fill this gap by correlating proteomic, metabolic, and regulatory data for the methionine transmethylation (Met → Hcy), remethylation (Hcy → Met), transsulfuration (Hcy → Cys) and glutathione synthesis (Cys → GSH) pathways of yeast.

Description of yeast study

For their study, Lafaye et al. (2005a) used yeast cells (*Saccharomyces cerevisiae*) grown in a modified minimum medium. Control medium contained 1 mM sulfate and 20 mM ammonium. Treatments included supplementation with 500 μM methionine, reduction of sulfate to 50 μM, and treatment with 50 μM Cd^{2+} to induce the glutathione (GSH) synthesis pathway. A new method based on liquid chromatography-electrospray-mass spectrometry was used for the quantitative analysis of N-containing metabolites (Lafaye et al., 2005b). By using an internal reference extract from yeast grown for 25 generations with 20 mM [^{15}N]ammonium sulfate instead of [^{14}N]ammonium sulfate, the $^{14}N/^{15}N$ ratio could be measured by mass spectrometric analysis for each metabolite and the absolute levels of ^{14}N metabolites deduced by comparison with calibration curves.

The study revealed that overall sulfur metabolite concentrations varied greatly and were dependent upon growth conditions. However, within a given metabolic pathway, the concentration of intermediate metabolites was correlated with changes in flux. What the authors found to be surprising, however, was that the correlation of data from proteome and metabolite profiling was not always consistent. Indeed, both positive and negative correlations were observed depending on the evaluated growth conditions. For example, when yeast were treated with cadmium, an increase in the enzymes involved in the Cys → GSH path-

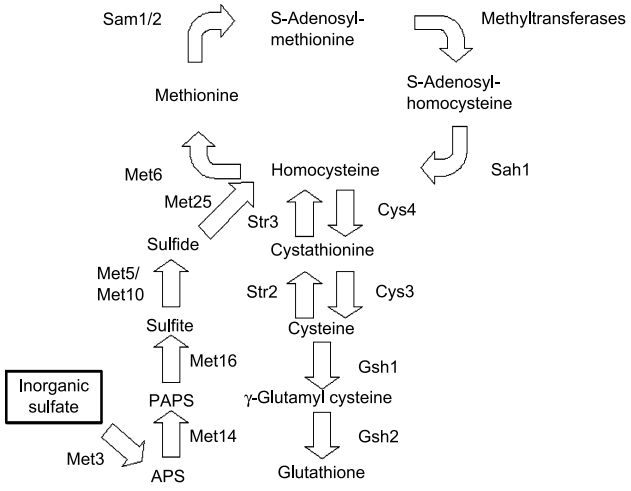

Fig. 1. A diagram of the pathways involved in yeast sulfur metabolism. Yeast are able to synthesize all of their sulfur amino acids *de novo* from mineral sources of sulfur (inorganic sulfate, indicated in box). APS, adenosyl-5′-phosphosulfate; Cys3, cystathionine γ-lyase; Cys4, cystathionine β-synthase; Gsh1, γ-glutamylcysteine synthetase; Gsh2, glutathione synthetase; Met3, ATP-sulfurylase; Met5/Met10, sulfite reductase; Met6, methionine synthase; Met14, APS kinase; Met16, PAPS reductase; Met25, homocysteine synthase; PAPS, 3′-phosphoadenosyl-5′-phosphosulfate; Sah1, S-adenosylhomocysteine hydrolase; Sam1/2, S-adenosylmethionine synthetases; Str2, cystathionine γ-synthase; Str3, cystathionine β-lyase

way was associated with an increase in GSH production (a positive correlation). Cadium also increased flux through the Cys → GSH pathway by redirecting sulfur utilization away from protein synthesis and the methionine cycle towards the production of glutathione. On the other hand, methionine supplementation resulted in repressed expression of all enzymes of sulfur metabolism. Nevertheless, the sulfur metabolite levels and flux to GSH were all strongly increased by methionine supplementation (a negative correlation). Conversely, under conditions of sulfur starvation, there was a general drop in all sulfur metabolite pools, particularly GSH, despite little or no change in expression of enzymes in the pathways (a negative correlation).

Apparently these results were "surprising" because "it is usually considered that induction of enzymes in a metabolic pathway indicates an increased flux in the pathway" when interpreting proteomic and gene expression data. Given the results of their metabolic analyses, the authors argued that proteomic (as well as gene expression) data alone are insufficient pieces of information to adequately predict the flux of metabolic pathways.

In light of this paper's findings, researchers seeking to model whole cell/organism metabolism should give further reconsideration to some aspects of the relationships connecting enzyme concentration, enzyme activity, substrate concentration, and metabolic flux. In the past century, these concepts might have been fundamental to any study of metabolism and not been seen as "novel and unanticipated." This recent yeast metabolomic study, however, illustrates the need to not discard fundamental concepts of nutrition and enzyme kinetics as we embrace the newer and powerful methods being developed in the fields of genomics, proteomics, and metabolomics.

Fundamental concepts of metabolism

1. How enzyme levels (or activity) are regulated in response to an increase in substrate or precursor availability is likely to be different for enzymes whose primary purpose is catabolism or removal of substrate than for enzymes whose primary purpose is synthesis of an essential metabolite. We might expect positive correlation of substrate or precursor and metabolite concentrations and enzyme levels for pathways involved in removal of excess compounds (e.g., cysteine dioxygenase in animals). On the other hand, we might expect a negative correlation between substrate or precursor and metabolite concentrations and enzyme levels for pathways involved in synthesis of essential compounds whose synthesis must be conserved when substrate supply is limiting (e.g., glutamate-cysteine ligase).
2. Gene expression, proteomic, and metabolomic profiles will not replace the need for at least a simple understanding of enzyme kinetics, which in the previous century were fundamental to any study of metabolism. As clearly shown by Heinrich and Rapoport (1974), the capacity of an enzyme to mediate the control of flux through a metabolic pathway is partially linked to its kinetic responses to changes in metabolite concentrations as well as its capacity to influence the metabolite concentrations in the pathway. In mathematically describing the simple kinetics of a single substrate enzyme, the reaction rate or velocity (V) can be expressed as the product of the catalytic rate constant (k) of the enzyme times the enzyme concentration [E] times the substrate concentration [S].

$$V = k[E][S]$$

An increase in either k, E or S will increase flux through the reaction, unless the E is saturated with S – something that is commonly approached in *in vitro* assays of enzyme activity but rarely approximated *in vivo*. Many enzymes involved in metabolism operate below or near their K_m values. These enzymes are not saturated with substrate under physiological conditions. Thus, any change in substrate concentration [S] will

result in a corresponding change in the conversion of substrate to product (V).

Likewise, changes in the reaction rate (k) will impact metabolism. Levels of expressed proteins, as observed on western blots or protein arrays, are not always identical with levels of functional protein or of enzyme activity. Changes in the rate constants of many enzymes are known to be regulated via allosteric effectors, post-translational modification, association with regulatory subunits, and competitive inhibitors.

3. Although intrinsic kinetic properties play an important role in determining the capacity of an enzyme to influence the rate of flux within the immediate vicinity of its position in a metabolic pathway, the fractional change in total pathway flux that can be produced by altering the enzyme's level of expression is a more complex systems property. Metabolic control analysis has revealed that the total flux change, δJ, that occurs in response to a change in the amount of enzyme, δE, can be represented by the equation:

$$\delta J = C \cdot \delta E$$

where C is the flux control coefficient, a value that can vary from 0 (changes in active enzyme level have no effect on the change in total flux) to 1 (changes in active enzyme level have an effect on the total change in flux that is perfect unity). The summation of all the control coefficients for enzymes of a given metabolic system is equal to 1. The determination of flux control coefficients must therefore be empirically determined *in vivo* within the context of the metabolic pathway of interest. Most enzymes studied thus far have flux control coefficients that are far less than one, a reflection of the fact that the control of total pathway flux is distributed amongst multiple enzymes. Estimations of C based on comparisons of the *in vitro* kinetics of the enzymes that form the metabolic pathway can be misleading. For example, phosphofructokinase classically has been considered to be the flux regulating (i.e., rate-limiting) step in yeast on the basis of its large disequilibrium ratio *in vitro*. Indeed, many biochemistry texts still cite this argument. Nevertheless, increasing the expression of this enzyme ~3.5-fold *in vivo* had no significant effect on the rate of glycolysis in yeast (Heinish, 1986). Caution must therefore be used when attempting to extrapolate changes in total pathway flux solely on the basis of a change in the concentration of a single constitutive enzyme. For further reading on the use of metabolic control analysis in the quantitative modeling of whole cell metabolism, consult "Understanding the Control of Metabolism" by Fell (1997).

4. Nutrition matters. The availability of substrate, from the diet or from stores in various tissues, cannot be ignored when considering the metabolic reactions that occur in the cells of higher animals and humans. Dietary composition and nutrient bioavailability, nutrient absorption, nutrient transport, nutrient storage and release, and the regulation of these processes play important roles in determining what substrates are available to a particular cell and in determining what metabolites are removed from the cell for use by other tissues or for excretion. As observed in the yeast cells treated with cadmium, an increase in the utilization of a substrate for one pathway, without compensatory changes in nutrient uptake or export, will result in a decrease in that substrate's availability for a competing pathway. This information is not directly provided by proteomic, transcriptome, or metabolic profiles but is nonetheless critical for a full understanding of cellular metabolism.

Mammalian enzymes of sulfur amino acid metabolism as illustrations of these concepts

Let us apply these insights or principles to several enzymes of mammalian sulfur amino acid metabolism that have been studied extensively. Figure 2 shows a schematic

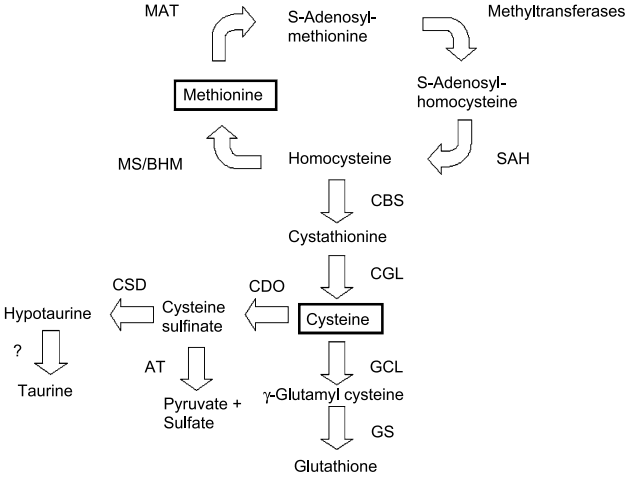

Fig. 2. A diagram of the quantitatively important pathways of sulfur utilization in mammals. Note that unlike yeast, mammals are unable to assimilate inorganic sulfur into the sulfur amino acid pool and thus are dependent upon the dietary intake of methionine/cysteine to meet their sulfur requirements. Mammals also possess an enzymatic pathway for the sulfoxidation of cysteine (CYS → Taurine). AT, cysteinesulfinate aminotransferase; BHM, betaine-homocysteine methyltransferase; CBS, cystathionine β-synthase; CDO, cysteine dioxygenase; CGL, cystathionine γ-lyase; CSD, cysteinesulfinate decarboxylase; GCL, glutamate-cysteine ligase; GS, glutathione synthetase; MAT, methionine adenosyltransferase; MS, methionine synthase; SAH, S-adenosylhomocysteine hydrolase; ?, unknown enzyme

representation of mammalian sulfur amino acid metabolism, which uniquely differs from that of yeast in that mammals do not have a sulfate assimilation pathway but are dependent upon methionine and cyst(e)ine in dietary protein as the initial source of substrate for metabolism by the various pathways (Stipanuk, 2004).

Cysteine dioxygenase (CDO) catalyzes the oxidation of cysteine to cysteinesulfinate, which is the first step in the degradation of excess cysteine to sulfate and pyruvate. CDO is upregulated when cysteine levels are high (e.g., a high protein diet), and the combination of high enzyme and high substrate concentration facilitate the rapid removal of excess cysteine by converting it to cysteinesulfinate and, hence, pyruvate and inorganic sulfate via the cysteinesulfinate transamination pathway (Bella et al., 1999a, b). In response to an elevation of plasma sulfate levels, sulfate excretion in the urine is increased. Thus, in this example, enzyme levels, substrate levels, and product levels are expected to be positively coordinated. Because cysteine concentrations are generally below the K_m of CDO (<0.1 mmol/kg cysteine in liver vs. a K_m of 0.45 mM cysteine for CDO) the flux at this step in the mammalian sulfur pathway should be dependent on both S and E concentrations (Bagley et al., 1995). The dependence of Cys $\rightarrow$ SO_4 flux has been clearly demonstrated in experiments in which the effects of [E] and [S] could be separated (Stipanuk, et al., 1992; Bagley and Stipanuk 1994, 1995; Bella et al., 1996). Cells with different levels of CDO were obtained by isolating hepatocytes from rats fed high or low protein diets that altered hepatic CDO levels or by culturing cells in medium containing low or high sulfur amino acid concentrations to alter the CDO levels. These cells were then incubated or cultured in medium with different concentrations of cysteine to vary [S] independently of [E]. These experiments demonstrated that (1) at a fixed [S], cells with higher CDO levels converted more substrate to product and (2) at a fixed [E] cells incubated with a higher substrate concentration converted more substrate to product. Thus, both [E] and [S] have been shown to modify flux through the Cys $\rightarrow$ SO_4 pathway in hepatocytes.

Cysteinesulfinate decarboxylase (CSD) acts to convert some of the cysteinesulfinate that is produced by CDO to hypotaurine (the precursor of taurine, an essential metabolite). The K_m of CSD for cysteinesulfinate is ~0.1 mM, far above the physiological levels of cysteinesulfinate, which is rapidly removed by transamination via the Cys $\rightarrow$ SO_4 pathway (Guion-Rain and Chatagner, 1972). Hepatic CSD is much less robustly regulated than is CDO, but it decreases when cysteine and cysteinesulfinate availability are increased (e.g., high protein diet). Nevertheless, taurine formation is increased in hepatocytes from rats fed a high protein diet (which have decreased CSD expression) because the increase in concentration of cysteinesulfinate [S], which is a consequence of either or both higher CDO activity and higher cysteine concentration, has a larger effect on overall velocity of the CSD reaction than does the decrease in CSD enzyme concentration. In studies with isolated hepatocytes (Bagley and Stipanuk, 1994, 1995), the effect of CSD concentration on flux through the individual reaction was demonstrated by incubating cells with a fixed concentration of cysteinesulfinate; cells from rats fed high protein diets had a lower [CSD] and produced less hypotaurine/taurine. However, when these same hepatocytes were incubated with a fixed concentration of cysteine to evaluate the overall flux of Cys $\rightarrow$ Hypotaurine/Taurine, the over-riding effect of [CDO] became apparent: hepatocytes from rats fed high protein diets actually produced more hypotaurine/taurine when cysteine was used as the initial substrate, reflecting the higher concentration of cysteinesulfinate that stemmed from the higher CDO activity in these cells.

Glutamate-cysteine ligase (GCL) catalyzes the first step in the pathway for GSH synthesis from cysteine (Cys $\rightarrow$ GSH pathway). Regulation of GCL activity is complex, with regulation involving changes in the concentration of both the catalytic and modifier subunits of the enzyme and by changes in the activity state of both the holoenzyme and the catalytic subunit alone. The activity of GCL is downregulated in response to an increase in cysteine availability (e.g., high protein diet). Despite this decrease in enzyme concentration, flux through the GSH production pathway increases because of the increase in substrate (cysteine) concentration. The K_m of glutamate-cysteine ligase for cysteine is ~0.1 mM, near the upper end of the physiological range of hepatic cysteine concentrations, but low enough that changes in [S] still have a marked effect on V (Chen et al., 2005). In studies with intact rats, the increase in [S] has a bigger overall effect than the decrease in [E] in determining the V of the reaction or tissue GSH levels (Bella et al., 1999a, b; Lee et al., 2004). Feeding a high protein diet resulted in a decrease in GCL activity but an increase in cysteine concentration and an increase in GSH production despite the decrease in GCL activity. Thus, as for CSD, we see that, while both the change in [S] and [E] concentration have some effect, the change in [S] has a greater effect than the change in [E] under typical physiological conditions.

GCL is a good enzyme to use as an example of how changes in activity state can influence velocity indepen-

dently of [*E*]. GCL activity is extensively regulated by association of the catalytic subunit (GCLc) with the modifier subunit (GCLm), which markedly increases the turnover or k_{cat} of GCLc but does not change its K_m for cysteine. Our work has shown that cysteine deprivation is associated with an increase in the ratio of GCLm to GCLc, and a consequent increase in GCLc activity state, in liver of intact rats and also in human hepatoma cells in culture (Lee et al., 2005). The increase in k_{cat} (i.e., due to holoenzyme formation) complements the increase in [GCLc] in rats or cells under conditions of sulfur amino acid limitation. Both the increase in [*E*] and the increase in reaction rate (*k*) help to off-set the effects of low cysteine concentration [*S*] and preserve synthesis of GSH in the face of substrate limitation. In this particular case, however, the effect of [*S*] dominates over the effect of *k*[*E*] and the overall rate of GSH production is lower than in animals or cells that receive adequate amounts of exogenous sulfur amino acids. As in the case of CSD, we see a negative association between *k*[*E*] and product formation under physiological conditions in which [*S*], [*E*], and *k* are all changing in response to sulfur amino acid supply.

Another good example to consider is cystathionine β-synthase (CBS). CBS is a highly regulated enzyme that sits at the branch point for remethylation of homocysteine, S-adenosylhomocysteine synthesis from homocysteine, and transsulfuration via cystathionine β-synthase and cystathionine γ-lyase (Stipanuk, 2004). Regulation of CBS by *S*-adenosylmethionine (SAM) results in increased cystathionine β-synthase activity when methionine is present in excess (resulting in high SAM) and homocysteine needs to be removed from the methionine cycle by converting it to cysteine (Stipanuk and Benevenga, 1977; Taoka et al., 1999; Janosik et al., 2001). A high level of SAM also inhibits *de novo* methyl group synthesis (and, hence, the Hcy → Met flux) and favors methyltransferase activities (and, hence, the Met → Hcy flux) such that SAM promotes an increase in both [Hcy] and CBS activity state. In this example, precursor and metabolite levels in the Met → Hcy → Cys pathway will be high and positively associated with CBS activity, although CBS protein levels may not change.

This small sample of illustrations taken from sulfur amino acid metabolic pathways adequately demonstrates that flux through any reaction can be affected by changes in *k*, [*S*] or [*E*], and sometimes by changes in all three at the same time. Metabolic flux is exquisitely regulated and multiple mechanisms ensure that homeostasis is maintained despite large fluctuations in diet or physiologic state. Although the independent effect of each change is easy to predict for many enzymes and can be easily demonstrated experimentally, it is generally more difficult to predict what changes will occur under physiological conditions where changes in rate constant, [*E*], or [*S*] are interdependently regulated. For example, a change in [*S*] may produce a change in [*E*] or *k*, and a change in *k* or [*E*] will bring about a change in [*S*] over time (as in the example of CBS). Because regulation is not simply linear – with mRNA level determining protein levels which in turn determine metabolic flux – we should remember that changes in an enzyme or a metabolite concentration is not sufficient information for a sound prediction of overall metabolic flux.

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