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Vitamin B₆ Nutritional Status and Cellular Availability of Pyridoxal 5'-Phosphate Govern the Function of the Transsulfuration Pathway's Canonical Reactions and Hydrogen Sulfide Production via Side Reactions

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Abstract

The transsulfuration pathway (TS) acts in sulfur amino acid metabolism by contributing to the regulation of cellular homocysteine, cysteine production, and the generation of H₂S for signaling functions. Regulation of TS pathway kinetics involves stimulation of cystathionine β-synthase (CBS) by S-adenosylmethionine (SAM) and oxidants such as H₂O₂, and by Michaelis-Menten principles whereby substrate concentrations affect reaction rates. Although pyridoxal phosphate (PLP) serves as coenzyme for both CBS and cystathionine γ-lyase (CSE), CSE exhibits much greater loss of activity than CBS during PLP insufficiency. Thus, cellular and plasma cystathionine concentrations increase in B₆ deficiency mainly due to the bottleneck caused by reduced CSE activity. Because of the increase in cystathionine, the canonical production of cysteine (homocysteine→cystathionine→cysteine) is largely maintained even during vitamin B₆ deficiency. Typical whole body transsulfuration flux in humans is 3-7 μmol/h per kg body weight.

The *in vivo* kinetics of H₂S production via side reactions of CBS and CSE in humans are unknown but they have been reported for cultured HepG2 cells. In these studies, cells exhibit a pronounced reduction in H₂S production capacity and rates of lantionine and homolantionine synthesis in deficiency. In humans, plasma concentrations of lantionine and homolantionine exhibit little or

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no mean change due to 4-wk vitamin B₆ restriction, nor do they respond to pyridoxine supplementation of subjects in chronically low-vitamin B₆ status. Wide individual variation in responses of the H₂S biomarkers to such perturbations of human vitamin B₆ status suggests that the resulting modulation of H₂S production may have physiological consequences in a subset of people.

Keywords

one-carbon metabolism; transsulfuration; cystathionine β -synthase; cystathionine γ -lyase; lanthionine; homolanthionine; hydrogen sulfide; vitamin B₆

1. Background

Two pyridoxal 5'-phosphate (PLP)-dependent enzymes, cystathionine β -synthase (CBS) and cystathionine γ -lyase (CSE), constitute the transsulfuration pathway. The canonical reactions of the transsulfuration pathway (homocysteine + serine $\rightarrow$ cystathionine $\rightarrow$ α -ketobutyrate + NH₃ + cysteine) provide a means of homocysteine catabolism and regulation, while also contributing to the supply of cysteine needed for the synthesis of proteins, glutathione and taurine. In addition to the canonical reactions, side reactions of CBS and CSE generate the physiologically active signaling molecule H₂S. Consequently, the activity of each enzyme, the profiles of substrates and products, and the kinetics of the canonical and side reactions, are ultimately dependent on vitamin B₆ nutritional status and the supply of intracellular PLP.

Recent data from the Centers for Disease Control and Prevention [1] suggests that 10-12% of the US population is below the 20 nmol/L threshold level of plasma PLP considered to indicate inadequate vitamin B₆ status, with at least another 10-15% of the population in the marginal range of 20-30 nmol/L PLP inadequacy. The importance of these observations is that low plasma PLP is associated with higher rates of cardiovascular disease and stroke [2-7]. Whether the low apparent B₆ status is due to dietary insufficiency or an inflammatory response, low vitamin B₆ status can lead to either systemic or tissue-specific insufficiency of cellular PLP [8, 9]. Alterations in metabolite patterns occurring during experimentally induced vitamin B₆ insufficiency [10-16] illustrates the potential for changes in PLP-dependent pathways (including transsulfuration) which could contribute to the pathogenesis. In this mini-review, we will (a) review the basic literature regarding the coenzymatic role of PLP for the transsulfuration enzymes, (b) evaluate the consequences of vitamin B₆ insufficiency on CBS and CSE enzymes, and (c) assess the metabolic consequences on the canonical reactions and consider the initial evidence regarding effects on H₂S production.

2. PLP dependence of transsulfuration enzymes

The involvement of PLP in transsulfuration has been recognized since the initial characterization of the pathway. For example, Finkelstein and Chalmers compared vitamin B₆-adequate versus B₆-deficient rats and reported reduced activity of hepatic CBS and CSE by 57% and 85%, respectively. *In vitro* addition of PLP partially restored the activity of each, which confirmed the association with PLP insufficiency. Later studies confirmed and extended these observations [17, 18].

2.1. Cystathionine β -synthase

The dependence of CBS on vitamin B₆ can be inferred by the pyridoxine responsiveness of some forms of homocystinuria [19]. Additionally, observations from Finkelstein and Chalmers showed partial restoration of CBS activity in liver of vitamin B₆-deficient rats by exogenous PLP [20] and others reported evidence of similar restoration using purified rat CBS [21]. Kraus et al. [22] directly demonstrated a PLP requirement by observing a loss of activity of purified holo-CBS from human liver when PLP was removed by hydroxylamine treatment. Full enzymatic activity of the resulting apo-CBS could be restored by preincubation with PLP and/or assay in the presence of PLP. These studies provided evidence that holo-CBS existed as a dimer, although PLP-binding stoichiometry was not directly determined.

Later studies established that the functional CBS enzyme exists as a tetramer (reviewed by Kruger [23]), with 4 potential binding sites for PLP. Studies by Taoka et al. from the Banerjee group [24] examined the roles of PLP and heme cofactors in human CBS activity. Treatment of the tetrameric holo-enzyme with hydroxylamine for 24 hr yielded removal of two PLP molecules per tetramer, with loss of another two PLPs following longer treatment with hydroxylamine. These findings suggested the existence of non-equivalent PLP-binding sites differing in binding affinity. Reconstitution with PLP yielded only 34% recovery of catalytic activity following removal of only two of the PLP molecules, with no recovery of activity when all four PLPs had been removed [24]. Thus, the four PLP coenzymes per tetramer of human CBS exhibit unequal binding affinities, all of which are very tightly bound, and appear to be necessary for enzyme stability. This loss of CBS activity following experimental PLP removal was further investigated by Kery et al., who showed a concurrent loss of secondary structure [25]. These researchers [25] reported a K_d of 0.7 μ M for PLP for human CBS, which was consistent with the value of 1 μ M reported earlier for rat CBS [26]. These values, along with the findings of Taoka et al., indicate tight binding of PLP by CBS.

2.2. Cystathionine γ -lyase

Oh and Churchich [27] conducted spectral evaluations of the binding of PLP to the apo-enzyme form of purified rat liver cystathionase (CSE). Assessment of the quenching of protein tryptophan fluorescence during titration with various concentrations of PLP allowed observation of the tight binding of PLP with an association coefficient (K_a) of $7 \times 10^5 \text{ M}^{-1}$ (equivalent to a dissociation constant, K_d , of $1.4 \times 10^{-6} \text{ M}$), which appears to be the only available quantitative estimate of the PLP-binding affinity of CSE. They also reported evidence of one PLP bound per protein monomer, which underwent further association to form a homotetramer with four bound PLP molecules. PLP binding to the apo-enzyme yielded 95% restoration of activity. It is noteworthy that this K_d for PLP binding by human CSE is similar to those reported for CBS [24, 26]. Thus, the fact the CSE is far more sensitive to loss of activity during vitamin B₆ insufficiency cannot be explained solely on the basis of dissociation constants or affinity constants.

Further studies of human CSE by Kraus et al. [28] by molecular structural modeling of apo and holo-enzyme forms. These studies indicated that the active enzyme had two PLP-binding active sites per heterotetramer comprised essentially of two similar dimers (A+B

subunits and C+D subunits). Modeling of the active sites indicated that bound PLP was stabilized as the Schiff base in the holo-enzyme by hydrogen bonding in addition to π - π interactions of the aromatic PLP with a neighboring tyrosine. Kraus et al., also examined the activity of various mutant forms of human CSE that exhibited varying extents of reduced catalytic activity, although no affinity constants were determined in this study. Zhu et al. [29] examined the affinity of PLP for wild type CSE, polymorphic variants and mutant forms of the enzyme by determining the rate of reconstitution of the apo-enzymes with PLP. The reconstitution of the wild type to restore activity was instantaneous.

3. *In vivo* observations: Vitamin B₆-dependence of the canonical reactions of the transsulfuration pathway

Biochemical studies of the types described do not appear to answer the question of whether PLP can be retained by CBS and CSE during conditions of very low concentrations of free cellular PLP that would occur in vitamin B₆ deficiency. Furthermore, existing data do not allow prediction of the position of CBS and CSE in the hierarchy of PLP binding protein affinities. Such data would be needed to allow prediction of the consequences of varying degrees of cellular PLP depletion in vitamin B₆ insufficiency.

3.1. Animal studies

Several rat studies have provided insights into the effects of vitamin B₆ nutritional adequacy on transsulfuration function [17, 18, 30, 31]. Stabler et al., [30] employed a pyridoxine dose-response rat feeding study to examine the relationship between vitamin B₆ intake, liver PLP concentration, and the serum concentration of relevant metabolites including homocysteine, cystathionine and aminobutyrate. Although homocysteine was only weakly related to dietary vitamin B₆ intake, deficiency yielded reduced concentration of aminobutyrate and markedly increased cystathionine. These results suggested that there was little effect of vitamin B₆ deficiency on the function of CBS but that CSE function was impaired.

Martinez et al. [17] examined the *in vivo* kinetics of one-carbon metabolism and transsulfuration reactions in vitamin B₆ adequate and severely deficient rats using enzyme activity assays and a bolus injection protocol of [²H₃]serine and [1-¹³C]methionine tracers. Deficiency yielded a statistically insignificant 7.4% reduction of hepatic CBS activity (CSE activity was not determined). However, based on the ratios of plasma [²H₃]cysteine / [²H₃]cystathionine, which reflected the CSE step in transsulfuration, we inferred that the CSE flux of whole body transsulfuration in vitamin B₆ deficiency was only 8.8% of the flux detected in vitamin B₆ replete rats. This suggests that CSE, determined by the conversion of cystathionine to cysteine, was significantly affected in the deficiency state.

The sensitivity of CBS and CSE to graded levels of vitamin B₆ status from adequacy through deficiency was examined in a rat study by the Lima et al. [18]. The variation in dietary vitamin B₆ yielded a gradation of liver PLP concentrations. Liver CBS activity was unrelated to liver PLP concentration, while CSE activity (with and without PLP) showed a linear relation with liver PLP. Liver cysteine concentrations were only modestly affected by vitamin B₆ insufficiency, whereas liver cystathionine (the transsulfuration intermediate)

increased by a factor of ~4. These findings indicate that a metabolic bottleneck exists at the level of CSE in the transsulfuration pathway in vitamin B₆ insufficiency, but that there is sufficient cysteine production to prevent a large reduction of liver cysteine concentration. Marked increases in liver cystathionine concentration coincided with the status of marginal vitamin B₆ deficiency in which CSE activity was reduced approximately 50%, relative to vitamin B₆-adequacy.

Further evaluation of CSE kinetics clarified the mechanism by which liver cysteine pools experience little effect of vitamin B₆ deficiency [18]. Crude rat liver CSE exhibited a K_m for cystathionine of 2.1 ± 0.2 mM. However, hepatic cystathionine ranged from 20-90 nmol/g (~20-90 μ M) and, thus, liver cystathionine concentration was far lower than the K_m at which CSE flux would be a linear function of cystathionine concentration. Consequently, the loss of CSE activity (i.e., reduction of its V_{max}) due to PLP insufficiency would at least partially be compensated by the corresponding increase in cystathionine concentration. In other words, the $([S]/K_m) \times V_{max}$ product (reflecting CSE rate in this first-order kinetic region of cystathionine concentration) would remain approximately constant at many levels of vitamin B₆ deficiency to maintain cysteine production via a Michaelis-Menten kinetic effect [18].

Miller, Selhub and colleagues [31] conducted a similar rat feeding study in which they assessed transsulfuration function using a methionine load test in which a bolus dose of methionine is administered to assess the function of homocysteine metabolism. Vitamin B₆ deficiency yielded a modest response of plasma homocysteine following the methionine bolus dose, which indicated that remethylation and CBS were not seriously altered by the degree of B₆ insufficiency. However, they did not measure liver or plasma cystathionine, so the effect on the CSE step in transsulfuration was not evaluated.

3.2. Human studies

Initial studies by Park and Linkswiler [10, 32] using controlled dietary restriction and repletion of vitamin B₆ in human subjects indicted a large response of cystathionine, providing further evidence of CSE restriction caused by B₆ deficiency. These findings were extended by Leklem et al. in further investigations using the methionine load technique monitoring cystathionine [33].

The metabolic effects of vitamin B₆ insufficiency in human subjects have been extensively characterized using stable isotopic kinetics approaches. Controlled 28-day dietary B₆ restriction protocols had no effect on cysteine flux [12] or overall transsulfuration flux [34]. The observed cysteine flux (26.1 ± 1.2 μ mol/(kg $\times$ hr) before and 26.5 ± 0.9 μ mol/(kg $\times$ hr) after B₆ restriction) indicated that cysteine turnover was not altered in moderate B₆ insufficiency, nor was transsulfuration flux (5.2 ± 0.3 μ mol/(kg $\times$ hr) before and 5.0 ± 0.3 μ mol/(kg $\times$ hr) after restriction). These data are consistent with other results (e.g., [35]) that showed that the flux through the CBS reaction was not altered by vitamin B₆ restriction (fractional synthesis rate for cystathionine: $15.7 \pm 2.4\%$ /hr before restriction and $16.1 \pm 1.3\%$ /hr after restriction).

It should also be noted that the data from these studies indicate that the human transsulfuration pathway accounts for approximately 20% of cysteine flux. These findings

contrast with the conclusion of Mosharov et al. [36] who reported that the transsulfuration pathway accounts for approximately half of the cysteine needed for the glutathione pool in cultured human hepatoma cells (HepG2). Our findings suggest that cysteine synthesis from transsulfuration plays a somewhat lesser role in humans for supporting cysteine demands such as glutathione synthesis than predicted by their cultured cell model. Irrespective of this quantitative difference, the acceleration of transsulfuration pathway in oxidative conditions observed by Mosharov et al. [36] has clear relevance to human metabolism. The evaluation of erythrocyte glutathione synthesis using a [$^{13}\text{C}_2$]glycine infusion tracer kinetic protocol, showed modest reduction in the mean fractional synthesis rates (mean fractional synthesis rates: $54 \pm 5\%/d$ before vitamin B₆ restriction and $43 \pm 4\%/d$ after 28-d vitamin B₆ restriction, $P = 0.10$), although individual responses varied considerably [37]. Severe vitamin B₆ deficiency has been associated with oxidative stress in animal studies; thus, similar oxidative conditions in some individuals after dietary vitamin B₆ restriction may account for some of the inter-subject variability observed in the kinetic measures of cystathionine synthesis rate, transsulfuration flux and glutathione synthesis rate [12, 34, 37].

Complementing the studies of metabolite patterns in vitamin B₆ restriction in rats [18, 30], targeted plasma metabolite profile analysis and global metabolomic analysis in humans have extended the understanding of metabolic effects of vitamin B₆ insufficiency [15, 16]. These studies confirmed the pronounced elevation of cystathionine and identified other more subtle effects on glycine, other amino acids and organic acids due to vitamin B₆ restriction. A recent study of oral contraceptive using women with chronically low plasma PLP, showed no change in plasma cystathionine concentration or its fractional synthesis rate (reflecting CBS flux) after vitamin B₆ supplementation [38, 39].

3.3. Mathematical modeling of transsulfuration

The development of mathematical models based on biochemical parameters governing the relevant enzymatic reactions, regulatory mechanisms and transport processes can provide useful tools for predicting metabolic effects of nutritional, genetic and physiological variables that sometimes cannot be directly determined by in-vivo experimentation. Several models have been developed that include aspects of the transsulfuration pathway along with other components of one-carbon metabolism [40-44]. The comprehensive mathematical model of one-carbon metabolism including the transsulfuration pathway [44] adapted to include glutathione synthesis was used to evaluate the effects of moderate vitamin B₆ deficiency, which is more relevant to the level of vitamin B₆ insufficiency observed in humans [43]. This model predicted the fluxes and changes in metabolite concentrations attributable to various degrees of inactivation of PLP-dependent enzymes (glycine cleavage system, serine hydroxymethyltransferase, CBS and CSE). This model also predicted the observed increase in liver total glutathione associated with the degree of vitamin B₆ insufficiency [18]. The model also predicted metabolite changes associated with vitamin B₆ restriction in cultured cells [45].

4. Influence of Vitamin B₆ Status on Hydrogen Sulfide Production by CBS and CSE

The physiological roles of H₂S as a vasorelaxant, neuromodulator, signaling molecule and possible antioxidant have attracted extensive interest. Side reactions of the transsulfuration enzymes CBS and CSE are major contributors to H₂S production in mammals [46-49]. In addition, the mechanisms by which CBS and CSE produce H₂S have been characterized quite thoroughly [23, 24, 50-53]. As with the canonical reactions of these enzymes, PLP plays an obligatory role as coenzyme in these H₂S-producing side reactions [51], with the relative rates dependent primarily on substrate concentrations and the respective K_m and V_{max} values. In physiological conditions the CBS-catalyzed β-replacement reaction (*homocysteine + cysteine* → *H₂S + cystathionine*) [54, 55] is favored based on kinetic simulation data. The production of cystathionine from this reaction is suspected to account for 5-20% of total cystathionine [55, 57]. A less efficient, yet quantifiable, route of H₂S generation (*2 cysteine* → *H₂S + lanthionine*) is also catalyzed by CBS. Currently unpublished observations of plasma lanthionine concentrations by the authors and the Kozich group suggest that the CBS-catalyzed condensation of two molecules of cysteine provides greater H₂S production than the condensation of cysteine and homocysteine to produce cystathionine *in vivo*. This is in contrast to kinetic simulation experiments. In terms of H₂S equivalent production, the concentration of cystathionine originating from cysteine + homocysteine is lower than lanthionine concentration. - In normal metabolic conditions (i.e., without elevation of homocysteine), lesser amounts of H₂S are produced by the CSE-catalyzed reaction: *2 homocysteine* → *H₂S + homolanthionine*. This reaction is sensitive to elevations in homocysteine, and can exceed CBS-catalyzed production of H₂S in conditions of hyperhomocysteinemia or homocystinuria. Because of the concurrent production of these thioether amino acids and their absence in other metabolic reactions in the transsulfuration pathway, lanthionine and homolanthionine may be useful as indicators of the relative magnitude of H₂S production catalyzed by CBS and CSE [58]. The potential usage of lanthionine and homolanthionine as biomarkers reflecting H₂S production will require additional validation and determination of the relative turnover rates and distribution of H₂S, lanthionine, and homolanthionine.

Because the activity of CSE but not CBS is highly sensitive to the cellular supply of PLP, we infer that H₂S production by CSE would be most extensively affected by vitamin B₆ insufficiency. As the CSE-catalyzed process is of secondary significance in terms of PLP-dependent H₂S production at normal cellular concentrations of homocysteine, we postulate that moderate B₆ deficiency in humans would only mildly affect H₂S production. We have examined the relative concentrations of lanthionine and homolanthionine in plasma of healthy adults while in adequate vitamin B₆ status and following 28-d dietary B₆ restriction. As expected, the plasma concentration of lanthionine exceeded that of homolanthionine. The absolute concentrations and response to B₆ restriction varied considerably among individuals human participants (to be reported elsewhere). Further work is needed to fully assess the relationships among vitamin B₆ status, H₂S production in health and disease, and the physiological effects of altered H₂S production.

DeRatt et al. examined the effect of intracellular PLP in cultured HepG2 cells grown in varying concentrations of pyridoxal [59]. Severe vitamin B₆ deficiency decreased the intracellular and extracellular concentrations of both lanthionine and homolanthionine and their rates of synthesis. The H₂S production capacity of in-vitro lysates of these cells confirmed that H₂S production is impaired by vitamin B₆ inadequacy and supported the validity of lanthionine and homolanthionine as surrogate markers of H₂S production.

A schematic model of the transsulfuration pathway (Figure 1) illustrates the relative magnitude of the major reactions catalyzed by CBS and CSE in vitamin B₆ replete and deficient conditions.

5. Summary and Conclusions

In healthy human beings, the function of the transsulfuration pathway is maintained by a homeostatic mechanism involving CSE by which cysteine production is largely maintained even in PLP insufficiency. CBS is very resilient, maintaining much of its activity in all but severe states of vitamin B₆ deficiency. Although this review has focused primarily on nutritional modulation of transsulfuration, effects of vitamin B₆ insufficiency are predicted to be accentuated if the deficiency occurred in an individual with loss-of-function mutations, especially in the CBS gene. Milder CSE mutations and a relatively common CSE polymorphism also may influence the homeostatic regulation of transsulfuration. Although the assessment of *in vivo* kinetics of transsulfuration and related pathways requires cumbersome tracer protocols, further use of metabolite profiling methods and metabolomics will be useful in further assessment of nutritional modulation of these processes.

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Abbreviations

PLP	pyridoxal 5'-phosphate
CBS	cystathionine β-synthase
CSE	cystathionine γ-lyase
H ₂ S	hydrogen sulfide

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Highlights

- The transsulfuration enzymes, cystathionine β -synthase (CBS) and cystathionine γ -lyase (CSE) bind the coenzyme pyridoxal 5'-phosphate (PLP) with similarly high affinity
- The transsulfuration pathway accounts for approximately 20% of cysteine flux in humans.
- During vitamin B₆ insufficiency, cellular PLP concentrations decline, with corresponding reduction in the activity of CSE. PLP insufficiency has less effect on CBS activity.
- The concentration of cystathionine increases in plasma, tissues and cultured cells during vitamin B₆ insufficiency. This cystathionine elevation tends to maintain the flux of cysteine production by CSE even during low cellular PLP conditions.
- PLP-dependent side reactions of CBS and CSE produce hydrogen sulfide (H₂S). Production of lanthionine and homolanthionine can serve as surrogate markers of H₂S production.
- Low cellular PLP reduces H₂S production capacity and corresponding synthesis of lanthionine and homolanthionine.

Figure 1.

Schematic model of the transsulfuration pathway illustrating relative concentrations and fluxes of reactions during vitamin B₆ replete and deficient conditions. Major effects shown: (a) preferred reactions (canonical) are bold lines; (b) vitamin B₆ deficiency causes a reduction in CSE activity from the reduced availability of cellular PLP (reduced quantity of active holo-CSE is designated by its reduced size); (c) CBS undergoes much less loss of activity during vitamin B₆ deficiency; (d) cystathionine concentration is increased (greater font size) during vitamin B₆ deficiency because of the bottleneck caused by reduced CSE activity, whereas cysteine production undergoes little change in deficiency because the higher cystathionine concentration compensates for the lower activity of CSE; (f) production of lanthionine and H₂S is catalyzed mainly by CBS, which appears to exceed the CBS catalyzed production of H₂S + cystathionine from homocysteine and cysteine. (g) production of H₂S + homolanthionine is impaired in vitamin B₆ deficiency due to the reduction in CSE activity (designated by smaller font); (e) CBS and CSE actually exist as tetramers (shown as monomeric holoenzymes in the figure).