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Kinetics of microbial methionine metabolism in continuous cultures administered different methionine sources

J. L. Firkins,*[1] C. M. Fowler,*[2] E. Devillard,† and B. J. Bequette‡[3]
*Department of Animal Sciences, The Ohio State University, Columbus 43210
†Adisseo Inc., 03600 Commentry, France
‡Department of Animal and Avian Sciences, University of Maryland, College Park 20742

ABSTRACT

The Met precursor 2-hydroxy-4-(methylthio) butanoic acid (HMB) is expected to be more extensively degraded in the rumen than its isopropyl ester (HMBi). A control and 3 isomolar treatments—0.097% DL-methionine, 0.11% HMBi (HMBi), and 0.055% HMBi plus 0.048% Met (Met + HMBi)—were dosed every 8 h simultaneously with 3-times-daily feeding into continuous cultures. Starting on d 9, for 6 consecutive doses, both [1-^{13}C]-L-Met and [methyl-$^{2}H_3$]-L-Met replaced part of the unlabeled DL-Met, [$^{13}C_5$]-DL-HMBi replaced a portion of the unlabeled DL-HMBi, and [1-^{13}C]-L-Met plus [$^{13}C_5$]-DL-HMBi replaced a portion of the respective unlabeled doses for the Met + HMBi treatment. After the sixth dose (d 11), unlabeled Met or HMBi provided 100% of the doses to follow elimination kinetics of the labels in HMBi, free Met, and bacterial Met compartments. The free [1-^{13}C]-L-Met recycled more and was recovered in bacterial Met to a lesser extent than was the free [methyl-$^{2}H_3$]-L-Met recycling and that was recovered in bacterial Met. Increasing HMBi inclusion (0, 50, and 100% substitution of the exogenously dosed Met on a molar equivalent basis) tended to increase HMBi escape from 54.7 to 71.3% for the 50 and 100% HMBi treatments, respectively. Despite HMBi substituting for and decreasing the dosage of Met, increasing HMBi increased accumulation of free Met in fermenter fluid. The HMBi (after de-esterification of the isopropyl group) presumably produces Met through the intermediate α-ketomethylthyiobutyrate with an aminotransferase that also has high affinity for branched-chain AA. We provide evidence that the HMBi-derived Met is likely released from bacterial cells and accumulates rather than being degraded, potentially as a result of lagging D-stereoisomer metabolism. More research is needed to evaluate racemization and metabolism of stereoisomers of HMBi, Met, and other AA in ruminal microbes.
Key words: methionine, hydroxymethylthiobutanoic acid, microbial methionine metabolism

INTRODUCTION

In our companion study, Fowler et al. (2015) reasoned that the isopropyl ester (**HMBi**) of 2-hydroxy-4-(methylthio) butanoic acid (**HMB**) would increase the efficiency of bacterial protein synthesis through sustained higher availability of Met and other AA synthesized through linked pathways. Using batch cultures from ruminal fluid inoculum to determine which, if any, single AA could stimulate growth compared with ammonia alone or with a complete mix of all 20 protein-forming AA, only Glu and Gln (precursors for transamination reactions) significantly increased bacterial growth rate compared with ammonia in batch culture of mixed ruminal microbes (Kajikawa et al., 2002). However, removal of Leu, Trp, Tyr, Glu, Met, Phe, and Val from the 20-AA supplement significantly decreased the growth stimulation. Threonine, Ile, and Phe were particularly inhibitory when in excess by disrupting the balance of the branched-chain amino acids (**BCAA**) and aromatic AA, respectively. In a subsequent study, Kajikawa et al. (2005) reported that removal of all 3 of the BCAA was less inhibitory than removal of just Leu and Val (making Ile imbalanced), and removal of all 3 of the aromatics was less inhibitory than removal of just Trp and Tyr (making Phe imbalanced). Bacterial growth was inhibited by excess Thr, but this inhibition could not be reversed by adding more Lys or Met even though those 3 share the precursor Asp, which is produced from oxaloacetate diverted from fermentation of glucose. However, the Thr imbalance was mitigated by addition of several other AA that were not in the Asp family, presumably allowing better coordination of availabilities of all of the protein-coding AA. In these studies, Met addition did not cause an imbalance (Kajikawa et al., 2002). Among the ^{15}N- and ^{13}C-labeled AA tested (unfortunately not Met), Thr was extensively

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[1]Corresponding author: firkins.1@osu.edu
[2]Current address: Provimi North America, Lewisburg, OH 45338.
[3]Deceased.

degraded by mixed ruminal bacteria in vitro, whereas Phe and the BCAA were much less degraded and more directly incorporated into bacterial protein (Atasoglu et al., 2004). Thus, within families, common control points can be rooted to branches for groups of similar AA but also back to partitioning of carbon for de novo synthesis of AA and other cellular components needed for growing bacterial cells.

The curious relationship for Met not causing an imbalance or not preventing a Thr imbalance (Kajikawa et al., 2005) might be related to Met's unique need for various functions in rumen bacteria. Besides its derivative *N*-formyl Met initiating protein synthesis in bacteria, the conversion of L-Met to *S*-adenosylmethionine (**SAM**) provides a critical methyl donor and regulator of transcription in various bacterial models (Tomsic et al., 2008), presumably conserved in rumen bacteria. In most bacteria, L-Met (hereafter referred to as simply Met unless there is reference to the D-isoform) can be synthesized from glucose and inorganic sulfur (Figure 1A). Or-Rashid et al. (2001) documented that rumen bacteria also can synthesize Met from homocysteine. Flow of glucose carbon into VFA production versus de novo synthesis of AA must be coordinated to prevent glycogen accumulation (Matheron et al., 1999) and energy spilling (Hackmann et al., 2013). In bacteria, an increasing cellular concentration of Met allosterically inhibits its own synthesis, but feedback is convoluted because other AA (i.e., Lys, Thr, and Ile) are synthesized from Asp (Kalcheva et al., 1994); the feedback by Thr and its potential effect on Met synthesis are depicted in Figure 1A. When intracellular concentration of Met increases, it is not readily converted to Cys (Or-Rashid et al., 2001), as might be expected in animal nutrition, but Met is fermented to propionate, butyrate, or 2-aminobutyrate (Smith and MacFarlane, 1997). Methionine permeases or transporters are also presumably inhibited or downregulated by increasing intracellular Met concentration in rumen bacteria, as in *Bacillus subtilis* (Zhang et al., 2003). Intracellular accumulation of homocysteine can be toxic to *Escherichia coli*, presumably by depleting the intracellular BCAA, particularly Ile and Val (Tuite et al., 2005). Feedback is critical to prevent homocysteine accumulation and resultant toxicity in *E. coli* (Roe et al., 2002). However, the route for Met synthesis differs for *E. coli* compared with other bacteria (Ferla and Patrick, 2014), so modeling of Met metabolism in mixed rumen bacteria can provide important information.

In contrast to shorter term allosteric inhibition by Met itself, which has been well characterized for bacteria (Kalcheva et al., 1994; Kajikawa et al., 2002), SAM has emerged as a critical controller of expression of operons or regulons (genomic DNA containing a series of genes that are regulated by a single promoter) in Met metabolism (Tomsic et al., 2008), including both branches of SAM in Figure 1A. One of the branches through decarboxylated SAM yields α-ketomethylthiobutyrate (**KMB**; also known as α-ketomethylthiobutanoate), which provides a critical juncture for the current study's hypotheses. The aminotransferase(s) converting KMB to Met probably use(s) Glu or BCAA (Leu is depicted in Figure 1A) as amino donors and probably is(are) not regulated by SAM, as verified in many nonrumen bacterial strains (Berger et al., 2003). The enzymes typically have varying affinity constants for different substrates and products (which become substrates in reversible reactions) such that some concentration-dependent flux control would be expected. Increasing provision of HMB is hypothesized to sustain increased concentrations of KMB and therefore Met. Because HMBi is slowly converted to HMB, we reasoned that there would be a more sustained supply of Met, less de novo Met synthesis, less cycling of Met, and less swing in the activation and repression of Met biosynthesis and catabolism. We reasoned that more sustained entry of preformed Met would potentially circumvent an interrupted supply of AA rooted from the Asp branch and thereby improve the efficiency of bacterial protein synthesis. Our hypothesis was based on the expectation of approximately 50% degradation of HMBi (St-Pierre and Sylvester, 2005). If BCAA are preferred amino donors for the aminotransferase in the last step of Met synthesis from HMBi (via HMB), we questioned whether depletion of BCAA might also negatively affect cellular function or if their product (the branched-chain VFA) might stimulate bacterial function. We assumed that reductive carboxylation of the branched-chain VFA could regenerate BCAA in rumen bacteria (Allison et al., 1984), but changing the intracellular concentration of BCAA through competing usage and regeneration could affect global transcription of hundreds of enzymes, as documented for gram-positive bacterial models (Brinsmade et al., 2010). Therefore, we reasoned that a dose-response of HMBi substitution for DL-Met would be nonlinear if bolus-dosed.

This study was conducted simultaneously with that of Fowler et al. (2015) and used the isotopes shown in Figure 1B to address our limited understanding of Met metabolism in mixed rumen bacteria. The first aim was to dose with 2 positional isotope labels of Met ([1-^{13}C]-L-Met and [methyl-$^{2}H_3$]-L-Met, termed **M1** and **M3**, respectively) to assess kinetics of cycling of Met through the decarboxylated SAM and homocysteine routes, respectively (Figure 1A). Assuming that HMBi could affect exogenous entry of Met into the intracellular Met pool in a longer term, the second aim was to