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Evidence that glutamine transaminase and omega-amidase potentially act in tandem to close the methionine salvage cycle in bacteria and plants

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ABSTRACT

S-Adenosylmethionine is converted enzymatically and non-enzymatically to methylthioadenosine, which is recycled to methionine (Met) via a salvage pathway. In plants and bacteria, enzymes for all steps in this pathway are known except the last: transamination of α-ketomethylthiobutyrate to give Met. In mammals, glutamine transaminase K (GTK) and ω-amidase (ω-Am) are thought to act in tandem to execute this step, with GTK forming α-ketoglutaramate, which ω-Am hydrolyzes. Comparative genomics indicated that GTK and ω-Am could function likewise in plants and bacteria because genes encoding GTK and ω-Am homologs (i) co-express with the Met salvage gene 5-methylthioribose kinase in Arabidopsis, and (ii) cluster on the chromosome with each other and with Met salvage genes in diverse bacteria. Consistent with this possibility, tomato, maize, and *Bacillus subtilis* GTK and ω-Am homologs had the predicted activities: GTK was specific for glutamine as amino donor and strongly preferred α-keto-methylthiobutyrate as amino acceptor, and ω-Am strongly preferred α-ketoglutaramate. Also consistent with this possibility, plant GTK and ω-Am were localized to the cytosol, where the Met salvage pathway resides, as well as to organelles. This multiple targeting was shown to result from use of alternative start codons. In *B. subtilis*, ablating GTK or ω-Am had a modest but significant inhibitory effect on growth on 5-methylthioribose as sole sulfur source. Collectively, these data indicate that while GTK, coupled with ω-Am, is positioned to support significant Met salvage flux in plants and bacteria, it can probably be replaced by other aminotransferases.

1. Introduction

The methionine (Met) (**1**) salvage pathway – also called the Yang cycle – occurs in bacteria, animals, and plants (Albers, 2009; Miyazaki and Yang, 1987; Sekowska et al., 2004) (Fig. 1A). This pathway recycles Met (**1**) from 5′-methylthioadenosine (MTA) (**3**), which is formed from *S*-adenosylmethionine (AdoMet) (**2**) as a by-product of the synthesis of polyamines, ethylene, and other compounds (Albers, 2009), and also comes from spontaneous AdoMet (**2**) breakdown (Hoffman, 1986). In essence, the pathway, which has several variants, rebuilds Met (**1**) from the methylthio and ribose moieties of MTA plus a transamination-derived amino group (Fig. 1A).

For bacteria and plants, enzymes and genes are well defined for all the steps in Met (**1**) salvage except the last one, the transamination that converts α-ketomethylthiobutyrate (KMTB) (**10**) to Met (**1**) (Pommerrenig et al., 2011; Sekowska et al., 2004). Assays of cell extracts and of recombinant aminotransferases suggest that several enzymes could be responsible (Albers, 2009; Berger et al., 2003; Pirkov et al., 2008; Pommerrenig et al., 2011; Sekowska et al., 2004). However, some of these studies did not test all potential amino donors (most notably omitting glutamine (**11**) (Fig. 1B) and asparagine) and none of them attempted to show which if any of the measured activities carry the bulk of the salvage flux in vivo.

The KMTB (**10**) transamination step is somewhat better characterized in mammalian tissues, where enzymological

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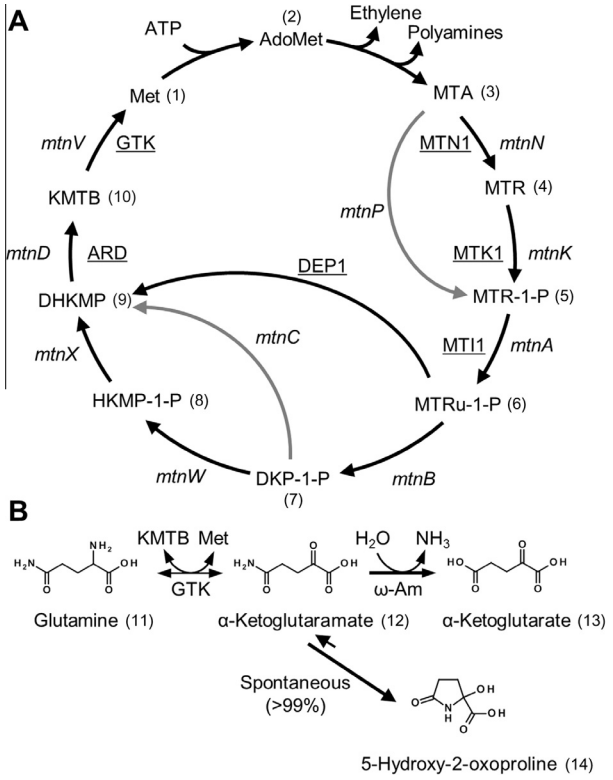

Fig. 1. The methionine (**1**) salvage pathway. (A) Steps in the Met (**1**) salvage pathways in bacteria (gene names in italics) and plants (gene names underlined). Alternative bacterial steps are shown as gray arrows. Abbreviations: MTA (**3**), methylthioadenosine; MTR (**4**), methylthioribose; MTR-1-P (**5**), 5-methylthioribose-1-phosphate; MTRu-1-P (**6**), methylthioribulose-1-phosphate; DKP-1-P (**7**), 2,3-diketo-5-methylthiopentyl-1-phosphate; HKMP-1-P (**8**), 2-hydroxy-3-keto-5-methylthiopentenyl-1-phosphate; DHKMP (**9**), 1,2-dihydroxy-3-keto-5-methylthiopentene; KMTB (**10**), α-ketomethylthiobutyrate. (B) The tandem transamination and deamination reactions mediated by glutamine transaminase K (GTK) and ω-amidase (ω-Am) that are thought to close the salvage cycle in mammals. ω-Am is also known as Nit2. Note that α-ketoglutaramate (**12**) spontaneously cyclizes to 5-hydroxy-2-oxoproline (**14**) and that at neutral pH the equilibrium favors the ring form (99.7%) over the open-chain form (0.3%) (Cooper, 2004).

(Backlund et al., 1982; Cooper, 2004) and in vivo ^{15}N tracer (Hoskin et al., 2001) data implicate a distinctive glutamine-dependent aminotransferase, glutamine transaminase K (GTK) [E.C. 2.6.1.64]. The glutamine transamination product, α-ketoglutaramate (KGM) (**12**), is potentially toxic but is removed by an ω-amidase (ω-Am) [E.C. 3.5.1.3], which hydrolyzes it to α-ketoglutarate (**13**) and ammonia (Cooper, 2004; Jaisson et al., 2009; Krasnikov et al., 2009) (Fig. 1B). In this tandem GTK/ω-Am arrangement, KGM (**12**) removal by ω-Am 'pulls' the transamination in the direction of Met (**1**) formation and so lowers KMTB (**10**) levels (Cooper, 2004). As KMTB (**10**) is unstable and yields toxic products (Cooper, 2004; Gao et al., 1998), keeping its level low may be beneficial.

Certain bacteria have homologs of mammalian GTK and ω-Am that respectively possess glutamine aminotransferase and ω-Am activity (Berger et al., 2003; Cobzaru et al., 2011; Hosono et al., 2003;), and plant genomes encode GTK and ω-Am homologs (Gerdes et al., 2011; Hudson et al., 2006). Moreover, the activities of both enzymes have been detected in plants (Huang and Ireland, 1991; Lloyd and Joy, 1978; Streeter, 1977), although the ω-Am substrate tested was α-ketosuccinamate (the transamination product of asparagine) rather than KGM (**12**) (Lloyd and Joy, 1978; Streeter, 1977). There are thus indications that GTK and ω-Am could potentially mediate KMTB (**10**) transamination in bacteria and plants as well as in mammals.

The importance of Met (**1**) salvage in bacteria and plants (Miyazaki and Yang, 1987; Sekowska et al., 2004) led us to revisit the enigmatic KMTB (**10**) transamination step, beginning with comparative genomics and transcriptomics analyses to identify candidate aminotransferase genes. Such analyses have a strong track record in identifying genes encoding metabolic functions in prokaryotes and plants (Hanson et al., 2009). At the outset, it was predicted that plant KMTB aminotransferase genes would encode cytosolic proteins because other plant Met salvage enzymes lack obvious targeting signals and so are most probably cytosolic (Sauter et al., 2004, 2005). Having obtained comparative genomic evidence associating bacterial and plant GTK and ω-Am genes with Met (**1**) salvage, representative plant and bacterial enzymes were characterized, and established that plant GTK and ω-Am proteins localize both to the cytosol and to organelles. The effects of inactivating the *Bacillus subtilis* GTK and ω-Am genes were also reinvestigated as earlier work on this (Sekowska and Danchin, 2002) was inconclusive.

2. Results

2.1. Comparative genomics

Genes encoding GTK and ω-Am homologs (henceforth GTK, ω-Am) are adjacent in the *B. subtilis* genome and flanked by Met (**1**) salvage genes (Sekowska et al., 2004). These arrangements point to possible functional relationships between GTK and ω-Am, and between these enzymes and Met (**1**) salvage. To assess the prevalence of such arrangements, the SEED database and its tools were used (Overbeek et al., 2005) to compare the distribution and chromosomal clustering of GTK, ω-Am, and Met (**1**) salvage genes in 588 representative bacterial genomes. Of these genomes, 426 (72%) encode ω-Am and 204 (35%) encode GTK. Of the 204 with GTK, 202 have ω-Am and only two do not. Thus, GTK almost never occurs without ω-Am, whereas ω-Am often occurs without GTK (Fig. 2A and B). Moreover, when GTK and ω-Am genes are present, they are often neighbors (Fig. 2B and C). These distribution and clustering data suggest that GTK action requires ω-Am but that ω-Am can act independently of GTK.

The occurrence of a Met (**1**) salvage pathway was assessed by the presence of genes encoding methylthioribose-1-phosphate isomerase (*mtnA*) and acireductone dioxygenase (*mtnD*), the two enzymes common to all variants of the pathway (Fig. 1A). Of 82 genomes having *mtnA* and *mtnD*, 64 also have GTK and ω-Am, and in 28 of these cases GTK and ω-Am cluster with the Met (**1**) salvage genes (Fig. 2A, 2B, and 2C). However, of the 82 gen omes with *mtnA* and *mtnD*, 16 lack GTK (Fig. 2B). These patterns indicate (i) that GTK and ω-Am could potentially close the Met (**1**) salvage cycle in a majority (78%) of the bacteria surveyed; (ii) that in other bacteria, a different enzyme(s) must close the cycle, and (iii) that because 202 genomes have GTK and ω-Am, and only 64 also have Met (**1**) salvage, GTK and ω-Am must have other, widely occurring roles besides Met (**1**) salvage.

Possible associations between GTK, ω-Am, and Met (**1**) salvage in plants were probed using the co-expression tools for Arabidopsis genes in the ATTED-II database (Obayashi et al., 2011). Consistent with such an association, the GTK and ω-Am genes occurred together in a network with the Met (**1**) salvage gene 5-methylthioribose kinase, as well as genes related to glutamate or aspartate metabolism (Fig. 2D).

2.2. Characterization of recombinant GTK and ω-Am enzymes

The tomato and maize GTKs (Solyc11g013170 and GRMZM2G067265) were expressed as His-tagged N-terminal Nus fusions, and further purified by Ni^{2+} affinity chromatography, followed by a size-exclusion step to remove degradation products

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