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Biochemical Analysis on the Parallel Pathways of Methionine Biosynthesis in *Corynebacterium glutamicum*

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Abstract Two alternative pathways for methionine biosynthesis are known in *Corynebacterium glutamicum*: one involving transsulfuration (mediated by *metB* and *metC*) and the other involving direct sulfhydrylation (mediated by *metY*). In this study, MetB (cystathionine γ-synthase) and MetY (*O*-acetylhomoserine sulfhydrylase) from *C. glutamicum* were purified to homogeneity and the biochemical parameters were compared to assess the functional and evolutionary importance of each pathway. The molecular masses of the native MetB and MetY proteins were measured to be approximately 170 and 280 kDa, respectively, showing that MetB was a homotetramer of 40-kDa subunits and MetY was a homohexamer of 45-kDa subunits. The K_m values for the *O*-acetylhomoserine catalysis effected by MetB and MetY were 3.9 and 6.4 mM, and the maximum catalysis rates were 7.4 (k_{cat}=21 s^{-1}) and 6.0 (k_{cat}=28 s^{-1}) μmol mg^{-1} min^{-1}, respectively. This suggests that both MetB and MetY can be comparably active *in vivo*. Nevertheless, the K_m value for sulfide ions by MetY was 8.6 mM, which was too high, considering the physiological condition. Moreover, MetB was active at a broad range of temperatures (30 and 65°C) and pH (6.5 and 10.0), as compared with MetY, which was active in a range from 30 to 45°C and at pH values from 7.0 to 8.5. In addition, MetY was inhibited by methionine, but MetB was not. These biochemical data may provide insight on the role of the parallel pathways of methionine biosynthesis in *C. glutamicum* with regard to cell physiology and evolution.

Keywords: *Corynebacterium glutamicum*, cystathionine γ-synthase, *metB*, methionine, *metY*, *O*-acetylhomoserine sulfhydrylase

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Biosynthetic pathways and regulatory mechanisms for methionine have been studied extensively in *Escherichia coli* and *Salmonella typhimurium*. Although current knowledge regarding methionine biosynthesis in *C. glutamicum* and related species remains fairly limited [37], the genetic background of methionine biosynthesis has been studied relatively profoundly [10, 26; see 18 for review]. The biosynthetic pathways by which methionine is synthesized differ among organisms in many aspects. First, in Gram-negative facultative aerobic and anaerobic bacteria, homoserine is acylated to *O*-succinylhomoserine in the presence of succinyl-CoA, which is catalyzed by the homoserine *O*-succinyl transferase encoded by *metA* [3, 6, 31, 34]. In contrast, homoserine is acetylated to *O*-acetylhomoserine in the presence of acetyl-CoA, which is catalyzed by the homoserine *O*-acetyltransferase encoded by *metX* in Gram-positive bacteria (Fig. 1), including *Corynebacterium* [12, 23], *Bacillus* [38], and *Brevibacterium* [20], as well as many yeast and fungi [see 19, 35 for review]. Second, the subsequent formation of homocysteine from acylated homoserine can be achieved *via* two alternative routes. One of these routes is referred to as the transsulfuration pathway (Fig. 1), in which cysteine is the sulfur donor, and is incorporated into the acylated homoserine to form cystathionine. This reaction requires the cystathionine γ-synthase encoded by *metB* [9]. Cystathionine is subsequently converted into homocysteine, which is catalyzed by the cystathionine β-lyase encoded by *metC* (*aceD*) [14, 28]. The other route is referred to as the direct sulfhydrylation pathway (Fig. 1), in which sulfide serves as the sulfur donor, and is incorporated into the acylated homoserine to form homocysteine by the *O*-acetylhomoserine sulfhydrylase encoded by *metY* or *metZ* [10]. Both *E. coli* and *S. typhimurium* have been shown to employ the transsulfuration pathway

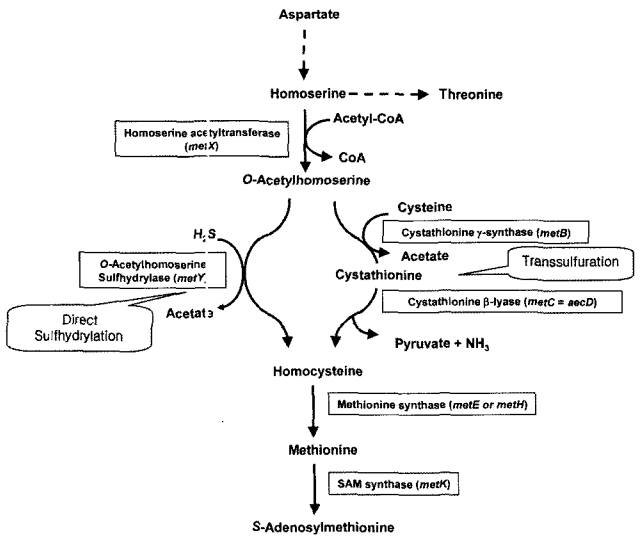

Fig. 1. Schematic diagram of the methionine biosynthetic pathway in *C. glutamicum*.
Alternative pathways of the transsulfuration pathway mediated by MetB and MetC, and the direct sulfhydrylation pathway mediated by MetY are shown.

[15, 31]. In contrast, *Rhizobium etli* [34], *Pseudomonas aeruginosa* [6], and *Pseudomonas putida* [1] are known to utilize the direct sulfhydrylation pathway. Some eukaryotes, including yeasts, fungi, and green plants, employ both the functional transsulfuration and direct sulfhydrylation pathways, and prokaryotes such as *C. glutamicum* [10] and *Leptospira meyeri* [24], and *M. tuberculosis* [32] can also utilize both pathways. *S. cerevisiae*, *Neurospora*, and green plants evidence enzyme activity for both pathways, although only one of the pathways may be physiologically active in methionine biosynthesis.

In this study, the proteins of cystathionine γ-synthase and *O*-acetylhomoserine sulfhydrylase, which catalyze the transsulfuration and direct sulfhydrylation pathways, respectively, were purified from *C. glutamicum*, and their biochemical characteristics were directly compared. On the basis of the biochemical results, functional and evolutionary roles of the transsulfuration and direct sulfhydrylation pathways were assessed with regard to methionine biosynthesis in the cell.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strains and plasmids employed in this study have been previously described [10]. *C. glutamicum* cells were cultured at 30°C in MB [7]. The minimal medium for *C. glutamicum* was MCGC [36], and glucose was added to a final concentration of 1%. The sulfur-free minimal medium (pH 7.4) for *C. glutamicum* was composed of 1% glucose, 50 mM Na_2HPO_4, 25 mM KH_2PO_4, 20 mM NaCl, 30 mM NH_4Cl, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1 mM $FeCl_2$, 0.01 mM $MnCl_2$, and 1 mg/l biotin. All amino acids were added to a final concentration of 40 mg/l. The antibiotics, ampicillin and kanamycin, were added to final concentrations of 50 and 25 mg/l, respectively.

Enzyme Activity Assay

O-Acetylhomoserine was synthesized by the method of Nagai and Flavin [21]. The activities of *O*-acetylhomoserine sulfhydrylase were assayed by measuring homocysteine formation, using the nitroprusside reaction [6]. Cystathionine γ-synthase activity was assayed in accordance with the methods of Ravanel *et al.* [25], which measure the reduction of cysteine.

Purification of Enzymes

The purification of cystathionine γ-synthase was conducted as follows. *C. glutamicum* HL921 harboring pSL123 was cultured in MCGC at 30°C for 50 h with shaking at 250 rpm. The cells were harvested when the OD_{600} was approximately 12, and then washed twice and resuspended in Buffer Y (20 mM Tris·Cl, pH 7.5, 50 μM pyridoxal 5'-phosphate). The cells were disrupted by sonication using an Ultrasonic Processor (VCX400, Sonic and Materials Inc., CT, U.S.A.), and centrifuged for 30 min at 15,000 ×*g* four times. The supernatants (crude extract) were treated with ammonium sulfate (60–90%) to collect proteins. The pellets were then dissolved in Buffer A (50 mM Tris, pH 7.5, 20 mM KCl, 10 mM $MgCl_2$, and 5% glycerol) and applied to a Sephacryl S-200 HR column (1.5×59 cm) equilibrated with Buffer Y. Fractions showing high enzyme activity were combined and applied to a DEAE-cellulose column (2.5×10 cm) equilibrated with Buffer Y. Proteins were eluted with a linear gradient of NaCl from 0 M to 0.5 M at a rate of 1 ml/min. The fractions with peak enzyme activity were pooled, and 30% ammonium sulfate was added, after which the pooled sample was applied to a Butyl-Toyopearl column (2.5×6 cm) equilibrated with Buffer Y containing 30% ammonium sulfate. The proteins were eluted by a linear reduction in ammonium sulfate concentration, from 30% to 0%, at a rate of 1 ml/min. Fractions exhibiting enzyme activity were combined and concentrated with 90% ammonium sulfate. The proteins were then applied to a Sephacryl S-200 HR column (1.5×59 cm), and eluted at a rate of 1 ml/min with Buffer Y. The active fractions from this process were again combined, concentrated with 90% ammonium sulfate, and dialyzed against Buffer Y containing 5% glycerol. The purified enzyme was then maintained at −70°C until use.

O-Acetylhomoserine sulfhydrylase was purified according to the following procedure. *C. glutamicum* HL938 harboring pSL191 was cultured in MCGC at 30°C for 50 h with shaking at 250 rpm [5, 13, 33]. The cells were harvested

when the OD_{600} was approximately 15, and then washed twice and resuspended in Buffer Y. The crude extracts were prepared by sonication, as described in the purification of cystathionine γ-synthase. Proteins were precipitated with ammonium sulfate (30%) and the pellets were dialyzed against Buffer A. The proteins were then applied to a Q-Sepharose chromatography column (2.5×10 cm) equilibrated with Buffer Y and eluted with a linear gradient of NaCl from 0 M to 1 M, at a rate of 1 ml/min. Fractions exhibiting high enzyme activities were then combined, and ammonium sulfate was added to a final concentration of 20%. The proteins were applied to a Butyl-Toyopearl column (2.5× 6 cm) equilibrated with Buffer Y containing 20% ammonium sulfate. The proteins were eluted at a rate of 1 ml/min by a linear reduction in ammonium sulfate concentrations, from 20 to 0%, in Buffer Y. The fractions exhibiting peak enzyme activity were pooled, dialyzed against Buffer A, and concentrated with 90% ammonium sulfate. The proteins were then injected into a Sephacryl S-200 HR column (1.5×59 cm) equilibrated with Buffer Y. The proteins were eluted with Buffer Y at a rate of 1 ml/min, and the active fractions were concentrated using polyethylene glycol (MWCO 8,000). The enzyme was dialyzed against Buffer Y containing 5% glycerol, and maintained at −70°C until use.

Kinetic Parameters

K_m and k_{cat} values for cystathionine γ-synthase activity were determined using *O*-acetylhomoserine and cysteine as substrates. The K_m and k_{cat} values for *O*-acetylhomoserine sulfhydrylase were determined using *O*-acetylhomoserine and Na_2S as substrates. Initial velocity data were fitted to the Michaelis-Menten equation by nonlinear least-squares regression.

Biochemical Measurements

The temperature stability of MetY was assessed by incubating the protein at 30, 37, or 42°C for up to 40 min at time intervals, followed by measuring enzyme activity. Protein concentrations were determined by the method of Bradford [4], using bovine serum albumin as the standard. The proteins were separated on SDS-PAGE in 12% separating gel and 4% stacking gel [16], and visualized by staining with Coomassie Brilliant Blue R-250.

RESULTS

Cross-talks in Enzyme Activities Between Cystathionine γ-Synthase and *O*-Acetylhomoserine Sulfhydrylase

The cystathionine γ-synthase of *E. coli* has been shown to exhibit *O*-acylhomoserine sulfhydrylase activity to some extent [8, 30]. This was tested in *C. glutamicum* (Table 1). In a *metY* mutant strain, the cystathionine γ-synthase (MetB) activity was almost unaffected, whereas the *O*-acetylhomoserine sulfhydrylase (MetY) activity was decreased to 6% of the wild-type activity. In a *metB* mutant strain, the MetY activity was almost unaffected, whereas the MetB activity was decreased only to a 40% level as compared with the parental strain. To elucidate the nature of the basal activity observed in the mutant strains, we introduced the *metB* (pSL123) or *metY* (pSL191) genes into the mutants and analyzed the increase of activity in the crude extracts. As shown in Table 1, MetB appeared to contribute to 6–7% of MetY activity in the E12/pMT1 strain and *vice versa* (see below for data with purified enzymes). On the basis of knowledge regarding cross-talk in enzyme activities between cystathionine γ-synthase and *O*-acetylhomoserine sulfhydrylase in *C. glutamicum*, MetB and MetY were purified from the *metY*-disrupted *C. glutamicum* harboring a *metB* clone in a plasmid (HL921/pSL123) and the *metB*-disrupted *C. glutamicum* harboring a *metY* clone in a plasmid (HL938/pSL191), respectively.

Purification and Size Determination

Cystathionine γ-synthase purification was accomplished in five successive steps (Table 2). This purification scheme yielded homogeneous enzymes, as was confirmed by

Table 1. Cross-talks in enzyme activities between cystathionine γ-synthase and *O*-acetylhomoserine sulfhydrylase[a].

Strains/Plasmids	Phenotype[b]		CGS activity (nmol min^{-1} mg^{-1})	OAHSH activity (nmol min^{-1} mg^{-1})
	metB	*metY*		
E12/pMT1	+	+	44.5	57.1
HL921/pMT1	+	-	46.5	3.5
HL938/pMT1	-	+	19.8	63.5
HL921/pSL123	+++	-	186	27.9
HL938/pSL191	-	+++	46.1	481

[a]The enzymes were induced by growing *C. glutamicum* cells to stationary phase on MCGC minimal medium containing 1% glucose. The cells were harvested, disrupted, and assayed for activity, as was described in the Materials and Methods. Enzyme reactions were carried out for 10 min at 30°C. The enzyme activities are expressed as the means of three independent experiments.
Abbreviations: E12, AS019E12 [7]; CGS, cystathionine γ-synthase; OAHSH, *O*-acetylhomoserine sulfhydrylase.
[b]+ and +++ indicate the relative copy numbers of each gene.

Table 2. Purification of cystathionine γ-synthase and *O*-acetylhomoserine sulfhydrylase from *C. glutamicum*.

Steps	Protein (mg)	Total activity (U)	Sp. activity (U/mg)	Recovery (%)	Purification (fold)
Cystathionine γ-synthase					
Crude extracts	840	171[a]	0.20	100	1.0
60–90% AS[c]	108	79	0.73	46	3.6
Sephacryl S-200	56	59	1.1	35	5.4
DEAE-cellulose	24	56	2.3	33	11.3
Butyl-Toyopearl	13	50	3.8	29	18.7
Sephacryl S-200	5	24	4.8	14	23.6
O-Acetylhomoserine sulfhydrylase					
Crude extracts	920	550[b]	0.60	100	1.0
30% AS[c]	565	355	0.63	65	1.1
Q-Sepharose	140	315	2.3	57	3.8
Butyl-Toyopearl	43	213	5.0	39	8.3
Sephacryl S-200	19	139	7.3	25	12.2

[a]One unit of enzyme activity is defined as the amount of enzyme required to convert 1 mg of *O*-acetylhomoserine into 1 μmol cystathionine per min.
[b]One unit of enzyme activity is defined as the amount of enzyme required to convert 1 mg of *O*-acetylhomoserine into 1 μmol homocysteine per min.
[c]AS, ammonium sulfate precipitation.

SDS-PAGE (Fig. 2). Cystathionine γ-synthase was purified 24-fold with a recovery of 14%, yielding a protein with a specific activity of 4.8 μmol/min/mg. *O*-Acetylhomoserine sulfhydrylase was purified in four steps (Table 2, Fig. 2). The protein was purified by 12-fold with 25% recovery, and yielded a protein with a specific activity of 7.3 μmol/min/mg.

The molecular masses of *C. glutamicum* cystathionine γ-synthase and *O*-acetylhomoserine sulfhydrylase, deduced from their own genes, were 41.7 and 46.7 kDa, respectively [9, 10]. Consistent with the data, the sizes of the subunits of cystathionine γ-synthase and *O*-acetylhomoserine sulfhydrylase were approximately 40 and 45 kDa, respectively, as determined by SDS-PAGE analysis (Fig. 2). The molecular masses of the native cystathionine γ-synthase and *O*-acetylhomoserine sulfhydrylase were estimated to be approximately 170 and 280 kDa, respectively, which were determined by gel filtration chromatography (data not shown). The results indicated that cystathionine γ-synthase was a homotetramer and *O*-acetylhomoserine sulfhydrylase was a homohexamer.

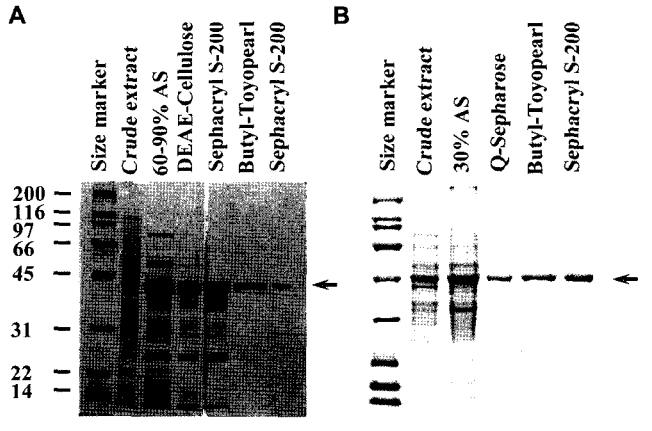

Fig. 2. Overall purification of cystathionine γ-synthase (**A**) and *O*-acetylhomoserine sulfhydrylase (**B**) from *C. glutamicum* after each step of the purification procedure, as described in Materials and Methods.
Arrows indicate the positions of cystathionine γ-synthase (**A**) and *O*-acetylhomoserine sulfhydrylase (**B**). Approximate molecular masses (in kilodaltons) are shown on the left.

Catalytic Characteristics

First of all, the phenomenon of cross-talk in enzyme activities between cystathionine γ-synthase and *O*-acetylhomoserine sulfhydrylase was analyzed in detail with purified enzymes. As shown in Table 3, both MetB and MetY demonstrated to have dual enzyme activities of cystathionine γ-synthase and *O*-acetylhomoserine sulfhydrylase activity. However, for both enzymes, the V_{max} values turned out to be significantly low when the alternative activities were measured. With regard to the utilization of sulfide as a substrate, MetY is analogous to *O*-acetylserine sulfhydrylase, which uses *O*-acetylserine and sulfide as substrates, and is involved in cysteine biosynthesis. However, the purified MetY was not capable of utilizing *O*-acetylserine as a substrate (data not shown), exhibiting no detectable *O*-succinylhomoserine sulfhydrylase activity.

As the purified MetB and MetY were determined to be bifunctional enzymes, we determined the steady-state kinetic constants and catalytic efficiency of MetB and MetY for both activities, and conducted a comparison of these values (Table 3). The K_m value for cysteine could not be determined, because the assay employed in this study measures the amount of disappearing cysteine. Although the K_m values for the catalysis of *O*-acetylhomoserine or sulfide by MetB or MetY for each activity were of the same order of magnitude (between 3.9 and 5.8 mM, and 5.7 and 8.6 mM, respectively), it can be concluded that MetB and MetY mainly function as cystathionine γ-synthase

Table 3. Comparison of the biochemical properties of cystathionine γ-synthase (CGS) and *O*-acetylhomoserine sulfhydrylase (OAHSH).

Proteins	Enzyme activity	Substrates	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
MetB	CGS	*O*-Acetylhomoserine	3.9	7.4	21.0	5.38×10^3
	OAHSH	*O*-Acetylhomoserine	4.1	1.4	4.0	0.98×10^3
		Na_2S	5.8	1.6	4.5	0.78×10^3
MetY	OAHSH	*O*-Acetylhomoserine	6.4	6.0	28.0	4.38×10^3
		Na_2S	8.6	8.0	37.3	4.34×10^3
	CGS	*O*-Acetylhomoserine	5.7	1.5	7.0	1.23×10^3

K_m and k_{cat} values for cystathionine γ-synthase were determined using *O*-acetylhomoserine and cysteine as the substrates. K_m and k_{cat} values for MetY were determined using *O*-acetylhomoserine and Na_2S as the substrates. Initial velocity data were fitted to the Michaelis-Menten equation by nonlinear least-squares regression.

and *O*-acetylhomoserine sulfhydrylase *in vivo*, respectively, comparing their catalytic efficiency for each activity: The k_{cat}/K_m value of MetB was definitely higher for cystathionine γ-synthase activity than for *O*-acetylhomoserine sulfhydrylase activity. Similarly, the k_{cat}/K_m value of MetY was considerably higher for *O*-acetylhomoserine sulfhydrylase activity than for cystathionine γ-synthase activity. On the other hand, the k_{cat}/K_m value of MetB for cystathionine γ-synthase activity (5.4×10^3 $M^{-1}s^{-1}$) was about 20% higher than that of MetY for *O*-acetylhomoserine sulfhydrylase activity (4.4×10^3 $M^{-1}s^{-1}$), which suggests that the transsulfuration pathway may be a favorable route in *C. glutamicum*.

Inhibition by Metabolic End Products

The inhibitory effects of the end products of sulfur metabolism on cystathionine γ-synthase and *O*-acetylhomoserine

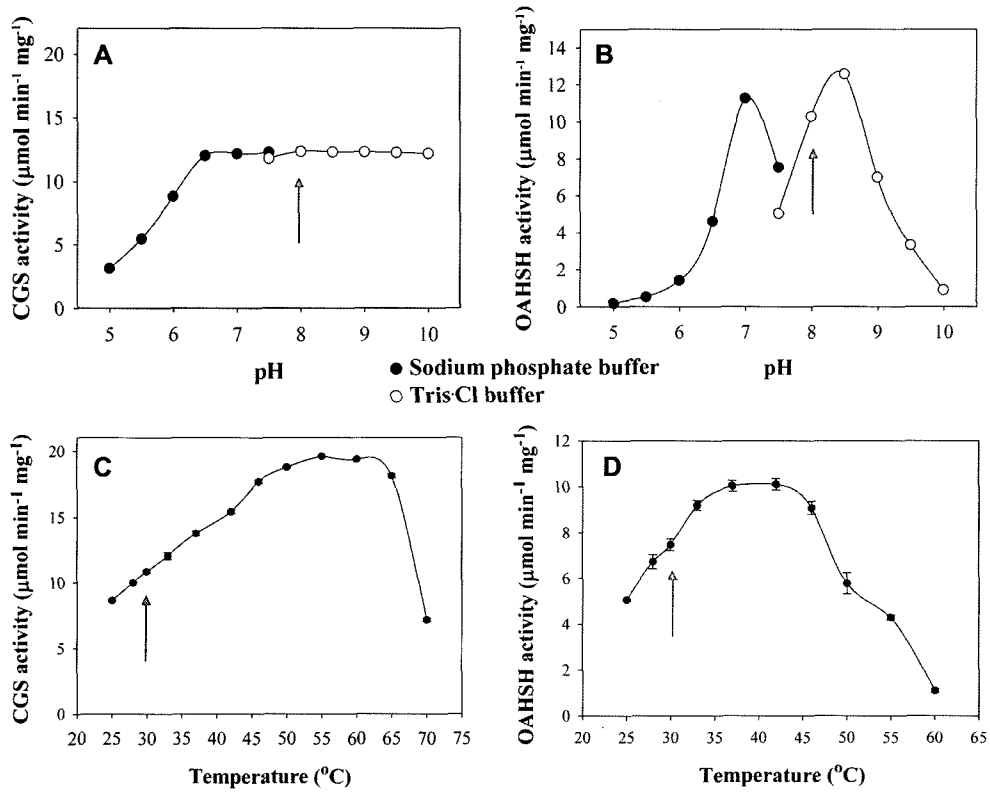

Fig. 3. Effect of pHs (**A**, **B**) and temperature (**C**, **D**) on cystathionine γ-synthase (**A**, **C**) and *O*-acetylhomoserine sulfhydrylase (**B**, **D**) activities of *C. glutamicum*.

The optimal pH values of cystathionine γ-synthase and *O*-acetylhomoserine sulfhydrylase activity were determined by incubating the reaction mixtures in sodium phosphate buffer (closed circles) or Tris·Cl buffer (open circles) at the indicated pHs, after which the enzyme activity was measured. The optimal temperature values were determined by incubating the reaction mixtures at the indicated temperature for 30 min, after which the enzyme activities were measured. Arrows denote the standard assay condition (30°C or pH 8.0).

sulfhydrylase activities were assessed. The cystathionine γ-synthase activity of MetB was inhibited completely by 10 mM cystathionine, but not inhibited by exposure to other end products, including methionine, lysine, threonine, isoleucine, leucine, or *S*-adenosylmethionine (data not shown). In contrast, the *O*-acetylhomoserine sulfhydrylase activity of MetY was inhibited by about 50% upon exposure to 10 mM methionine or 10 mM cystathionine, but was not notably affected by any of the other tested end products.

Biochemical Properties

The optimal temperatures for cystathionine γ-synthase and *O*-acetylhomoserine sulfhydrylase activity were determined by incubating the reaction mixture at varying temperatures. Whereas the cystathionine γ-synthase activity of the purified MetB remained higher until 65°C than was observed at 30°C, with the highest level at 55°C (Fig. 3C), the *O*-acetylhomoserine sulfhydrylase activity of the purified MetY was more active up to 47°C, as compared with the activity observed at 30°C, with the maximal level at temperatures between 37 and 42°C (Fig. 3D).

The optimal pH values for cystathionine γ-synthase and *O*-acetylhomoserine sulfhydrylase activities were determined. The purified MetB exhibited the full activity at a broad pH range of 6.5 to 10.0 (Fig. 3A). The enzyme retained 25% of full activity even at a relatively low pH value (pH 5.0). In contrast, the purified MetY represented the optimal activity at pH 7.0 in sodium phosphate buffer or at pH 8.5 in Tris·Cl buffer and retained only minimal activity at pH 5.0 or pH 10.0 (Fig. 3B), probably indicating the intrinsic sensitivity of the protein to the ions present in the buffer and/or reflecting the dependence of enzyme activity on buffer substance not related to pH change. The data relating to enzyme activities at variable temperatures and pH values suggest that cystathionine γ-synthase is considerably stable over a broad range of temperature and pH, and can retain its levels of activity even under relatively harsh conditions.

Temperature Stability of MetY

In our former study, it was determined that the *metX* (formerly *metA*) and *metY* gene products of *C. glutamicum* may be temperature-sensitive [10], similarly to the *metA* of *E. coli* and *Bacillus polymyxa* [27, 38]. Therefore, in this study, in order to identify the temperature-sensitivity of the *O*-acetylhomoserine sulfhydrylase, the activity of the purified MetY was measured after the incubation of the protein at 37°C or 42°C up to 40 min. It was observed that MetY retained over 80% of its full activity (data not shown), showing that MetY is not temperature-sensitive. This indicates that MetX of *C. glutamicum* is probably the temperature-sensitive enzyme.

DISCUSSION

In this report, we purified the two enzymes cystathionine γ-synthase and *O*-acetylhomoserine sulfhydrylase in *C. glutamicum* to homogeneity, respectively, and compared their biochemical properties in order to clarify the functional and evolutionary roles of the transsulfuration and the direct sulfhydrylation pathways for methionine biosynthesis in *C. glutamicum*.

As the purification steps proceeded, the enzyme activities of MetB and MetY were detected to decrease rapidly, resulting in low recovery rates as the result of their instability. Addition of protease inhibitors to the partially purified proteins at each purification step proved to be ultimately unhelpful, but PLP (pyridoxal 5'-phosphate), which functions as a cofactor for corynebacterial MetB and MetY [2, 10, 17, 22], allowed for a significant retention of activities of MetB and MetY. The N-terminal amino acid sequences of the purified MetB and MetY were shown to be SFDPN and MPKYDNS (data not shown), respectively, findings that are also consistent with the genomic database information regarding *C. glutamicum* [9, 10].

Cystathionine γ-synthase appears to be closely related to *O*-acetylhomoserine sulfhydrylase evolutionarily. It has been reported that the cystathionine γ-synthase of *E. coli* displays some *O*-acylhomoserine sulfhydrylase activity [11, 30], and that *E. coli* and *Leptospira meyeri* cystathionine γ-synthase utilize both *O*-acetylhomoserine and *O*-succinylhomoserine, and are able to participate in the direct sulfhydrylation pathway, as well as in their inherent transsulfuration pathways [8]. *C. glutamicum* MetB and MetY have several features in common and may also be evolutionarily related: First, MetY was found to exhibit 33% sequence identity with MetB (data not shown). Second, both MetB and MetY harbor PLP-binding motifs and require PLP as a cofactor. Third, they exhibit both cystathionine γ-synthase and *O*-acetylhomoserine sulfhydrylase activities. Fourth, they are able to use *O*-acetylhomoserine as a substrate, but not *O*-succinylhomoserine, although MetY can use *O*-succinylhomoserine poorly [10]. Therefore, it is probable that, in *C. glutamicum*, an ancestral gene was duplicated at some point, and a subsequent accumulation of mutations has resulted in the cystathionine γ-synthase and *O*-acetylhomoserine sulfhydrylase within the organism.

In *C. glutamicum*, the transsulfuration pathway may operate preferentially under normal physiological conditions. Besides the data presented in this study, as was pointed out previously, the *metY* gene was unusually highly expressed under methionine-starved conditions, but was repressed strongly at low methionine concentrations, and MetY activity dramatically declined when *C. glutamicum* was grown in complex media [10]. However, the repression of

the *metB* gene and the inhibition of cystathionine γ-synthase by methionine occurred much less profoundly. Based on these findings, Hwang *et al.* [10] proposed preferential synthesis of methionine *via* cystathionine γ-synthase. Kinetic data presented in this study using purified enzymes strongly support the hypothesis.

Although the transsulfuration pathway may be the favorable route for methionine biosynthesis in *C. glutamicum*, choice of the direct sulfhydrylation pathway, which utilizes inorganic sulfide ion as a substrate, may be related to available sulfur sources, together with sulfur assimilation and cysteine biosynthesis, in a manner analogous to lysine biosynthesis in *C. glutamicum* in which the succinylase or dehydrogenase pathways are utilized depending on the availability of the ammonium ion [29]. Direct entry of sulfide into the methionine biosynthetic pathway may provide energetic and evolutionary benefits to the cells. Therefore, the presence of two independent enzymes for the transsulfuration and direct sulfhydrylation pathways, with different regulatory mechanisms, regardless of preferential route, will provide *C. glutamicum* with marked metabolic flexibility.

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