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Regulation of *O*-Acetylserine Sulfhydrylase B by L-Cysteine in *Salmonella typhimurium*

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A technique based on resistance to azaserine was used to isolate mutants lacking *O*-acetylserine sulfhydrylase B, one of two enzymes in *Salmonella typhimurium* capable of synthesizing L-cysteine from *O*-acetyl-L-serine and sulfide. The mutant locus responsible for this defect has been designated *cysM*, and genetic mapping suggests that *cysM* is very close to and perhaps contiguous with *cysA*. Strains lacking either *O*-acetylserine sulfhydrylase B or the second sulfhydrylase, *O*-acetylserine sulfhydrylase A (coded for by *cysK*), are cysteine prototrophs, but *cysK cysM* double mutants were found to require cysteine for growth. *O*-Acetylserine sulfhydrylase B was derepressed by growth on a poor sulfur source, and derepression was dependent upon both a functional *cysB* regulatory gene product and the internal inducer of the cysteine biosynthetic pathway, *O*-acetyl-L-serine. Furthermore, a *cysB*^{c} strain, in which other cysteine biosynthetic enzymes cannot be fully repressed by growth on L-cystine, was found to be constitutive for *O*-acetylserine sulfhydrylase B as well. Thus *O*-acetylserine sulfhydrylase B is regulated by the same factors that control the expression of *O*-acetylserine sulfhydrylase A and other activities of the cysteine regulon. It is not clear why *S. typhimurium* has two enzymes whose physiological function appears to be to catalyze the same step of L-cysteine biosynthesis.

The final step of L-cysteine biosynthesis in *Salmonella typhimurium* and *Escherichia coli* consists of the sulfhydration of *O*-acetyl-L-serine by either free sulfide (3, 12) or by protein-bound hydrodisulfide (17). In *S. typhimurium* two enzymes have been described which are capable of carrying out this reaction using free sulfide as a substrate. *O*-Acetylserine sulfhydrylase A is the better characterized of the two and is coded for by the *cysK* gene (8). Several lines of evidence indicate that *O*-acetylserine sulfhydrylase A participates in cysteine biosynthesis in vivo. In addition to its high in vitro enzyme activity, this protein readily binds to serine acetyltransferase (EC 2.3.1.30) (which is coded for by *cysE* and synthesizes *O*-acetyl-L-serine [12]) to form a complex designated cysteine synthetase (11). The importance of serine acetyltransferase itself in in vivo cysteine biosynthesis has been clearly established (10, 12), and its physical association with *O*-acetylserine sulfhydrylase A strongly implies a similar physiological role for the latter. Furthermore, *O*-acetylserine sulfhydrylase A is a part of the cysteine regulon, since its full expression requires the same factors that are necessary for derepression of other activities of the biosynthetic pathway, i.e., growth on a poor sulfur source such as L-djenkolate or glutathione, the presence of the internal "inducer" *O*-acetyl-L-serine, and a functional *cysB* gene (10). The *cysB* gene (5) codes for a regulatory protein (18) of positive control (15) which is necessary for the derepression of sulfate permease, ATP sulfurylase (EC 2.7.7.4), adenylylsulfate kinase (EC 2.7.1.25), 3′-phosphoadenosine-5′-phosphosulfate reductase, and sulfite reductase (EC 1.8.1.2), in addition to *O*-acetylserine sulfhydrylase A (9, 10).

Although a large number of *cysK* mutants have been isolated and characterized in our laboratories, none has shown a nutritional requirement for cysteine, even though many totally lack *O*-acetylserine sulfhydrylase A activity. However, all such strains, as well as wild type, have small amounts of an enzyme activity which by chromatographic behavior and lack of reactivity with antibody specific for *O*-acetylserine sulfhy-

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drylase A has been identified as *O*-acetylserine sulfhydrylase B (4). It is the presence of this enzyme that is thought to be responsible for the Cys^+ phenotype of all *cysK* mutants. In this communication we describe the isolation and characterization of mutant strains lacking *O*-acetylserine sulfhydrylase B activity, and we present evidence indicating that the normal physiological function of this enzyme too is one of cysteine biosynthesis.

MATERIALS AND METHODS

Bacterial strains and genetic methods. The strains of *S. typhimurium* LT2 used in these studies are listed in Table 1. The *cysB1352* allele causes high-level derepression of all the enzymes of the cysteine regulon regardless of the sulfur source used for growth (10). Therefore, it is designated a *cysB*^{c} (constitutive) allele and gives a Cys^+ phenotype. *cysB484* does not allow expression of the enzymes of the sulfate reduction pathway and gives a Cys^- phenotype, but this allele is *cysB*^{c} for *O*-acetylserine sulfhydrylase A, causing constitutive expression of this enzyme (10). The *cysA* locus is required for sulfate permease activity (6), and *cysK* is the structural gene for *O*-acetylserine sulfhydrylase A (8). Mutations in *cysA* and *cysK* are selected for by resistance to chromate (14) and 1,2,4-triazole (7, 8), respectively.

Transduction was performed as described (7) using the L4 strain of phage P22 (16). Growth on mannitol was used to select for Pts^+.

TABLE 1. *Bacterial strains*

Strain	Genotype	Source
cysA3	*cysA3*	Demerec collection[a]
cysA20	*cysA20*	Demerec collection[a]
cysA197	*cysA197*	Demerec collection[a]
cysA272	*cysA272*	Demerec collection[a]
cysB403	*cysB403*	Demerec collection[a]
DW130	*cysK1751*	(8)
DW18	*cysE2*	(10)
DW42	*trpA160 cysB15*	(10)
DW48	*trpA160 cysB1352*	(10)
SB3751	*trpB223 Δ(cysK-ptsHI182)*	C. Cordaro
TK181	*trpC109 cysK1772*	Triazole-resistant (7, 8) derivative of *trpC109*
DW378	*trpC109 cysK1772 cysM1770*	Cys^- azaserine-resistant derivative of TK181
DW379	*trpC109 cysK1772 cysM1771*	Cys^- azaserine-resistant derivative of TK181
DW384	*trpC109 cysM1770*	Cys^+ revertant of DW378
DW385	*trpC109 cysM1771*	Cys^+ revertant of DW379
DW393	*trpC109 cysK1772 cysB1773*	Nitrosoguanidine-induced Cys^- from TK181
DW164	*pyrE125 cysA*	Chromate resistant (14) Cys^- from *pyrE125*
DW382	*pyrE125 cysK1751*	DW130 × DW164[b]
DW383	*cysE2 cysK1751*	DW18 × DW382[b]
TK1115	*pyrF cysA*	Chromate-resistant (14) Cys^- from *pyrF*
TK1129	*pyrF cysK1751*	DW130 × TK1115[b]
TK1190	*cysB1352 cysK1751*	DW48 × TK1129[b]
TK1191	*cysB484 cysK1751*	*cysB484* × TK1129[b]

[a] Obtained from either P. Hartman or K. E. Sanderson.
[b] Constructed by transduction (donor × recipient).

Media and culture conditions. Bacteria were grown at 37°C with rotary shaking in the minimal salts medium C of Vogel and Bonner (19) in which the sulfur source was either 0.5 mM L-cystine, 0.5 mM L-djenkolate, 0.5 mM reduced glutathione, or 0.8 mM sulfate (10). Glucose (5 g/liter) was added as the usual carbon source; mannitol (2 g/liter) was substituted for selection of Pts^+ recombinants. L-Tryptophan (40 mg/liter) and uracil (10 mg/liter) were included when required for the growth of auxotrophs, and agar (15 g/liter) was added for solid media. Cells grown for enzyme assays were harvested by centrifugation during exponential growth, washed once with 0.1 M Tris-hydrochloride (pH 7.6), and usually frozen overnight as a pellet before processing.

Azaserine selection for cysteine auxotrophs. Approximately 10^8 cells are spread on a minimal agar plate containing 40 mg of L-tryptophan per liter and 0.5 mM glutathione. A 1-cm filter-paper disk containing 1 μmol of freshly prepared azaserine is placed in the center of the plate, and after 48 h resistant colonies appearing in the zone of inhibition are picked and cloned several times on plates lacking azaserine. L-Tryptophan itself gives considerable resistance to azaserine, presumably owing to competition with the *aroP* permease system (1, 2). For this reason we routinely use L-tryptophan even with Trp^+ strains, since in its absence a large percentage of resistant colonies appear to be *aroP* mutants rather than cysteine auxotrophs. With this technique, the majority of azaserine-resistant isolates are cysteine auxotrophs of various different genotypes. Nutritional and genetic tests have shown that these carry mutations either in the regulatory gene *cysB* or in any of the structural genes involved in the pathway of sulfate reduction. The rationale for the use of azaserine in selecting for cysteine auxotrophs is discussed in Results.

Assay for *O*-acetylserine sulfhydrylase. *O*-Acetylserine sulfhydrylase activity was measured by a previously described method (3) which was adapted for use in an autoanalyzer. The enzyme reaction mixture was formed by mixing 0.3 M Tris-hydrochloride (pH 7.2) containing 1 mM Na_2EDTA (0.32 ml/min) with air bubbles (0.23 ml/min), 10 mM Na_2S (0.10 ml/min), 0.1 M *O*-acetyl-L-serine (0.10 ml/min), and enzyme (0.05 ml/min) in a 2.4-mm-ID glass coil at 23°C. After a transit time of 5 min, the reaction was terminated by the addition of 10 mM $NaNO_2$ in 2 N H_2SO_4 at 0.16 ml/min. After retention in a mixing coil for 4 min, 0.17 M ammonium sulfamate was added at 0.23 ml/min, and 3.4 min later a solution composed of 4 parts of 0.4 M sulfanilamide in 0.4 N HCl, 2 parts of 7.7 mM *N*-naphthylethylenediamine dihydrochloride in 0.4 N HCl, and 1 part of 74 mM $HgCl_2$ in 0.4 N HCl was added at 1.00 ml/min. Four minutes later the stream was debubbled and directed through a 1-cm-light path flow cell in which absorbance at 540 nm was measured in a Gilford model 2000 recording spectrophotometer. A 1-U/ml solution of *O*-acetylserine sulfhydrylase, as determined by our manual assay in which a unit catalyzes the formation of 1 μmol of L-cysteine per min, gives an absorbancy at 540 nm of 2.2 in the autoanalyzer assay.

The two O-acetylserine sulfhydrylases were resolved by a modification of the method of Becker and Tomkins (4). Cells (1 to 2 g) were suspended in 3 volumes of 0.1 M Tris-hydrochloride (pH 7.6) and disrupted by sonic oscillation. After centrifugation at 27,000 × *g* for 30 min, the supernatant was mixed with 0.5 volume of neutralized 10% streptomycin sulfate, and the resulting precipitate was removed by centrifugation and discarded. The supernatant was then treated with solid ammonium sulfate to obtain the protein fraction precipitating between 0.40 and 0.70 saturation. This material, containing virtually all the O-acetylserine sulfhydrylase activity, was dissolved in a small volume of 0.1 M Tris-hydrochloride (pH 7.6) containing 1 mM 2-mercaptoethanol (buffer A) and desalted by gel filtration on Sephadex G-25 equilibrated in the same buffer. From 10 to 20 mg of protein was then applied to a column (8 cm by 1.8 cm^2) of DE23 anion-exchange cellulose equilibrated in buffer A, and protein was eluted at 23°C with a 250-ml gradient of 0 to 0.3 M NaCl in buffer A at a flow rate of 0.15 ml/min. Fractions of 3 ml were collected and assayed with the autoanalyzer for O-acetylserine sulfhydrylase both before and after treatment (8) with a 5- to 20-fold excess of rabbit antibody, which inactivates O-acetylserine sulfhydrylase A but not O-acetylserine sulfhydrylase B (4).

With this technique, cystine-grown wild type gives a profile of enzyme activity with three distinct but incompletely resolved peaks (Fig. 1). The first peak is free O-acetylserine sulfhydrylase A; the second is cysteine synthetase, a complex of serine acetyltransferase and O-acetylserine sulfhydrylase A; and the third is O-acetylserine sulfhydrylase B. After treatment with antibody to O-acetylserine sulfhydrylase A, only the O-acetylserine sulfhydrylase B peak is noted. O-Acetylserine sulfhydrylase levels quantified in this manner are expressed in terms of units per milligram of protein

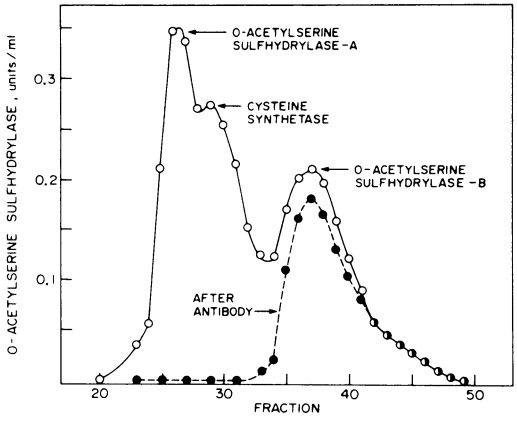

FIG. 1. *Elution of O-acetylserine sulfhydrylase activities from a DE23 anion-exchange column. Enzyme was assayed before and after preincubation with a 5- to 20-fold excess of antibody, which specifically inactivates O-acetylserine sulfhydrylase A but not O-acetylserine sulfhydrylase B. Cysteine synthetase is a complex of O-acetylserine sulfhydrylase A and serine acetyltransferase.*

applied to the DE23 column, giving specific activities that are about twice that expected in unfractionated crude extracts.

Although this method for assaying O-acetylserine sulfhydrylase B is useful for repressed wild-type cells, it is unsatisfactory for derepressed cells, where an almost 100-fold increase in O-acetylserine sulfhydrylase A obscures the O-acetylserine sulfhydrylase B peak. Antibody is of no help in this regard because it is capable of inactivating no more than 95% of O-acetylserine sulfhydrylase A. Therefore, to examine the regulation of O-acetylserine sulfhydrylase B, many of our experiments were performed in strains carrying either the *cysK1751* or *cysK1772* alleles, which code for normally regulated O-acetylserine sulfhydrylase A proteins having less than 2% of wild-type specific activity (8).

Materials. Azaserine was purchased from Calbiochem, and autoanalyzer glassware and tubing were obtained from Evergreen Scientific. Rabbit antibody to purified O-acetylserine sulfhydrylase A was prepared as described (11). The sources of other chemicals have been cited (8).

RESULTS

Isolation of mutant strains lacking O-acetylserine sulfhydrylase B. Mutant strains lacking O-acetylserine sulfhydrylase B activity were first isolated by mutagenizing a Cys^+ *cysK* strain lacking detectable O-acetylserine sulfhydrylase A activity and then scoring for Cys^- colonies after penicillin selection. Several of these mutants grew on L-cystine but would not grow on sulfate, sulfite, sulfide, O-acetyl-L-serine, or combinations of the latter compounds. Enzyme assays confirmed that these isolates contained no O-acetylserine sulfhydrylase activity. A more convenient method based on azaserine resistance was used for the isolation of the strains described here. Azaserine (O-diazoacetyl-L-serine) is an analog of O-acetyl-L-serine, and we find that it is an excellent substrate for both O-acetylserine sulfhydrylase A and O-acetylserine sulfhydrylase B, giving L-cysteine and diazoacetic acid as products of its reaction with sulfide (data not shown). Mutant strains unable to reduce sulfate to sulfide are resistant to azaserine when grown on minimal agar containing sulfate and a poor sulfur source such as L-djenkolate or glutathione. Presumably, azaserine toxicity in *S. typhimurium* results in large part from the generation of diazoacetic acid, which in wild type is derived from the reaction of azaserine with sulfide. *cysK* mutants are also relatively resistant to azaserine, and we assume that this is because they lack O-acetylserine sulfhydrylase A, one of the two enzymes which catalyze the reaction between azaserine and sulfide. By selecting for a higher level of azaserine resistance in a *cysK* strain on plates containing L-djenkolate (to permit growth of Cys^- mutants) it is

relatively easy to obtain mutants which lack both *O*-acetylserine sulfhydrylase A and *O*-acetylserine sulfhydrylase B activities.

Strains DW378 and DW379 were derived from the *cysK* strain TK181 by this technique, and they were found by both nutritional phenotype and enzyme assay to lack any *O*-acetylserine sulfhydrylase activity. Their Cys$^+$ revertants DW384 and DW385 were selected for further study because they were found to contain normal levels of *O*-acetylserine sulfhydrylase A and no detectable *O*-acetylserine sulfhydrylase B. We have given the designation *cysM* to the mutant locus responsible for the absence of *O*-acetylserine sulfhydrylase B activity in these strains. The *trpC cysK*$^+$ *cysM* strains DW384 and DW385 have no nutritional requirements other than tryptophan and grow as well on sulfate as sole sulfur source as do wild type or *cysK cysM*$^+$ strains. Identical results have been found in *trpC*$^+$ *cysM* derivatives, which are Trp$^+$. It appears, therefore, that either of the two *O*-acetylserine sulfhydrylase enzymes is sufficient for cysteine prototrophy under our usual conditions of aerobic growth and that neither enzyme is required for some other essential metabolic function.

Genetic mapping of *cysM*. Preliminary experiments indicated that *cysK* and *cysM* are cotransducible by P22. Since *cysK* is known to be very close to the *pts* locus we measured the linkage between *cysM* and *pts*. Using the Cys$^+$ Δ(*cysK-ptsHI*) strain SB3751 as a recipient and two different Cys$^-$ *cysK cysM* donors, we selected for Pts$^+$ and scored for Cys. A *cysK*$^+$ recombinant is expected very rarely or not at all from such a cross, depending on whether the recipient's *cysK* deletion includes the site in which the donor *cysK* locus is mutated. Therefore all or virtually all Cys$^+$ recombinants should be *cysM*$^+$ *cysK*, and all Cys$^-$ recombinants of course are *cysK cysM*. In the experiments summarized in Table 2, *cysM* was cotransduced with *pts* at a frequency of 52 to 55%.

The linkage between *pts* and *cysM* is very close to that known for *pts* and *cysA* (8). Three-point tests could not be performed to determine the relative order of these three loci owing to the lack of a reliable scoring method for *cysM* in Cys$^+$ strains. However, direct assay of a strain carrying *cysA20*, a deletion which covers all tested *cysA* point mutations (13), showed complete absence of *O*-acetylserine sulfhydrylase B activity. Three *cysA* point mutants, *cysA3*, *cysA197*, and *cysA272*, had normal levels of *O*-acetylserine sulfhydrylase B, suggesting that the loss of this enzyme activity in *cysA20* is due to a deletion extending through *cysA* into *cysM*. *O*-Acetylserine sulfhydrylase A was present in all four *cysA* strains.

TABLE 2. *Genetic mapping of cysM*[a]

Donor[b]	Recombinants		
	Total no. of Pts$^+$	No. of Cys$^-$ Pts$^+$	Percent co-transduction
DW378 (*cysM1170 cysK1772*)	94	49	52
DW379 (*cysM1171 cysK1772*)	97	53	55

[a] Transduction was performed with the L4 strain of P22. Recipient was SB3751 [Δ(*cysK-ptsHI*)]. Selection was for Pts$^+$ on plates containing mannitol as a carbon source and L-cystine.

[b] Only the relevant genotypes are given.

Regulation of *O*-acetylserine sulfhydrylase B activity. In cystine-grown, repressed wild type, *O*-acetylserine sulfhydrylase B comprises about 30% of the total *O*-acetylserine sulfhydrylase activity; the remainder is *O*-acetylserine sulfhydrylase A, approximately 25% of which is found complexed to serine acetyltransferase as cysteine synthetase. Levels of free *O*-acetylserine sulfhydrylase A are increased 50- to 100-fold during derepression by growth on a limiting sulfur source such as L-djenkolate or glutathione, making it impossible to estimate accurately the *O*-acetylserine sulfhydrylase B peak from a DE23 column even with the aid of antibody to *O*-acetylserine sulfhydrylase A. Therefore, to study the regulation of the latter enzyme it was often necessary to use strains with mutant *cysK* alleles which code for *O*-acetylserine sulfhydrylase A proteins having little or no activity. In the *cysK* strains DW130, TK181, and TK1129, growth on a poor sulfur source gives *O*-acetylserine sulfhydrylase B levels 4- to 14-fold higher than those found in L-cystine-grown cells (Table 3). This derepression of enzyme activity also requires a functional *cysB* gene product, as deduced from the fact that sulfur-deprived cultures of *cysB403*, DW42, and DW393 (carrying three separate *cysB* alleles) all show little or no increase in *O*-acetylserine sulfhydrylase B activity over the low levels found in L-cystine-grown cultures.

In contrast, the *cysB*^{c} allele *cysB1352*, which is present in TK1190, causes high-level expression of *O*-acetylserine sulfhydrylase B in both L-cystine-grown and sulfur-deprived cells. This effect is similar to that observed for other enzymes of the cysteine regulon (10). *cysB484*, which is *cysB* (negative) for the enzymes of sulfate reduction but *cysB*^{c} for *O*-acetylserine sulfhydrylase A, was found to be *cysB* (negative)

TABLE 3. *O-Acetylserine sulfhydrylase B expression in various cysteine mutants*

Strain	Relevant genotype	O-Acetylserine sulfhydrylase B (units/mg of protein)	
		Grown on L-cystine	Grown on L-djenkolate
Wild type		0.199	—[a]
cysA20	Δ*cysA*	<0.02	—
DW384	*cysM*	<0.02	—
DW385	*cysM*	<0.02	—
DW130	*cysK*	0.158	0.580
TK181	*cysK*	0.045	0.713
TK1129	*cysK*	0.104	0.512
cysB403	*cysB*	0.072	0.079
DW42	*cysB*	0.062	0.122
DW393	*cysB cysK*	0.115	0.121
TK1191	*cysB484 cysK*	0.097	0.092
TK1190	*cysB*^{c} *cysK*	0.542	0.492
DW18	*cysE*	0.148	0.076
DW383	*cysE cysK*	0.046	0.188[b] (0.414)[c]

[a] —, Not measured owing to large excess of *O*-acetylserine sulfhydrylase A.

[b] Grown on 0.5 mM glutathione because of very poor growth on L-djenkolate.

[c] Grown on 0.5 mM glutathione plus 2 mM *O*-acetyl-L-serine.

with respect to *O*-acetylserine sulfhydrylase B expression.

O-Acetyl-L-serine is also required for derepression of the cysteine regulon in *cysB*$^+$ strains. Therefore *cysE* strains are not derepressed for these enzymes owing to the lack of serine acetyltransferase, the enzyme required for *O*-acetyl-L-serine synthesis. DW18, carrying *cysE2*, could not be derepressed for *O*-acetylserine sulfhydrylase B by growth on glutathione, and DW383, also carrying *cysE2*, was only partially derepressed for this enzyme. The addition of 2 mM *O*-acetyl-L-serine to a glutathione culture of the latter strain, however, gave a greater than twofold rise in *O*-acetylserine sulfhydrylase B activity.

DISCUSSION

The lack of measurable *O*-acetylserine sulfhydrylase B activity in *cysM* mutants and the Cys$^-$ phenotype of *cysK cysM* strains suggests that *cysM* is the structural gene for this enzyme. A more definite designation, however, must await the demonstration of an altered enzyme protein in a *cysM* strain. Although our genetic mapping data are insufficient to establish the precise chromosomal location of *cysM*, its linkage to *pts* and the absence of *O*-acetylserine sulfhydrylase B in the deletion strain *cysA20* suggest close linkage and perhaps even contiguity between *cysM* and *cysA*.

Since *cysM* mutants display no nutritional requirements other than the Cys$^-$ phenotype of *cysK cysM* double mutants, it seems unlikely that *O*-acetylserine sulfhydrylase B is simply a spurious activity of an enzyme with another physiological function. Of course the same argument might be made against the role of this enzyme in L-cysteine biosynthesis because *cysK*$^+$ *cysM* strains grow as wild type on sulfate. However, the apparent close linkage of *cysM* with *cysA*, and our finding that *O*-acetylserine sulfhydrylase B is regulated by the same nutritional and genetic factors that control expression of other cysteine biosynthetic activities, indicate that this enzyme is a part of the cysteine regulon and plays a role in cysteine biosynthesis other than that imposed upon it in a *cysK* strain.

Having used similar reasoning in concluding that the physiological function of *O*-acetylserine sulfhydrylase A is to synthesize L-cysteine, we are now confronted with the perplexing question of why there should exist two sulfhydrylase enzymes, either one of which is sufficient for normal growth on sulfate. In general it is very unusual for an organism such as *S. typhimurium* to carry and express genes for two different enzymes which carry out the same biochemical reaction. One obvious explanation for this situation is that they may not catalyze exactly the same reaction in vivo. For example, even though both enzymes use free sulfide as a reactant with *O*-acetyl-L-serine in our in vitro assay, the actual sulfur donor in vivo may be different for each enzyme. Perhaps one enzyme prefers free sulfide and the other is required for the direct utilization of carrier-bound sulfide, such as the thioredoxin hydrodisulfide intermediate proposed by Tsang and Schiff in *Escherichia coli* (17).

An interesting observation (our unpublished data) which may bear on this problem is that although *cysM* strains grow well aerobically on sulfate, they are cysteine bradytrophs under anaerobic conditions. In contrast, *cysK* strains, which lack the major portion of total *O*-acetylserine sulfhydrylase activity as measured by our in vivo assay, grow on sulfate as well as wild type both aerobically and anaerobically. Perhaps a careful examination of the pathway of L-cysteine biosynthesis and its regulation during anaerobiosis will eventually lead to an explanation for the existence of two different *O*-acetylserine sulfhydrylases in *S. typhimurium*.

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