*REGULATION OF HOMOSERINE BIOSYNTHESIS BY L-CYSTEINE, A TERMINAL METABOLITE OF A LINKED PATHWAY**

By Prasanta Datta

DEPARTMENT OF BIOLOGICAL CHEMISTRY, UNIVERSITY OF MICHIGAN, ANN ARBOR

Communicated by Martin D. Kamen, June 23, 1967

In bacteria, the amino acid homoserine is a key branch-point intermediate in the synthesis of several amino acids of the aspartic pathway.[1] On the one hand, homoserine is converted to threonine (and thus to isoleucine), and through a separate sequence of reactions is transformed to methionine.[1] In the latter sequence, a succinylated product of homoserine is condensed with cysteine to produce cystathionine,[2-5] a precursor of methionine. Recent studies (see refs. 6 and 7 for up-to-date reviews on the subject) with several microorganisms have revealed that the synthesis of homoserine is regulated by various combinations of repression and/or feedback inhibition controls of early biosynthetic enzymes of the aspartic pathway by several end-product metabolites. One of these enzymes, homoserine dehydrogenase, catalyzes the pyridine nucleotide-linked reduction of aspartate β-semialdehyde to homoserine. In the various bacteria that have been examined, this dehydrogenase as well as the two earlier enzymes of the pathway (namely, aspartokinase and aspartate β-semialdehyde dehydrogenase) are not subject to feedback inhibition control by the end product methionine.

This report describes the results of experiments on the control of activity of several bacterial homoserine dehydrogenases by L-cysteine, a terminal metabolite of a linked or connecting pathway, required for the synthesis of cystathionine.[2-5] The findings suggest that the size of the homoserine pool in bacterial cells must depend, at least in part, on complex interdependent regulatory interactions of both cysteine and threonine on homoserine dehydrogenase.

Materials and Methods.—Organisms and culture media: *Escherichia coli* K12, strain W3110 was a gift from Dr. R. L. Somerville. The organism was grown aerobically at 37° in the minimal glucose-salt medium of Vogel and Bonner.[8] *Rhodospirillum rubrum* S1 and *Rhodopseudomonas capsulatus* were from the collection of Dr. H. Gest; these were grown photosynthetically at 23° in a synthetic malate + glutamate medium[9] as described previously.[10, 11] A strain of *Rhodopseudomonas spheroides* M29.5, methionine- and leucine-requiring, was kindly provided by Dr. W. R. Sistrom. This organism was grown aerobically in darkness at 28° in the medium S of Lascelles[12] supplemented with 50 μg/ml each of L-methionine and L-leucine.

Homoserine dehydrogenase preparations: The *E. coli* homoserine dehydrogenase was partially purified from sonic extracts of washed cells by treatment with streptomycin, ammonium sulfate fractionation, and by heat treatment using the procedure outlined by Patte *et al.*[13] The preparation had a specific activity of 0.85, in terms of increase of absorbancy at 340 mμ min^{-1} mg^{-1} protein under standard assay conditions in the reverse direction (see below). The enzyme from *R. rubrum* was a 1500-fold purified preparation obtained by a slightly modified procedure of Datta and Gest.[10] A fivefold purified enzyme from *Rps. spheroides* was obtained as a by-product during the purification of threonine deaminase on a DEAE[15]

column (to be published). The *Rps. capsulatus* homoserine dehydrogenase was purified approximately 300-fold by ammonium sulfate fractionation and DEAE column chromatography (Datta, unpublished).

Reagents: All amino acids and their derivatives were purchased either from Calbiochem or from Mann Research Chemicals. DL-Aspartic β-semialdehyde was prepared according to the method of Black.[14] NAD, NADP, and NADPH were obtained from the Sigma Chemical Co. Solutions of cysteine, cysteine derivatives, and other reducing agents were freshly prepared and used within a few hours.

Enzyme assay: Homoserine dehydrogenase activity was measured spectrophotometrically at 25° using a Zeiss PMQ II spectrophotometer. For the *E. coli* enzyme, the standard reaction mixture in the forward direction assay (ASA + NADPH→HS + NADP) contained, in a final volume of 1 ml: potassium phosphate buffer pH 6.5, 100 μmoles; EDTA, 1 μmole; KCl, 400 μmoles; NADPH, 0.2 μmole; L-ASA, 0.17 μmole; and enzyme. For the reverse direction assay (HS + NADP→ASA + NADPH), the reaction mixture contained: Tris-phosphate buffer pH 9.0, 100 μmoles; EDTA, 1 μmole; KCl, 400 μmoles; NADP, 0.36 μmole; L-homoserine, 10 μmoles; and enzyme. The oxidation or reduction, respectively, of NADPH or NADP was measured by following absorbance changes at 340 mμ in cuvettes with 1-cm light path. Enzyme activities are expressed as $\Delta A_{340\ m\mu}\ min^{-1}$.

Activities of the other enzymes were measured as described above, except that the reaction mixtures specified by Datta and Gest[10] were used.

Protein determination: Protein was estimated either spectrophotometrically[16] or by the procedure of Lowry *et al.*[17]

Results.—Cysteine inhibition of homoserine dehydrogenase activity: The results presented in Table 1 show that L-cysteine is a strong inhibitor of the homoserine dehydrogenase activity of *E. coli*. With 10 mM L-cysteine the inhibition was about 85 per cent in the forward direction assay, and almost 95 per cent in the reverse direction. When NAD was used instead of NADP in the reverse direction assay, L-cysteine was similarly strongly inhibitory. Two other amino acids, namely homocysteine and cystathionine, showed some inhibition; in view of the possibility of nonspecific effects at the high concentrations used, these inhibitions have dubious significance. The striking effect of L-cysteine is quantitatively

TABLE 1

EFFECT OF VARIOUS AMINO ACIDS ON THE ACTIVITY OF *E. coli* HOMOSERINE DEHYDROGENASE

Addition	Enzyme Activity (Δ A 340 mμ min^{-1})		
	ASA → HS	HS → ASA	
None	0.044	0.036	(0.020)
L-isoleucine	0.047	—	—
L-methionine	0.039	0.033	—
DL-homocysteine	0.036	—	—
DL-allo, cystathionine	0.035	0.031	—
L-threonine	0.009	0.010	(0.003)
L-cysteine	0.007	0.002	(0.002)

Enzyme activity was measured using standard assay conditions (see text). For the forward direction assay, 5.2 μg protein were used; for the reverse direction assay, 36 μg. The figures in parentheses are for reverse direction assays using NAD as the coenzyme replacing NADP. All amino acids were 10 mM except DL-homocysteine and DL-allo, cystathionine, which were 20 mM. The latter amino acid was a mixture of all four isomers. Enzyme was added to start the reaction.

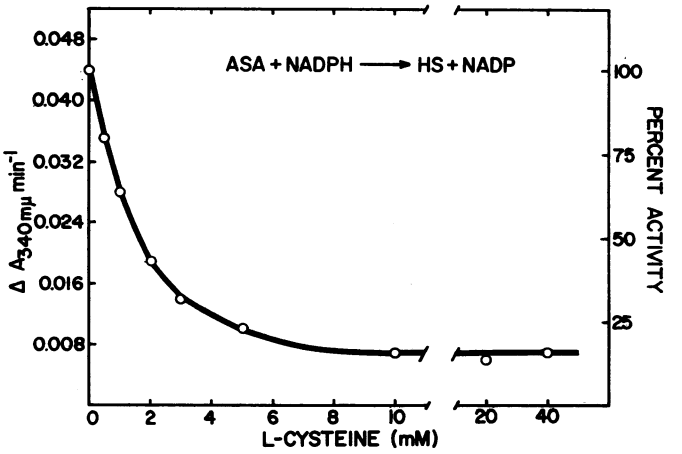

FIG. 1.—Progressive inhibition of *E. coli* homoserine dehydrogenase activity with increasing L-cysteine concentration. In each assay (forward direction), 5.2 μg protein were used under the conditions described in the text.

comparable to the well-known feedback inhibition of this enzyme by L-threonine.[7]

Figure 1 depicts the progressive inhibition of enzyme activity with increasing L-cysteine concentration, using ASA and NADPH as substrates. Since the inhibition reaches a limiting value and is not complete even at 40 mM L-cysteine, it appears that the inhibitor binding site is distinct from the substrate site. An alternative explanation of these results is that *E. coli* produces two kinds of homoserine dehydrogenase, one of which is repressible by methionine[18] and insensitive to L-cysteine inhibition.

The kinetic data presented in Figure 2 show that inhibition of *E. coli* homoserine dehydrogenase activity by L-cysteine is competitive with respect to homoserine. An apparent K_i of about 0.12 mM can be calculated from the data shown. Patte *et al.*[19] have reported that with the *E. coli* K12 enzyme, L-threonine is a noncompetitive inhibitor with respect to homoserine and ASA. These kinetic results suggest that L-cysteine and L-threonine may occupy separate sites on the enzyme molecule. More direct evidence to support this notion is given below.

Specificity experiments: The stereoisomer of L-cysteine and several derivatives of the amino acid were tested for their ability to influence *E. coli* homoserine dehydrogenase activity (Table 2). D-Cysteine at 10 mM concentration did not inhibit enzyme activity in either direction. Modification of either the −SH or the −COOH, or both, of L-cysteine resulted in abolition of the inhibitory property except in the instance of S-methyl L-cysteine, which at 10 mM concentration inhibited the enzyme activity appreciably (35%). The inhibition was apparent only when ASA was used as the substrate, suggesting that the cysteine derivative may act as an ASA analogue.

Control experiments with dithiothreitol and β-mercaptoethanol (Table 2) as well as reduced glutathione showed that these reducing agents did not significantly inhibit enzyme activity. Furthermore, no inhibition of homoserine dehydrogenase activity was observed when an enzyme solution was preincubated with 0.1 *M*

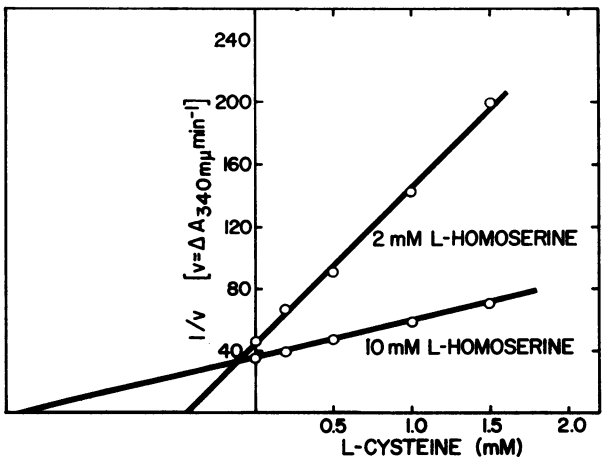

FIG. 2.—Competitive inhibition of *E. coli* homoserine dehydrogenase activity by L-cysteine. Data are plotted as the reciprocal of the initial rates against L-cysteine concentrations at two different concentrations of L-homoserine as indicated. Activity was measured in the reverse direction assay using 36 μg protein as described in the text.

NH_2OH or 10 mM EDTA. The latter results eliminate the possibility that cysteine inhibition may be due to nonspecific chelation of metal ions required for enzyme activity.

Separate regulatory sites for cysteine and threonine: *E. coli* homoserine dehydrogenase can be desensitized with respect to threonine by mild heat treatment.[19] In order to make a preparation of this kind, an enzyme solution (1.16 mg protein/ml in 0.02 *M* potassium phosphate buffer pH 7.2 containing 0.2 *M* KCl and 0.2 mM EDTA) was heated at 55° for five minutes and chilled immediately. Assay of samples diluted 100-fold showed that two thirds of the original enzyme activity was lost. Although the remaining activity was virtually insensitive to L-threonine inhibition, it was still susceptible to L-cysteine inhibition (65% inhibition at 10 mM L-cysteine in the forward direction assay). This observation strongly suggests the existence of at least two separate regulatory sites on the enzyme, one specific for the binding of L-cysteine and another specific for L-threonine binding.

TABLE 2

SPECIFICITY OF L-CYSTEINE INHIBITION OF HOMOSERINE DEHYDROGENASE ACTIVITY OF *E. coli*

Addition	Enzyme Activity (Δ A 340 mμ min^{-1}) ASA → HS	HS → ASA	
None	0.045	0.035	(0.020)
L-cysteine	0.007	0.002	(0.002)
D-cysteine	0.041	0.032	(0.017)
L-cysteic acid	0.048	0.036	—
L-cysteine ethyl ester	0.041	0.031	—
S-methyl L-cysteine	0.030	0.034	—
Taurine	0.048	—	—
β-Mercaptoethanol	0.036	0.030	—
Dithiothreitol	0.041	0.032	—

For experimental detail and explanations see legend of Table 1. All compounds were 10 mM.

TABLE 3

L-CYSTEINE INHIBITION OF ACTIVITY OF SEVERAL BACTERIAL HOMOSERINE DEHYDROGENASES

Source of Enzyme	Per Cent Activity in the Presence of L-cysteine (2 mM)	L-cysteine (10 mM)	D-cysteine (10 mM)
Rhodospirillum rubrum	70	40	140
Rhodopseudomonas capsulatus	57	22	97
Rhodopseudomonas spheroides	54	21	96
Escherichia coli	44	6	92

Enzyme activity was measured in the reverse direction using standard conditions as described in the text. Amounts of protein used for individual assays were as follows: *R. rubrum*, 20 μg; *Rps. capsulatus*, 17 μg; *Rps. spheroides*, 260 μg; and *E. coli*, 36 μg. In all cases enzyme was added to initiate the reaction. The effect of D-cysteine on *R. rubrum* enzyme activity is not considered unusual since a number of other L- and D-amino acids stimulate, particularly at high concentrations (see ref. 10).

Cysteine inhibition of other bacterial homoserine dehydrogenases: One common property of the bacterial homoserine dehydrogenases studied thus far (see ref. 7) is their susceptibility to L-threonine inhibition. The possibility that inhibition by L-cysteine is also a general property is supported by the data of Table 3. The results presented clearly show that enzyme activities from three photosynthetic bacteria are also subject to inhibition by L-cysteine.

Preliminary evidence indicating the physiological implications of L-cysteine effects was obtained from relevant growth experiments. Growth of *R. rubrum* was strongly inhibited by addition of 6.6×10^{-4} *M* L-cysteine to the culture medium presumably due to interference with methionine and threonine biosynthesis. The inhibition was partially relieved by further addition of L-methionine (4.4×10^{-4} *M*), and almost completely abolished by addition of L-methionine and L-threonine (5×10^{-4} *M*).

Discussion.—The first three enzymes of the branched aspartic pathway catalyze the formation of *common* intermediates required in the synthesis of four amino acid end products, namely, methionine, threonine, isoleucine, and lysine. Regulation of the rates of synthesis of these intermediates in bacteria is presumably facilitated, at least in part, by negative and positive feedback effects on activity of the first and third enzymes (aspartokinase and homoserine dehydrogenase, respectively) by three of the four end-product amino acids. From presently available information it seems that in bacteria, metabolites unique to the methionine branch do not exert feedback inhibition control on the first three enzymes of the aspartic pathway. Because of the absence of methionine feedback effects, it has been suggested[20] that a "slow" repression control of the synthesis of aspartokinase and homoserine dehydrogenase (superimposed on the other controls) may suffice to ensure regulation of the intracellular methionine pool. The regulatory plan is somewhat different in the yeast *Saccharomyces cerevisiae* in that methionine inhibits homoserine dehydrogenase activity,[21] and it is possible that this reflects differences between yeast and bacteria[22, 23] in respect to the actual carbon transformations involved in lysine synthesis.

Since homoserine is the branch-point precursor of *both* threonine and methionine, feedback modulation of homoserine dehydrogenase activity by both threonine and a metabolite involved in methionine synthesis would evidently provide a means of adjusting the rate of homoserine synthesis to appropriate values. In all the organisms examined thus far, L-threonine is, in fact, an effective feedback

inhibitor. The present findings suggest the following rationale for additional control by cysteine. A temporal accumulation of cysteine would presumably accelerate cystathionine (thus, methionine) synthesis if a sufficient amount of homoserine were available. Overproduction of methionine would be prevented by end-product control (by methionine) of enzymes unique for methionine biosynthetic reactions and/or by control of the homoserine concentration in the cell through L-cysteine inhibition of homoserine dehydrogenase activity. Similarly, overproduction of threonine would be avoided by analogous feedback controls exerted by this amino acid (on homoserine dehydrogenase[7] and homoserine kinase[24, 25]). According to this interpretation, the intracellular homoserine pool size would be controlled in large measure through complex interdependent regulatory interactions of cysteine *and* threonine on homoserine dehydrogenase.

It should be emphasized that cysteine, *per se*, is not a direct intermediate in bacterial methionine synthesis, but rather the "terminal" metabolite of a unique biosynthetic pathway which is subject to regulation by various controls including end-product inhibition control.[26] The pathway for cysteine biosynthesis is, however, linked to the aspartic pathway in that cysteine donates its three-carbon unit for a condensation reaction resulting in cystathionine synthesis.[1, 2–5] Thus, cysteine plays a dual regulatory role by controlling the activity of enzymes involved in its own biosynthesis and by functioning as a regulatory modifier of a critical enzyme of a linked, or interconnecting, pathway. One might predict that similar multiple regulatory effects of a "terminal" metabolite will be found in other interconnecting pathways of biosynthesis.

Summary.—In addition to feedback inhibition control by L-threonine, homoserine dehydrogenase activity of *E. coli* and of three representative photosynthetic bacteria is subject to inhibition by L-cysteine. The effect is specific for L-cysteine; D-cysteine and L-cysteine derivatives do not inhibit. Desensitization experiments indicate that L-threonine and L-cysteine occupy separate sites on the enzyme molecule. The significance of the L-cysteine effect on biosynthesis of homoserine and the regulation of the aspartic pathway are discussed.

I thank Lillian L. Lu for expert technical assistance.

* Supported by grants from the National Institutes of Health (GM-13804) and from the National Science Foundation (GB-4713).

[1] Umbarger, E., and B. D. Davis, *The Bacteria*, ed. I. C. Gunsalus and R. Y. Stanier (New York: Academic Press, 1962), vol. 3, p. 167.

[2] Flavin, M., C. Delavier-Klutchko, and C. Slaughter, *Science*, **143**, 50 (1964).

[3] Rowbury, R. J., and D. D. Woods, *J. Gen. Microbiol.*, **36**, 341 (1964).

[4] Kaplan, M. M., and M. Flavin, *Biochim. Biophys. Acta*, **104**, 390 (1965).

[5] Kaplan, M. M., and M. Flavin, *J. Biol. Chem.*, **241**, 4463 (1966).

[6] Cohen, G. N., *Ann. Rev. Microbiol.*, **19**, 105 (1965).

[7] Stadtman, E. R., *Advan. Enzymol.*, **28**, 41 (1966).

[8] Vogel, H. J., and D. M. Bonner, *J. Biol. Chem.*, **218**, 97 (1956).

[9] Ormerod, J. G., K. S. Ormerod, and H. Gest, *Arch. Biochem. Biophys.*, **94**, 449 (1961).

[10] Datta, P., and H. Gest, *J. Biol. Chem.*, **240**, 3023 (1965).

[11] Datta, P., and H. Gest, these PROCEEDINGS, **52**, 1004 (1964).

[12] Lascelles, J., *Biochem. J.*, **62**, 78 (1956).

[13] Patte, J.-C., P. Truffa-Bachi, and G. N. Cohen, *Biochim. Biophys. Acta*, **128**, 426 (1966).

[14] Black, S., *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, 1963), vol. 6, p. 622.

[15] The following abbreviations are used: DEAE, *O*-(diethylaminoethyl) cellulose; NAD, nicotinamide adenine dinucleotide; NADP and NADPH, oxidized and reduced forms, respectively, of nicotinamide adenine dinucleotide phosphate; ASA, aspartate β-semialdehyde; HS, homoserine; EDTA, ethylenediaminetetraacetate-sodium salt; Tris, Tris (hydroxymethyl) amino methane.

[16] Warburg, O., and W. Christian, *Biochem. Z.*, **310**, 384 (1941).

[17] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

[18] Patte, J.-C., G. LeBras, and G. N. Cohen, *Biochim. Biophys. Acta*, **136**, 245 (1967).

[19] Patte, J.-C., G. LeBras, T. Loviny, and G. N. Cohen, *Biochim. Biophys. Acta*, **67**, 16 (1963).

[20] Burlant, L., P. Datta, and H. Gest, *Science*, **148**, 1351 (1965).

[21] Karassevitch, Y., and H. de Robichon-Szulmajster, *Biochim. Biophys. Acta*, **73**, 414 (1963).

[22] Strassman, M., and S. Weinhouse, *J. Am. Chem. Soc.*, **75**, 1680 (1953).

[23] Strassman, M., and L. N. Ceci, *Biochem. Biophys. Res. Commun.*, **14**, 262 (1964).

[24] Wormser, E. H., and A. B. Pardee, *Arch. Biochem. Biophys.*, **78**, 416 (1958).

[25] Ning, C., and H. Gest, these PROCEEDINGS, **56**, 1823 (1966).

[26] Kredich, N. M., and G. M. Tomkins, *J. Biol. Chem.*, **241**, 4955 (1966).