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Cysteine Starvation, Isoleucyl-tRNA^{Ile}, and the Regulation of the *ilvGEDA* Operon of *Escherichia coli**

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The involvement of undermodified tRNA in the regulation of the *ilvGEDA* operon has been investigated using *Escherichia coli* C6, a *relA*$^-$, Cys$^-$, Met$^-$ mutant. This strain accumulates thionucleotide-deficient or methyl-deficient tRNA when starved for cysteine or methionine, respectively. The levels of threonine deaminase, the *ilvA* gene product, and transaminase B, the *ilvE* gene product, were both lower in cysteine-starved cells, as compared with either growing or methionine-starved cultures. When cysteine was added to cysteine-starved cells, growth ensued promptly and both enzyme activities returned to control levels. Treatment of recovering cultures with valine limited growth by isoleucine limitation, but did not cause a derepression of the *ilvGEDA* operon. Valine treatment of nonstarved or methionine-starved cells led to the expected increase in threonine deaminase and transaminase B activities. Cysteine-starved cells slowly regained the ability to derepress the operon after 3 h of recovery in complete medium. In contrast, the induction of the *lac* operon was normal in cysteine-starved cultures, even in the presence of valine. The loss of derepressibility of the *ilvGEDA* operon was correlated with the presence of a kinetically and chromatographically altered tRNA^{Ile} in cysteine-starved cells. No changes in tRNA^{Ile} were observed after methionine starvation. Using the periodate method, we found that the charging of tRNA^{Ile} increased from the normal level of 60 to 80% or greater after starvation for cysteine. Under conditions where the *ilvGEDA* operon was fully derepressed in nonstarved cells, the charging of tRNA^{Ile} fell to 27%. Unexpectedly, nearly identical results were obtained with cysteine-starved cells after an identical derepression test. These results suggest that factors other than the aminoacylation state of tRNA^{Ile} may be important in the regulation of this operon. In particular, modifications to tRNA which involve cysteine may be necessary for controlling the expression of the *ilvGEDA* operon in *E. coli*.

Transfer RNA has been implicated in the regulation of several amino acid biosynthetic enzyme operons in *Escherichia coli* (1, 2). The *ilvGEDA* operon of *E. coli* K-12 is multivalently controlled by the levels of aminoacylated tRNA^{Ile}, tRNA^{Leu}, and tRNA^{Val} (3, 4), presumably through an attenuation mechanism (5, 6). The regulatory region of this operon has been sequenced, and found to code for a leader RNA, within which is a message for a peptide rich in isoleucine, leucine, and valine residues (5, 6). According to a recent model, when the level of any or all of the aminoacylated regulatory tRNAs falls, translation of the leader RNA in the peptide region will be impeded and RNA polymerase will then transcribe the DNA containing the structural genes of the operon (7). When abundant levels of aminoacyl-tRNA are present, the leader peptide is translated, signalling RNA polymerase to terminate transcription at the attenuator site prior to the structural genes. Hence, any factor which alters the state of aminoacylation or function of the regulatory tRNAs may have an effect on the expression of the operon.

We have been investigating the role of thionucleotides in tRNA using *E. coli* C6, a *relA* mutant requiring both cysteine and methionine (8). Under conditions of cysteine starvation, this mutant produces tRNA deficient in thionucleotides (8–10). We observed a change in the aminoacylation behavior of tRNA^{Ile} from cysteine-starved cells (11), as compared to that found in cells grown on fully supplemented medium. Sulfur-deficient tRNA^{Ile} had a 4-fold increased rate of aminoacylation and an altered elution position on MAK[1] columns, relative to normal tRNA^{Ile}. We wanted to see if the presence of this undermodified tRNA^{Ile} in cysteine-starved cells would alter the regulation of the *ilvGEDA* operon. To accomplish this, threonine deaminase (*ilvA* gene product) and transaminase B (*ilvE* gene product) levels were measured in cysteine-starved and normal *E. coli* C6 cells. Our results show that cysteine-starved cells are unable to derepress the *ilvGEDA* operon, the effect being reversible by addition of the sulfur amino acid. Furthermore, we found a correlation between the regulation of this operon and the presence of the undermodified, kinetically altered tRNA^{Ile} present in cysteine-starved cells.

MATERIALS AND METHODS

Bacterial Strains and Cell Growth—*E. coli* HfrC, *relA* Met$^-$ Cys$^-$, λ(C6 mutant) was grown in M9 medium (12) in the presence of 0.16 mM cysteine and 0.13 mM methionine, as previously described (8, 11). Cells were maintained at 37 °C in either a gyratory shaking water bath or New Brunswick Microferm fermentor, growth being monitored at 650 nm. Cysteine starvation was carried out in 10- to 14-liter fermentor cultures in M9 medium supplemented with 0.016 mM cysteine and 0.13 mM methionine (11). Methionine starvation was carried out in a similar fashion but with 0.16 mM cysteine and 0.013 mM methionine.

Enzymatic Assays—Cells were harvested by centrifugation, washed once in cold M9 salts, and stored at −90 °C until needed. Cell extracts were prepared by sonication as previously described (13). Threonine deaminase was measured by the method of Ratzkin *et al.* (14), each 1.0-ml assay contained 0.1 M Tris-HCl, pH 8.0, 0.1 M NH_4Cl, 0.1 mM pyridoxal phosphate, and 0.04 M threonine. Transaminase B assays were carried out as described by Duggan and Wechsler (15) and contained 0.1 M Tris-HCl, pH 8.0, 0.08 mM pyridoxal phosphate, 15

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[1] The abbreviations used are: MAK, methylated albumin Kieselguhr; RPC-5, plaskon powder coated with adogen 464 (19).

mM α-ketoglutarate, 25 mM valine, and 0.1 ml of cell extract in a total volume of 1.0 ml. All assays were carried out under conditions where initial velocities were proportional to protein concentration. The amount of protein in cell extracts was determined by the Bio-Rad method (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as standard. β-Galactosidase activity in intact cells of *E. coli* strain C6 was determined as described by Miller (16).

Transfer RNA and Aminoacylation—Transfer RNA was prepared as described previously (8) using isopropanol fractionation as the final step in purification. An aminoacyl-tRNA synthetase mixture was prepared from *E. coli* B cells (Grain Processing Corp., Muscatine, IA) by a previously reported procedure (17). The aminoacylation assay (8, 11) contained the following components in a total volume of 0.5 ml: 20 mM Tris-HCl, pH 7.3, 10.0 mM magnesium acetate, 2.0 mM ATP, 0.5 to 1.0 μCi of L-[^{14}C]isoleucine or 2.5 to 5.0 μCi of L-[^{3}H]isoleucine (352 to 360 mCi/mmol), 1.0 A_{260} unit of tRNA, and an appropriate amount of synthetase mixture. All assays were carried out at 37 °C, 50-μl samples being removed at various times, applied to 2.3-cm Whatman paper discs, and the discs prepared for scintillation counting as previously described (8).

Aminoacyl-tRNA was isolated from reaction mixtures by extraction with an equal volume of redistilled phenol saturated with 0.05 M sodium acetate buffer, pH 5.0. The resulting emulsion was centrifuged at 15,000 × *g* for 20 min, and the aqueous layer removed. The phenol layer was extracted with ½ volume of 0.05 M sodium acetate buffer, pH 5.0, and the second aqueous layer obtained as above. Aminoacyl-tRNA was precipitated from the combined aqueous layers by adding 0.1 volume of 20% potassium acetate, pH 5.0, and 2 volumes of 95% ethanol, and was stored at −20 °C.

Determination of in Vivo Aminoacylation Levels—The fraction of $tRNA^{Ile}$ which is aminoacylated *in vivo* was determined by the method of Folk and Berg (18) with the following modifications. Cultures were rapidly treated with 5% trichloroacetic acid and sodium dodecyl sulfate at 0.1% prior to collection of the cell pellet. The pellet was extracted with phenol at pH 5 by sonication as described (18), but we found it necessary to include a 30-min shaking step to ensure quantitative extraction of RNA. All other steps were performed as described (18), the final dialysate being passed through a 0.2 mM Gelman Acrodisc filter. This step was needed to remove undissolved material which interfered with the subsequent assays. The per cent charging of $tRNA^{Ile}$ was measured with the standard aminoacylation system, using 0.5–1.0 A_{260} unit of periodate-treated or untreated RNAs (see legend to Table V). Per cent charging is defined as the incorporation of labeled isoleucine into periodate-treated RNA × 100 divided by the incorporation of isoleucine into untreated RNA.

RPC-5 Chromatography of tRNA—[^{3}H]- or [^{14}C]isoleucyl-tRNA was obtained as above and dissolved in 0.5 ml of 0.4 M NaCl, 0.01 M sodium acetate, pH 4.5, 0.01 M $MgCl_2$, and 0.002 M 2-mercaptoethanol. The sample was applied to an RPC-5 column (0.6 × 21 cm) (19) and washed with 100 ml of the same buffer at a flow rate of 1.5 ml/min at 150 p.s.i. (using a Milton Roy Model 396 minipump). tRNA was eluted using a linear gradient of 0.45 to 0.6 M NaCl in 0.01 M sodium acetate, pH 4.5, 0.01 M $MgCl_2$, and 0.002 M 2-mercaptoethanol. Fractions of 1.0 ml were collected, 3 ml of ACS scintillation fluid (Amersham) was added, and radioactivity was measured by the scintillation method.

Chemicals and Radioisotopes—Amino acids, pyridoxal phosphate, 2,4-dinitrophenylhydrazine, α-ketobutyrate, and α-ketoisovalerate were obtained from Sigma. RPC-5 column materials were kindly provided by Dr. G. D. Novelli of the Biology Division, Oak Ridge National Laboratory, Oak Ridge, TN. L-[^{14}C]isoleucine (360 mCi/mmol) and L-[^{3}H]isoleucine (1.5 Ci/mmol) were products of New England Nuclear. All other chemicals were of the highest quality commercially available.

RESULTS

Amino Acid Starvation and ilvGEDA Regulation—The importance of base modification to the regulatory functions of tRNA was first appreciated due to studies of the *hisT* mutant of *Salmonella typhimurium* (20). This mutant, unable to convert certain uridine residues to pseudouridine in tRNA, showed a derepression of both the histidine and *ilvGEDA* operons (21). We wanted to see if the regulation of the *ilvGEDA* operon was altered in the presence of other undermodified forms of tRNA. To accomplish this, *E. coli* strain C6 was cultured in the presence of reduced levels of cysteine or methionine, conditions which support less growth than when both amino acids are present in optimal amounts (Fig. 1). During the starvation period which follows, the cells accumulate either sulfur-deficient (8) or methyl-deficient tRNA (22) as a consequence of the *relA* mutation. To determine if the accumulation of undermodified tRNA altered the regulation of the *ilvGEDA* operon (5, 6), we measured the activity of threonine deaminase and transaminase B in fully supplemented (control), cysteine-starved, and methionine-starved *E. coli* C6 cells. In each case, the starvations were carried out for 6 h after growth had ceased due to depletion of the amino acid. The data of Table I show that cysteine starvation resulted in a lower specific activity of both threonine deaminase and transaminase B. This drop in the activity of both enzymes was consistently observed with cysteine-starved cells (see Table III also), but was not seen with *E. coli* strain C6 starved for methionine. It is clear that deprivation of cysteine or methionine did not lead to a derepression of the *ilvGEDA* operon.

The previous experiments showed that cysteine- or methionine-starved cells retained their ability to repress the *ilvGEDA* operon. However, a clear interpretation of these results is difficult, since deprivation of amino acids would also limit protein synthesis with the starved cultures (see Table IV, below). This prompted us to study the derepression of the *ilvGEDA* operon during recovery from cysteine or methionine starvation. The derepression condition involved treatment with valine, an inhibitor of acetohydroxyacid synthetase (23).

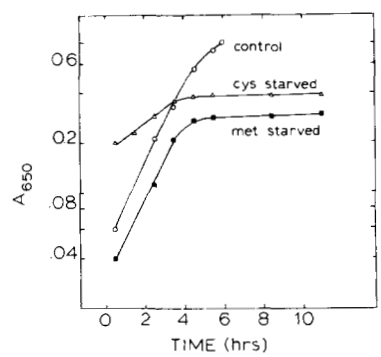

FIG. 1. **Effect of amino acid starvation on the growth of *E. coli* C6.** E. coli was grown in 10-liter fermentor cultures in medium containing 0.16 mM cysteine and 0.13 mM methionine (control), 0.016 mM cysteine and 0.13 mM methionine (cysteine-starved), or 0.16 mM cysteine and 0.013 mM methionine (methionine-starved). Each culture was inoculated in the evening using 5 ml of a freshly grown culture of strain C6 to yield a calculated initial A_{650} of 0.001. The first readings were taken about 10 h later, as shown. At the end of the growth study, cells were harvested by centrifugation, washed with M9 salts, and frozen at −80 °C.

TABLE I

Effect of amino acid starvation on isoleucine and valine biosynthetic enzymes

Control cultures were grown in complete medium and cells were harvested at A_{650} = 0.7. Starved cultures were incubated for 6 h after growth had ceased due to depletion of either cysteine or methionine (see Fig. 1). Cell extracts were prepared as described under "Materials and Methods." Enzyme assays were carried out in duplicate; the data are averages of two separate experiments.

Culture condition	Specific activity	
	Threonine deaminase	Transaminase B
	nmol/min/mg protein	
Control	46	67
Cysteine-starved	31	49
Methionine-starved	44	58

This treatment inhibits growth by causing an isoleucine limitation. Fig. 2 shows that the growth of *E. coli* C6 in complete medium was inhibited by the addition of 25 mM valine, and this inhibition was overcome by adding 12.5 mM isoleucine and 12.5 mM leucine along with valine (*top*). The high concentration of these amino acids was necessary due to an apparent defect in the uptake of amino acids by this mutant (13). The *lower two panels* of Fig. 2 show the results of experiments where starved cells were allowed to recover after the addition of either 0.16 mM cysteine or 0.13 mM methionine. Growth begins shortly after this addition in both cases and is limited by the presence of 25 mM valine. Cysteine-starved cells show a more pronounced inhibition of growth by valine, as discussed below. The effect of valine is overcome by also adding isoleucine and leucine as shown. At the conclusion of the growth experiments, the cells were harvested and washed, and extracts were assayed for threonine deaminase and transaminase B activities. Table II shows that treatment of control cultures with valine resulted in increased levels of both enzymes, indicating derepression of the operon. The effect is not coordinate as threonine deaminase levels were increased nearly 7-fold while transaminase B was elevated by 2-fold. Derepression was not observed with extracts from cells to which isoleucine and leucine were added along with valine. Cysteine-starved cells grew when cysteine was added (Fig. 2) and the levels of threonine deaminase and transaminase B returned to control levels (compare with Table I). However, although valine had a clear effect on growth, no derepression of the operon was observed. This is in contrast to the results with methionine-starved cells during recovery, in which valine caused derepression, and this was prevented by isoleucine and leucine. These results suggest that cysteine-starved cells are incapable of derepression of the *ilvGEDA* operon. The effect appears to be specific for cysteine as methionine-starved cells were similar to normal cells in their ability to derepress the operon.

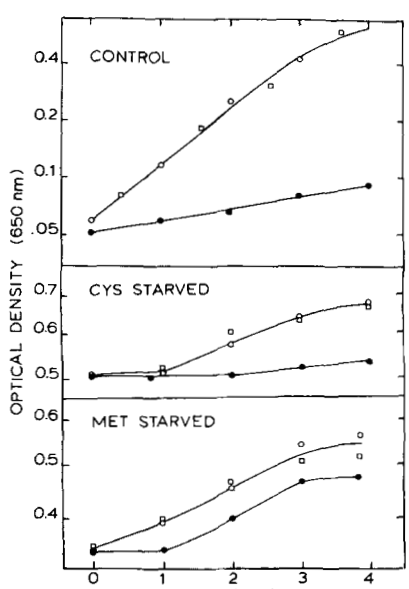

FIG. 2. **Effect of valine on the growth of *E. coli* C6.** Fully supplemented (control) and amino acid starved cultures were grown as described in the legend to Fig. 1. At 0 time, cells were resuspended in complete medium (0.16 mM cysteine and 0.13 mM methionine), in complete medium containing 25 mM valine, or in complete medium containing 25 ml of valine and 12.5 mM isoleucine and leucine. At the end of the 3-h growth study, cells were harvested, washed in M9 salts, and frozen at −80 °C until needed. The symbols are: ○, complete medium; ●, complete medium plus 25 mM valine; □, complete medium plus 25 mM valine and 12.5 mM isoleucine and leucine.

TABLE II

Regulation of the ilvGEDA operon after amino acid starvation

E. coli C6 cells were grown as described in the legend to Fig. 2, and suspended in complete medium with or without the addition of valine or valine, isoleucine, and leucine, as noted. Frozen cells were thawed and cell extracts prepared by sonication as described above. Threonine deaminase and transaminase B assays were carried out promptly as described under "Materials and Methods." Except where noted, all data are averages from two separate experiments. Within each experiment, each assay is carried out in duplicate.

Culture condition	Additions	Specific activity	
		Threonine deaminase	Transaminase B
		nmol/min/mg	
Control	None	55	70
	Valine	350	144
	Valine + isoleucine + leucine	50	60
Cysteine-starved	None	61	54
	Valine	52	62
	Valine + isoleucine + leucine	47	47
Methionine-starved	None	55	66
	Valine	220	110
	Valine + isoleucine + leucine	54[a]	76[a]

[a] The data are averages of duplicate enzyme assays from a single experiment.

TABLE III

Derepression of the ilvGEDA operon during cysteine starvation and recovery

Cysteine-starved and control cells of *E. coli* C6 were grown in a 12-liter fermentor culture as described in Fig. 1. At the indicated times before and during starvation, cells were removed and tested for derepression in complete medium as described in Table II. At the same time, cells were removed for tRNA isolation. After 6 h of starvation, cysteine was added at 0.16 mM and incubation was continued. Cells were taken at 1, 2, and 3 h after the addition of cysteine and tested for derepression and also saved for tRNA isolation. For the derepression assays, cells were incubated at 37 °C in complete medium or in complete medium containing 25 mM valine. After 3 h, the cells were harvested and washed and the enzyme assays were carried out as above.

Condition	Specific activity			
	Threonine deaminase		Transaminase B	
	Control	Valine added	Control	Valine added
	nmol/min/mg protein[a]			
Control	55	350	70	144
Prestarved	35	238	46	96
3-h starved	40	47	51	54
6-h starved	45	57	52	52
1-h recovery	30	71	48	55
2-h recovery	45	88	69	67
3-h recovery	71	247	74	87

[a] All data are averages of duplicate determinations from two separate experiments.

We determined the time course of the loss of derepressibility of the *ilvGEDA* operon during cysteine starvation. Table III gives the results of an experiment where cells were removed at various times during cysteine starvation and tested for derepression. The derepression test involves a 3-h incubation in complete medium, with or without 25 mM valine. Table III, line 1, repeats the results obtained with cultures grown in complete medium. Line 2 shows the result of the derepression

test with cells grown in cysteine-limited medium, taken at the point where growth first becomes limited by depletion of the sulfur amino acid. At this point, valine increased the doubling time of the culture from 60 to 120 min. The specific activities of both threonine deaminase and transaminase B were lower in this case (compare with line 1), but the magnitude of derepression in response to valine was similar. While cells at the onset of starvation were competent in derepression of the *ilvGEDA* operon, by 3 h of starvation no derepression was observed. Here, the effect of valine on growth was greater, increasing the doubling time to 280 min. No further changes in specific activities or derepression occurred as starvation continued to 6 h. Although the cells became more sensitive to growth inhibition by valine as starvation proceeded, no derepression of the *ilvGEDA* operon was observed.

The above experiments showed that the inability to derepress the operon occurred as early as 3 h after the onset of cysteine starvation. To see if this effect was reversible, cysteine at 0.16 mM was added to a culture after 6 h of starvation. A portion of this culture was removed at 1, 2, and 3 h of growth recovery and tested for the ability to derepress the *ilvGEDA* operon as above. With the control cultures, removed from the main culture and suspended in complete medium for an additional 3 h, the activities of threonine deaminase and transaminase B gradually increased from the values characteristic of cysteine-starved cells to those of cells grown in fully supplemented medium. Cells removed at 1, 2, and 3 h of recovery all showed some inhibition of growth when resuspended in complete medium with 25 mM valine, but the magnitude of this effect decreased markedly during recovery. However, after 1 or 2 h of recovery, valine elicited only a 2-fold increase in threonine deaminase activity with little change in the level of transaminase B. By the third hour of recovery, the ability to derepress the *ilvA* gene is nearly normal, while transaminase B was only slightly elevated in response to valine. The latter effect may be the result of a direct effect of cysteine on either the enzyme activity or expression of the *ilvE* gene (24). In summary, cysteine-starved cells recover their ability to derepress the *ilvGEDA* operon, but only after a considerable incubation period in the presence of cysteine.

The failure to derepress the *ilvGEDA* operon could be the result of restricted protein synthesis in cells containing tRNAs accumulated during cysteine starvation. To test this possibility, we measured the rate of protein synthesis (25) at various stages of recovery from cysteine starvation, the results being given in Table IV. The data show a dramatic drop in the overall rate of protein synthesis for cysteine-starved cells resuspended in complete medium. During recovery, the rate of protein synthesis increases gradually, but did not reach the exponential rate. The addition of 25 mM valine at various times during recovery dramatically lowered the rate of protein synthesis, but in no instance was total inhibition observed. These results show that cysteine-starved cells promptly resume protein synthesis when cysteine is added, although at lower than optimal rates.

TABLE IV

Protein synthesis during recovery from cysteine starvation

Exponential and cysteine-starved cultures were grown in complete or low cysteine medium as in Fig. 1. After 6 h of starvation, 0.16 mM cysteine was added and incubation was continued to allow growth recovery. One ml of cells were removed from exponential or recovering cultures and protein synthesis was determined after addition of 1 μCi of a mixture of 15 [^{14}C]aminoacids (New England Nuclear). The initial rate of protein synthesis was measured by removing 50-μl samples at 1-min intervals up to 5 min from each assay tube, application to 2.4-cm paper discs, and processing as described (25). Amino acid incorporation was linear for 5 min under each experimental condition. The data are corrected for differences in cell density as shown and are a composite from several different experiments.

Culture condition	Protein synthesis rate
	cpm/min/A_{650}
Exponential growth	120,000
5-min recovery	18,700
5-min recovery + 25 mM valine	4,800
30-min recovery	21,000
30-min recovery + 25 mM valine	3,620
90-min recovery	30,000
150-min recovery	50,000
180-min recovery + 25 mM valine	5,780

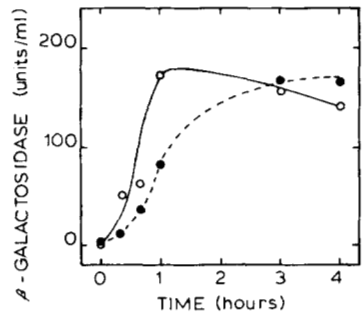

FIG. 3. **Effect of cysteine starvation on the expression of the *lac* operon.** *E. coli* strain C6 was starved 6 h for cysteine as described above. At the beginning of this experiment, cysteine (0.016 mM), isopropyl-β-D-thiogalactoside (0.25 mM) and cAMP (0.015 M) were added to each culture. Cells were incubated at 37 °C and 1-ml samples were removed at the indicated time for β-galactosidase analysis (16). The symbols are: ○, recovering cells; ●, recovering cells plus 25 mM valine.

To determine if cysteine starvation alters gene expression, we determined the induction of the *lac* operon in response to the gratuitous inducer, isopropyl-β-D-thiogalactoside (16). Fig. 3 shows that cysteine-starved cells of *E. coli* C6 can express the *lac* operon, as nearly a 100-fold increase in β-galactosidase activity was observed during 1 h of incubation in complete medium. The presence of 25 mM valine slowed the synthesis of this enzyme slightly, but a full induction was observed within 2 h of growth recovery. Cyclic AMP was required for the expression of this operon when valine was present, presumably to overcome catabolite repression. However, the effect of valine on the *lac* operon is complex, as exponential cells can induce the operon without the addition of cAMP. Finally, the presence of cAMP had no effect on the expression of the *ilvGEDA* operon during the first 3 h of recovery from cysteine starvation (data not shown). Hence, expression of the *lac* operon was observed under conditions where derepression of the *ilvGEDA* operon could not be demonstrated.

Properties of tRNA during Starvation and Recovery—Since aminoacyl-tRNA is involved in the regulation of the *ilvGEDA* operon, we determined the aminoacylation behavior of the relevant tRNAs in exponential and amino acid starved cultures of *E. coli* C6. Table V shows that cysteine-starved cells contain a $tRNA^{Ile}$ with a faster initial rate of aminoacylation than seen with either growing or methionine-starved cells. With $tRNA^{Leu}$, both the rate and extent of acylation were lower in cysteine-starved cells, and a similar drop in the extent of acylation was observed with methionine-starved cells as well. RPC-5 chromatography of $tRNA^{Leu}$ from cysteine-starved cells (data not shown) revealed isoacceptor profiles similar to those observed for amino acid starved *E. coli* (26). These effects were not further investigated since the alterations in $tRNA^{Leu}$ were not specific for cysteine-starved cells. Finally, little change in the rate or extent of $tRNA^{Val}$ acylation

TABLE V

Aminoacylation properties of tRNA from exponential and amino acid starved cultures

Transfer RNA was isolated as described under "Materials and Methods" from cultures of *E. coli* C6 grown in complete medium or with limited amounts of either cysteine or methionine. Starved cultures were maintained at 37 °C for 6 h after growth had ceased due to depletion of the limited amino acid. The initial rates of aminoacylation were determined using the standard aminoacylation assay, utilizing 4 μg of crude synthetase protein. Extents of aminoacylation were determined with the same system but with 0.8 mg of synthetase. The data are averages of duplicate determinations carried out with two separate tRNA preparations, except for the initial velocities of leucyl- and valyl-tRNA formation. In these cases, a single kinetic experiment was carried out.

Source of tRNA	Isoleucine		Leucine		Valine	
	Initial rate[a]	Extent[b]	Initial rate	Extent	Initial rate	Extent
Exponential cells	11.8	71.3	3.75	166.0	14.5	101.0
Cysteine-starved cells	52.3	82.5	1.55	109.0	14.5	95.0
Methionine-starved cells	10.4	71.0	ND	91.0	ND	96.0

[a] Initial rates are expressed as pmol of amino acid/A_{260}/min.
[b] Extents of aminoacylation are expressed as pmol of amino acid/A_{260}.
ND, not determined.

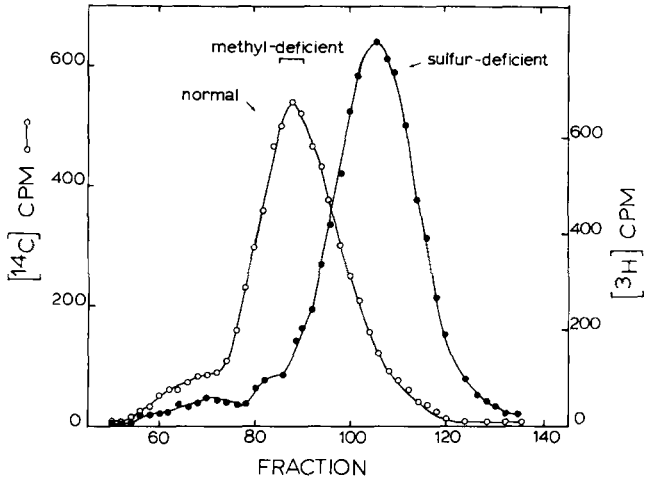

FIG. 4. **RPC-5 chromatography of isoleucyl-tRNA isolated from fully supplemented and amino acid starved cultures of *E. coli* C6.** Cell growth was as described in the legend to Fig. 1. Transfer RNA was isolated and aminoacylated as described under "Materials and Methods." Normal tRNA was acylated in the presence of [^{14}C]isoleucine while sulfur-deficient tRNA was aminoacylated using [^{3}H]isoleucine. The labeled tRNAs were isolated from reaction mixtures and co-chromatographed as described above. The position of the major tRNA^{Ile} for methyl-deficient tRNA was determined by co-chromatography with normal tRNA (data not shown). The symbols are: O, normal tRNA; ●, sulfur-deficient tRNA.

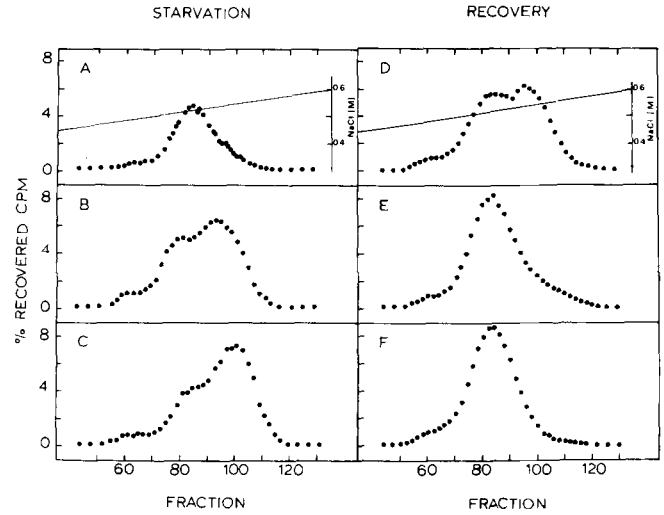

FIG. 5. **RPC-5 column chromatography of isoleucyl-tRNA at various times during cysteine starvation and recovery.** Two liters of cells were removed from the fermentor culture at the indicated times and tRNA was isolated as described under "Materials and Methods." Each sample was aminoacylated with [^{14}C]isoleucine, the labeled tRNA isolated by phenol extraction and chromatographed on an RPC-5 column as described. *A*, pre-starved cells; *B*, 3-h starved cells; *C*, 6-h starved cells; *D*, 1-h recovery; *E*, 2-h recovery; *F*, 3-h recovery.

was observed with tRNA from cysteine-starved cells. Hence, there is a correlation between the inability to derepress the *ilvGEDA* operon and a change in the aminoacylation properties of tRNA^{Ile} of cysteine-starved cells.

We then sought to determine whether the change in acylation behavior was specific to one isoaccepting tRNA^{Ile}. Normal, sulfur-deficient, and methyl-deficient isoleucyl-tRNAs were prepared *in vitro* and chromatographed on RPC-5 columns (19). Fig. 4 shows that the elution pattern for normal and methyl-deficient tRNAs were similar, while sulfur-deficient tRNA^{Ile} eluted at a higher salt concentration. The increased rate of aminoacylation observed for the latter tRNA is associated with an alteration in its chromatographic behavior, and these changes are both specific to tRNA from cysteine-starved cultures.

The kinetic and chromatographic properties of tRNA^{Ile} in cysteine-starved cells were investigated in more detail. We isolated tRNA at various times during starvation and recovery, and determined rates of aminoacylation and RPC-5 elution profiles (as in Table V and Fig. 4). The initial velocities of isoleucyl-tRNA formation for tRNA from prestarved, 3-h starved and 6-h starved *E. coli* C6 cells were 10.5, 38.0, and 40.4 pmol of Ile/A_{260}/min, respectively. Corresponding values for tRNA isolated after 1, 2, and 3 h of recovery from cysteine starvation were 33.5, 19.0, and 11.5 pmol of Ile/A_{260}/min. These results show a fairly rapid increase in the rate of isoleucyl-tRNA formation during starvation, which is nearly complete 3 h after growth ceases. After cysteine addition to starved cells, there is a gradual lowering of the rate of isoleucylation to normal values. Fig. 5*A* shows the isoacceptor pattern on RPC-5 for tRNA^{Ile} from pre-starved cells, demonstrating its normal behavior (also see Fig. 4). By 3 h of starvation, the amount of the major tRNA^{Ile} is much lower and is replaced by a new tRNA^{Ile} which elutes at higher salt concentrations. At 6 h, the normal tRNA^{Ile} is nearly absent and the altered tRNA^{Ile} predominates. This altered tRNA is responsible for the increased rate of aminoacylation observed for tRNA isolated during cysteine starvation (11). One hour after cysteine addition, there is a partial loss of the altered tRNA and an increase in normal tRNA^{Ile}. As recovery continues, the isoacceptor pattern returns to normal, as does the rate of aminoacylation. Hence, there is a correlation between

the presence of undermodified $tRNA^{Ile}$ in cysteine-starved cells, the kinetic properties of this tRNA, and the inability to derepress the *ilvGEDA* operon.

It is possible that the presence of the undermodified $tRNA^{Ile}$ in cysteine-starved cells, with its faster rate of aminoacylation, would increase the cellular level of isoleucyl-tRNA. This could explain the lower specific activities of threonine deaminase and transaminase B in cysteine-starved cells, and the failure to derepress the operon in response to valine. To test this possibility, we first measured the per cent charging of $tRNA^{Ile}$ *in vivo*, using the periodate method following pH 5 phenol extraction of the RNA (18). The data of Table VI show that, in *E. coli* strain C6 grown in complete medium, the charging of $tRNA^{Ile}$ was 62%. When cells are treated with 25 mM valine, one sees a dramatic drop in the charging of $tRNA^{Ile}$ to 27%. This is consistent with the inhibition of isoleucine synthesis by valine, and represents the amount of isoleucyl-tRNA present when the *ilvGEDA* operon is fully derepressed. Cysteine-starved cultures of *E. coli* strain C6 show a 20% increase in isoleucyl-tRNA levels, as compared to that of fully supplemented cells. In some experiments, the charging of tRNA in cysteine-starved cells approached 100% (see Fig. 6). Hence, the accumulation of the kinetically altered $tRNA^{Ile}$ during cysteine starvation does lead to an increase in the cellular level of isoleucyl-$tRNA^{Ile}$. The effect of valine on cysteine-starved cells during growth recovery was investigated. The per cent charging of $tRNA^{Ile}$ remains high even 3 h after the addition of cysteine to starved cells. However, treatment of starved cells with valine during recovery results in a decrease in the isoleucyl-tRNA level to 32%, a value similar to that observed with control cultures treated with valine. These results are quite unexpected and indicate that, with cysteine-starved cells, valine treatment causes a drop in the cellular level of charged $tRNA^{Ile}$, but this does not lead to a derepression of the *ilvGEDA* operon.

The derepression analysis used above measures enzyme levels after a 3-h treatment with valine. In contrast, the periodate method determines the % charging of tRNA at the time when the culture is harvested, i.e., is an instantaneous value. For this reason we determined the per cent charging of $tRNA^{Ile}$ in control and cysteine-starved cells at various times after suspension in complete medium with 25 mM valine. Fig. 6 shows that before addition of valine the per cent charging of $tRNA^{Ile}$ was higher in cysteine-starved cells than in control

TABLE VI

In vivo aminoacylation levels of $tRNA^{Ile}$

Fully supplemented cultures were grown in complete medium with or without 25 mM valine as described in the legend to Fig. 2. Cysteine- and methionine-starved cultures were grown as in Fig. 1 and incubated for 6 h after depletion of the amino acid. The recovery experiments utilized cysteine-starved cells to which cysteine at 0.16 mM (± valine) was added after 6 h of starvation. Incubation was continued for 3–4 h after addition of the sulfur amino acid. In all cases, RNA was prepared and per cent charging of $tRNA^{Ile}$ determined by the periodate method (19). Aminoacylation assays were performed as described under "Materials and Methods," except that a mixture of 15 unlabeled amino acids (no isoleucine) was included to prevent misacylation (24). The data are given as the mean ± S.E., the number of separate RNA preparations being given in parenthesis.

Culture condition	25 mM Valine	% Charging of $tRNA^{Ile}$
Control	0	61.8 ± 3.7 (5)
Control	+	26.6 ± 3.4 (5)[a]
Cysteine-starved	0	79.2 ± 7.6 (4)[b]
Recovery from cysteine starvation	0	79.7 ± 5.5 (4)
Recovery from cysteine starvation	+	32.4 ± 0.7 (5)[a]

[a] Significantly lower than control (no valine) at $p < 0.01$.
[b] Significantly higher than control (no valine) at $p = 0.07$.

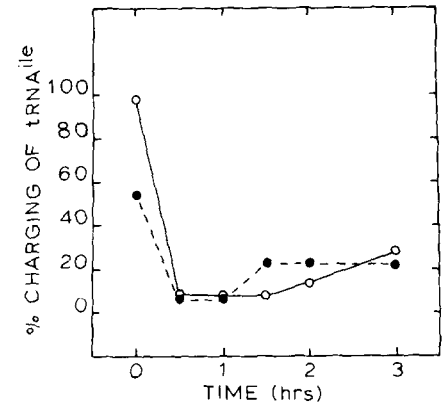

FIG. 6. **Per cent charging of $tRNA^{Ile}$ during valine treatment of *E. coli* C6.** Control cells were incubated in complete medium until the culture reached an A_{650} level of 0.2. Valine was added at 25 mM and incubation was continued, 1-liter samples being taken at various times for RNA isolation. With the starved culture, cysteine (0.16 mM) was added with valine (25 mM) to cells which had been starved for 6 h as in Fig. 1. RNA was isolated at various times and per cent charging of $tRNA^{Ile}$ was determined as described above. The symbols are: ●, control cells plus valine; ○, cysteine-starved cells plus valine.

cells. Thirty minutes after the addition of valine, the aminoacylation ratio fell to below 10% for both cultures. These ratios remained low for the next hour and then gradually rose in both cases as the growth effects of valine were overcome (data not shown). No significant differences in the isoleucyl-tRNA levels of control or starved cells were observed throughout the period of valine treatment. Therefore, the reason why cysteine starvation leads to an inability to derepress the *ilvGEDA* operon must be some other alteration in the regulatory tRNA(s) other than its aminoacylation state.

DISCUSSION

Cysteine starvation of *E. coli* strain C6 *relA*, Met^-, Cys^- was accompanied by a dramatic loss of the ability to derepress the *ilvGEDA* operon. This effect was reversed slowly as cysteine-starved cells recovered in complete medium. The loss of derepressibility was specific to cysteine starvation, as both growing and methionine-starved cells of the same strain were able to derepress the operon in response to an isoleucine limitation. Moreover, cysteine-starved cells were capable of a full induction of the *lac* operon under the same conditions where the *ilvGEDA* operon could not be expressed. This effect was correlated with the appearance of an altered $tRNA^{Ile}$ during cysteine starvation, which had a faster rate of aminoacylation and an altered elution position on RPC-5 chromatography. No changes in the activity or RPC-5 pattern were observed for $tRNA^{Ile}$ from methionine-starved cells of *E. coli* C6. During recovery from cysteine starvation, the altered $tRNA^{Ile}$ was gradually converted back to the normal isoacceptor, both in terms of aminoacylation and chromatographic behavior. Therefore, the loss of derepressibility of the *ilvGEDA* operon was correlated with an alteration of one of the tRNAs known to be involved in the regulation of this operon (3, 4).

The most likely explanation for the above findings would be an increase in the level of isoleucyl-tRNA in cysteine-starved cells of *E. coli* strain C6. Direct measurement of the per cent charging of $tRNA^{Ile}$ confirmed this hypothesis, as a 20% increase in isoleucyl-tRNA was observed after cysteine starvation. This may explain why threonine deaminase and transaminase B levels are somewhat lower after 6 h of cysteine starvation. Valine treatment was used to limit isoleucine

synthesis, which in turn generates a derepression signal. This treatment reduced the per cent charging of $tRNA^{Ile}$ from 62 to 27% in growing cells and from 80 to 32% in cysteine-starved cells during recovery. These values were determined 3 h after the addition of valine and measure the instantaneous per cent of aminoacylated $tRNA^{Ile}$ at the end of the derepression period. We also found no differences in the per cent charging of $tRNA^{Ile}$ under these two conditions throughout the 3-h treatment with valine (Fig. 6). Taken together, these results show that valine causes a drop in isoleucyl-tRNA levels, presumably the consequence of its inhibition of isoleucine synthesis at the level of acetohydroxy acid synthetase (23). On the other hand, the data do not allow us to conclude why the *ilvGEDA* operon is not derepressed by this addition of valine after cysteine starvation of *E. coli* strain C6.

In terms of the accepted model for the regulation of this operon (5, 6), failure to derepress could arise in two ways. First, the operon could be blocked at the level of transcription initiation, although little is known about the regulatory elements acting at this site. It is unlikely that ppGpp levels are too low in cysteine-starved cells to allow transcription of the operon since regulation is normal in methionine-starved cultures. Further, it was recently shown that, as opposed to *relA*$^+$ strains, ppGpp was low in amino acid starved cultures of *relA* mutants of *E. coli* but rose rapidly when the missing amino acid was added (27). The derepression test used here included cysteine in the medium along with valine. The second regulation of the *ilvGEDA* operon occurs at the several attenuators prior to structural genes (28, 29). The drop in the isoleucyl-tRNA level observed with cysteine-starved cells in the derepression test appears to be sufficient to cause a decrease in attenuation, but does not. The other major regulatory tRNAs are not involved, as we have shown that valine specifically lowers the per cent charging of $tRNA^{Ile}$ with no effect on the per cent charging of $tRNA^{Leu}$ or $tRNA^{Val}$ (24). Perhaps the $tRNA^{Ile}$ present in starved cells is more efficient in translation such that the leader peptide is translated even with less than 10% of the $tRNA^{Ile}$ in aminoacylated form. Alternatively, a thionucleotide-containing tRNA, which fails to translate some codon earlier in the leader, may cause a block in translation leading to transcription termination at the first attenuator (5, 6). Finally, nutritional deprivation of *relA*$^-$ strains of *E. coli* leads to mistranslation of mRNA (30–32). Hence, unmodified tRNA may mistranslate the leader preceding the attenuator site(s) of the *ilvGEDA* operon and signal RNA polymerase to terminate transcription. Further investigation will be necessary to resolve these issues.

Several lines of evidence suggest that the modification and aminoacylation of $tRNA^{Ile}$ are important for the regulation of the *ilvGEDA* operon. The *hisT* mutants of *S. typhimurium* (21) and *E. coli* (33), defective in pseudouridine synthesis, were found to have derepressed levels of the isoleucine-valine biosynthetic enzymes. Recent findings suggest that $tRNA^{Ile}$ in the *hisT* mutant may be defective in regulating the operon at the site of transcription initiation (34). The *trp* operon of *E. coli* was derepressed in a mutant defective in the synthesis of 6-(3-methyl-2-butenylamino)-2-methylthio-9-D-ribofuranosylpurine, further showing the importance of base modification to the regulatory roles of tRNA (35). The results with the C6 mutant reported here differ from these studies in that *no* derepression of the *ilvGEDA* operon occurs in the presence of the undermodified $tRNA^{Ile}$, and preliminary results suggest that sulfur-deficient $tRNA^{Ile}$ is normal in its pseudouridine content (36). It is of interest that the regulation of the expression of the gene for threonine deaminase was found to be more sensitive to changes in isoleucyl-tRNA than other enzymes of this operon (29, 37). Our results agree with this, as the derepression of the *ilvA* gene was the first to be observed as *E. coli* C6 recovered from cysteine starvation. Further, whatever alteration of $tRNA^{Ile}$ is responsible for the results given here, it is clear that the regulation of early and late genes is affected.

The changes in sulfur-deficient $tRNA^{Ile}$, which give rise to its altered aminoacylation and chromatographic behavior, are not completely understood at present. Normally, growing *E. coli* C6 cells contain three species of tRNA: $tRNA_1^{Ile}$, presently uncharacterized but comprising less than 5% of the total; $tRNA_2^{Ile}$, a slowly acylated 4-thiouridine-containing species comprising 45% of the total; and $tRNA_3^{Ile}$, a species with a fast rate of aminoacylation which is 50% of the total (38). The latter $tRNA^{Ile}$ is undermodified with respect to $tRNA_2^{Ile}$, but it is not yet clear if these species are products of different genes or are related through base modification. Cysteine-starved cells contain one major $tRNA^{Ile}$ whose rate of acylation is fast and is also similar to normal $tRNA_3^{Ile}$ in base composition (36). However, these two tRNAs are different, as they chromatograph distinctly on MAK and RPC-5 columns (Ref. 11 and Figs. 4 and 5). We report here that the $tRNA^{Ile}$ of cysteine-starved cells is converted back to the normal pattern after 3 h of recovery on complete medium. This had also been observed with sulfur-deficient tRNA treated with sulfurtransferase *in vitro*, namely, that $tRNA_2^{Ile}$ was reformed and the rate of aminoacylation was reduced to normal levels (8). While the latter experiments strongly suggest that sulfur-deficient $tRNA^{Ile}$ is an undermodified version of normal $tRNA^{Ile}$, a careful structural analysis of these tRNAs must precede any definite conclusions. These experiments are currently in progress.

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