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Derepression of Synthesis of the Aminoacyl-Transfer Ribonucleic Acid Synthetases for the Branched-Chain Amino Acids of *Escherichia coli*

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The kinetics of derepression of valyl-, isoleucyl-, and leucyl-transfer ribonucleic acid (tRNA) synthetase formation was examined during valine-, isoleucine-, and leucine-limited growth. When valine was limiting growth, valyl-tRNA synthetase formation was maximally derepressed within 5 min, whereas the rates of synthesis of isoleucyl-, and leucyl-tRNA synthetases were unchanged. Isoleucine-restricted growth caused a maximal derepression of isoleucyl-tRNA synthetase formation in 5 min and derepression of valyl-tRNA synthetase formation in 15 min with no effect on leucyl-tRNA synthetase formation. When leucine was limiting growth, leucyl-tRNA synthetase formation was immediately derepressed, whereas valyl- and isoleucyl-tRNA synthetase formation was unaffected by manipulation of the leucine supply to the cells. These results support our previous findings that valyl-tRNA synthetase formation is subject to multivalent repression control by both isoleucine and valine. In contrast, repression control of isoleucyl- and leucyl-tRNA synthetase formation is specifically mediated by the supply of the cognate amino acid.

Studies from our laboratory (6) and those of other investigators (7) have provided evidence that synthesis of valyl-transfer ribonucleic acid (valyl-tRNA) synthetase (EC 6.1.1.9, valine: soluble RNA ligase, adenosine monophosphate) isoleucyl-tRNA synthetase (EC 6.1.1.5, isoleucine: soluble RNA ligase, adenosine monophosphate), and leucyl-tRNA synthetase (EC 6.1.1.4, leucine: soluble RNA ligase, adenosine monophosphate) is controlled by a repression process similar to that for repression control of the isoleucine-, valine-, and leucine-forming enzymes (3). Furthermore, we have recently shown that repression control of valyl-tRNA synthetase (VRS) formation in both *Escherichia coli* and *Salmonella typhimurium* is multivalent in nature, requiring both valine and isoleucine for complete repression (6). In this respect, control of VRS formation is similar to the well-established multivalent control of isoleucine and valine biosynthesis as mediated by isoleucine, valine, and leucine (3). On the other hand, repression control of isoleucyl-tRNA synthetase (IRS) and leucyl-tRNA synthetase (LRS) is mediated by the supply of isoleucine and leucine, respectively (6).

That these two classes of branched-chain amino acid-specific enzymes (i.e., biosynthetic and synthetase) exhibit a common and parallel control pattern is supported by the recent studies of Levinthal et al. (4). They reported that a mutation in *ilvA*, the structural gene for threonine deaminase, resulted in altered regulation (i.e., a reduced capacity for derepression) in synthesis of two *ilv*-specific biosynthetic enzymes and that of the three branched-chain tRNA synthetases. In addition, certain regulatory mutants which possess mutations at loci other than the structural genes for the isoleucine-, valine-, and leucine-forming enzymes and the branched-chain tRNA synthetases exhibit alterations in repression control of *ilv* and *leu* gene products and the cognate tRNA synthetases (manuscript in preparation).

Despite the obvious similarities in repression control for the two classes of enzymes, the magnitude of the derepression response for the synthetases is generally less than that for the biosynthetic enzymes. Thus, the present study was undertaken to examine the kinetics of derepression of VRS, IRS, and LRS formation during specific amino acid limitation. We report (i) that the rate of formation of these three synthetases can vary over a considerable range; (ii) that derepression of VRS, LRS, and IRS

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formation required de novo protein synthesis; and (iii) that the derepressed differential rate of synthesis of these synthetases can be increased from 16- to 41-fold over the unrestricted rate of formation.

A preliminary report of some of these results has appeared (E. McGinnis and L. S. Williams, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 189, 1973).

MATERIALS AND METHODS

Organisms. Strain M4862-H-5 (obtained from M. Freundlich) is an isoleucine, valine, and leucine auxotroph of *E. coli* W and was used in all the experiments reported here.

Media and methods of cultivation. The minimal medium was the basal salts solution of Fraenkel and Neidhardt (2). For unrestricted growth, all media were as previously described (6). Valine, leucine, and isoleucine were made limiting by using 10 μg of DL-glycyl-amino acid per ml instead of the respective amino acid. Deuterium oxide medium was prepared by using deuterium oxide (80%) instead of water. Unless otherwise indicated, the cells were grown aerobically on a rotary-action shaker at 37 C. Procedures for removal of amino acid supplements, changes from deuterium oxide to water media, and other cultivation details were as described previously (6). Growth was measured by an increase in optical density at 420 nm with a 1-cm light path in a Hitachi-Perkin-Elmer model 101 spectrometer.

Preparation of cell extracts. Cells were subjected to sonic treatment with a Biosonik III Sonifier as described by Chrispeels et al. (1). The protein content was determined colorimetrically by the methods of Lowry et al. (5).

Enzyme measurement. The activity of the aminoacyl-tRNA synthetases was measured by the ^{14}C-labeled amino acid attachment assay system as described by Chrispeels et al. (1), except that incubation was for 5 min. For each assay, the values are corrected for a minus adenosine triphosphate blank and each value is the average of at least three determinations. One unit of activity is equal to 1 μmol of product formed per h. Specific activity is defined as units per milligram of protein.

Centrifugation in cesium chloride. All centrifugation procedures were as described by Williams and Neidhardt (9). The equilibrium centrifugation was performed in a Spinco model SW50.1 rotor with a Spinco-Beckman model L or L-4 ultracentrifuge.

Measurement of the de novo rate of enzyme synthesis. The methods for measuring enzyme synthesis were those of Williams and Neidhardt (9).

Chemicals. Uniformly ^{14}C-labeled L-amino acids were obtained from New England Nuclear, Boston, Mass. K-12 tRNA was purchased from General Biochemical Div., Chagrin Falls, Ohio. Cesium chloride was obtained from Pierce Chemical Co., Rockford, Ill. Glycyl-DL-amino acids were from Sigma Chemical Co., St. Louis, Mo., and Mann Research Laboratory, New York. Chloramphenicol was from Sigma Chemical Co., and rifampin was obtained from Calbiochem, Los Angeles.

RESULTS

Derepression of VRS formation. The cells were grown exponentially in unrestricted medium, washed twice with minimal medium, and suspended in prewarmed minimal medium containing unrestricted amounts of isoleucine and leucine and 10 μg of glycyl-DL-valine per ml. Upon the transfer from unrestricted to valine-limited growth, there was a twofold increase (derepression of synthesis) of VRS activity within the first 5 min of growth (Fig. 1). Thereafter, the steady-state derepressed level of VRS formation was maintained for the duration of the experiment. That this derepression of VRS formation is a rapid and immediate event is derived from the following consideration. The unrestricted growth rate (k) of the culture was 0.55 [ln2/mass doubling time (h)] compared to a k of 0.21 for valine-restricted growth. Thus, the differential rate of VRS formation was

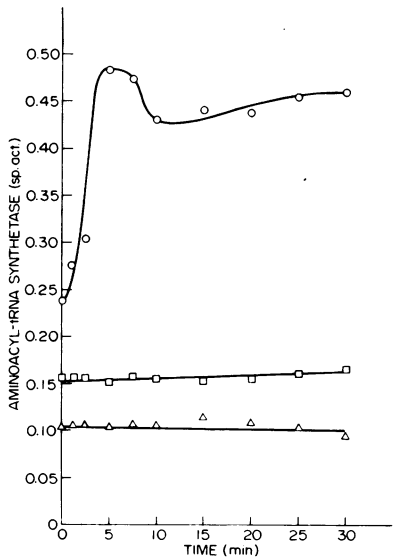

FIG. 1. *Derepression of VRS formation during valine restriction of an isoleucine, valine, and leucine auxotroph. The cells were grown exponentially in minimal medium supplemented with valine (100 μg/ml), leucine (50 μg/ml), and isoleucine (50 μg/ml) for two generations. Then the cells were chilled rapidly, collected by centrifugation, washed twice with minimal medium, and incubated in minimal medium with excess isoleucine and leucine and 10 μg of glycyl-DL-valine per ml. Samples taken at intervals throughout the growth period were assayed for VRS* (○), *LRS* (□), *and IRS* (△) *activities. The results are plotted as specific activity as a function of incubation time in limiting valine.*

increased 42-fold during the first 5 min of valine-restricted growth (Fig. 1).

In agreement with our previous findings (6), there was no change in the rate of synthesis of IRS and LRS during valine-limited growth.

Derepression of IRS formation. After several generations of unrestricted growth, the cells were washed twice with minimal medium and suspended in isoleucine-restricted (10 μg of glycyl-DL-isoleucine per ml) medium (Fig. 2). When isoleucine was limiting growth, there was a threefold increase in the specific activity of IRS in 10 min of growth. In fact, the activity of IRS was elevated twofold over the unrestricted level during the first 5 min of isoleucine-restricted growth. The apparent "overshoot" in IRS activity was observed in several identical experiments, even though it is not understood. When the rate of synthesis of IRS is expressed relative to the rate of total protein synthesis, it is apparent that differential rate of IRS formation was increased 48-fold over that of the unrestricted rate during isoleucine-mediated derepression (Fig. 2). Significantly, isoleucine restriction caused no measurable change in the rate of LRS formation relative to the rate of total protein synthesis (Fig. 2).

Conversely, isoleucine restriction caused a twofold increase in VRS activity within 15 min of cultivation. Since the growth rate of the culture was a k of 0.18 for isoleucine restriction as compared to 0.55 during unrestricted growth, the differential rate of VRS formation was increased 16-fold during isoleucine-restricted growth (Fig. 2). As we have previously reported (6), VRS formation is affected by both valine and isoleucine. However, the cells exhibited a greater and more immediate derepressive response to the absence of the valine-mediated repression signal than that for isoleucine. This can be seen by comparing the data shown in Fig. 1 and 2. VRS formation was maximally derepressed in 5 min during valine restriction, whereas 15 min of isoleucine restriction was required for a similar increase in VRS activity. Thus, while VRS formation is under multivalent control by both valine and isoleucine, valine is the most efficient end product effector.

Derepression of LRS formation. The same branched-chain amino acid auxotroph was grown with leucine limitation (Fig. 3). There was a twofold increase in the specific activity of LRS during 7.5 min of restricted growth. During leucine-limited growth, the cells grew at a k of 0.22 compared to 0.55 during unrestricted growth. Therefore, during derepression, the diffrential rate of LRS formation was increased 26-fold over the unrestricted rate (Fig. 3). Whereas the derepression of LRS formation was only affected by the supply of the cognate amino acid, leucine, to the cells, the rate and

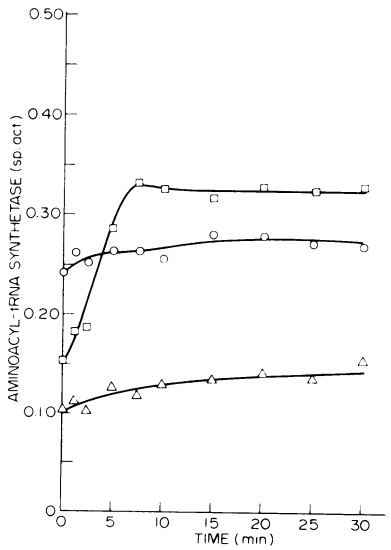

FIG. 2. *Derepression of IRS and VRS formation during isoleucine-restricted growth. All experimental details were as described in the legend to Fig. 1, except that the cells were grown with limiting isoleucine (10 μg of glycyl-DL-isoleucine per ml). The activities were determined for VRS (○), LRS (□), and IRS (△). The results are plotted as described in the legend to Fig. 1.*

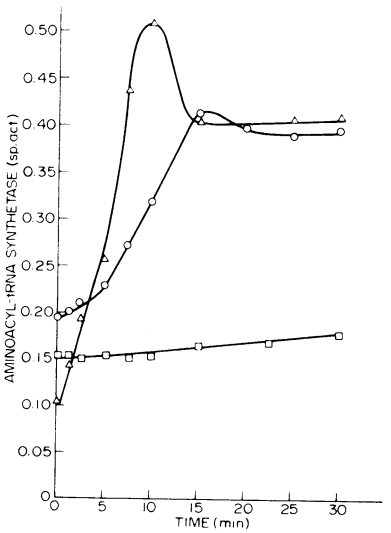

FIG. 3. *Effect of leucine restriction on derepression of LRS formation. All experimental details were as described in the legend to Fig. 1, except that the cells were grown with limiting leucine (10 μg of glycyl-DL-leucine per ml). The activities were determined for VRS (○), LRS (□), and IRS (△). The results are plotted as described in the legend to Fig. 1.*

magnitude of derepression of this synthetase were less than that for VRS and IRS during valine- and isoleucine-restricted growth, respectively (Fig. 1-3). This observation supports our previous findings (6; unpublished data) that the rate of VRS formation varies over the largest range whereas LRS formation exhibits the most restricted repression/derepression mode. Leucine restriction had essentially no affect on the rate of VRS and IRS formation, which is entirely consistent with our previous findings (6; Fig. 3).

Requirement for protein synthesis during derepression. Since the increases in synthetase activities shown in Fig. 1-3 were described as derepressive responses, we addressed the question of whether such changes in activity were, in fact, dependent upon active protein synthesis. We examined this question in two ways: determination of the sensitivity of derepression to inhibition of protein and RNA synthesis, and measurement of the rate of appearance of light enzyme (newly synthesized) after a shift from unrestricted deuterium oxide to amino acid-restricted water medium. The effect of chloramphenicol (CAP) and rifampin on derepression of VRS formation is shown in Fig. 4. The results

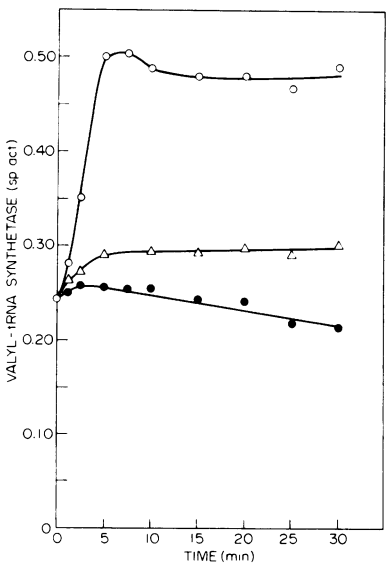

FIG. 4. *Effect of CAP and rifampin on derepression of VRS formation. The cells were grown with limiting valine as described in the legend to Fig. 1. The culture was divided into three flasks, one that was the control, another that received CAP (100 μg/ml), and another that received rifampin (150 μg/ml). The activity of VRS was determined for the control culture* (○), *the CAP-treated culture* (●), *and the culture to which rifampin was added* (△).

indicate that when CAP (100 μg/ml) was added at zero time, there was no derepression of VRS formation during valine-restricted growth. Also, the addition of rifampin (150 μg/ml) at zero time reduced the level of derepression to about 10% of the control value. Perhaps this small increase in VRS represents nascent RNA chains present in the cells at the time of rifampin addition to the culture (Fig. 4). Comparable studies were performed during isoleucine- and leucine-restricted growth, and similar results were obtained for IRS and LRS formation (data not shown).

Measurement of de novo synthesis of VRS and IRS during isoleucine-restricted growth. To further test the requirement for protein synthesis during derepression, we performed the following experiment. The cells were grown in unrestricted medium prepared in deuterium oxide (80%) for many generations, washed twice with minimal medium (water), and then transferred to two flasks containing prewarmed water medium with growth rate-limiting amounts of isoleucine (10 μg of glycyl-DL-isoleucine per ml). To one of the flasks CAP (100 μg/ml) was added at zero time, and the cultures were incubated until the mass of the control culture (minus CAP) had increased 12.5%. The samples were collected, centrifuged, and analyzed as described elsewhere (9; Fig. 5). Figure 5A shows the banding of VRS and IRS activities in CsCl gradients of samples taken during growth in D_2O-unrestricted medium. Figure 5B shows the banding of these two synthetase activities in CsCl gradients from samples taken after a 12.5% increase in mass after a shift from unrestricted D_2O to isoleucine-restricted water medium. There was a significant increase in light enzyme (newly synthesized) for both VRS and IRS (Fig. 5B). Given the amount of light relative to heavy enzyme in the gradients during a 12.5% mass increase, it was determined that VRS formation was derepressed eightfold, and IRS formation was derepressed about elevenfold during isoleucine-restricted growth. The addition of CAP at the zero time completely blocked the appearance of light enzyme, suggesting that the derepression of both VRS and IRS formation required de novo protein synthesis (Fig. 5C). Furthermore, the absence of any light enzyme in the CAP-inhibited culture adds validity to the significance of the accumulated light enzyme in the non-CAP-inhibited culture (Fig. 5B). This is to say that the presence of light enzyme in panel B is not accountable for by considerations such as nonspecific partitioning of heavy enzyme in a water medium in the CsCl gradients.

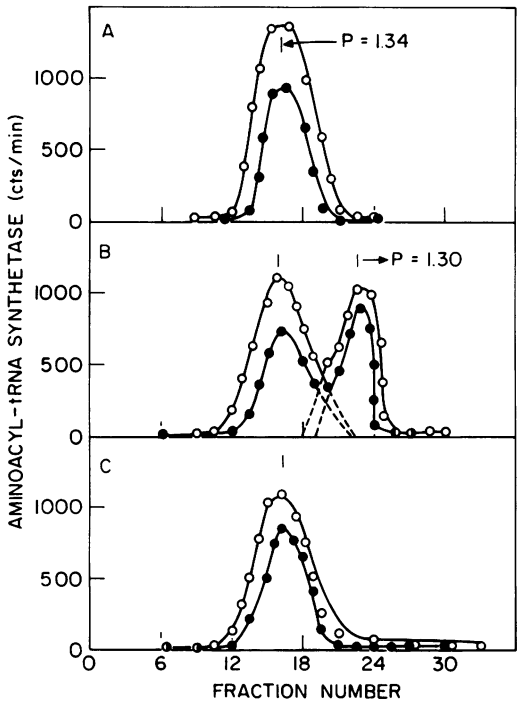

FIG. 5. *Banding in CsCl gradients of VRS and IRS of strain M4862 H-5, grown with isoleucine limitation after a shift from deuterium oxide to water medium. The three panels show banding of enzymes in sample A (80% D_2O), sample B (12.5% mass increase after the shift to water medium), and sample C (the time for a 12.5% mass increase after the shift from D_2O to water for a CAP-inhibited culture). The bottom of the gradient is to the left of each panel. The activities are shown as counts per minute of* L-[^{14}C]*valine* (○) *and isoleucine* (●) *attached to tRNA under the standard assay conditions.*

DISCUSSION

The data presented in this paper provide evidence that synthesis of VRS, IRS, and LRS is rapidly derepressed in response to specific branched-chain amino acid limitations.

Valine and isoleucine restrictions of this *E. coli* auxotroph derepress the rate of VRS formation. The density-labeling data provide clear evidence that this derepression required an increased rate of de novo synthesis of VRS. Although both valine and isoleucine restrictions allowed derepression of VRS formation, synthesis of this synthetase was preferentially affected by valine limitation. Thus, although regulation of VRS formation is similar to multivalent repression of isoleucine and valine biosynthesis (3), it differs from that of the biosynthetic enzymes in several important respects. For VRS, the multivalent repression signal is mediated by valine and isoleucine alone, valine has a greater affect than isoleucine, the noncognate amino acid, and significant derepression of the rate of VRS formation requires a reduction in the intracellular levels of isoleucine or valine or both below that maintained by a prototrophic cell grown in minimal medium.

That the effect of isoleucine restriction on VRS formation was not due to the valine sensitivity character of this *E. coli* strain is supported by our previous findings (6). Specifically, we have shown that VRS formation was derepressed by a valine-mediated isoleucine restriction of another *E. coli* strain and by isoleucine restriction of an auxotrophic strain of *S. typhimurium*. Furthermore, the addition of excess valine and isoleucine to such derepressed cultures specifically repressed VRS formation (6).

For IRS, only isoleucine restriction caused a derepression of the rate of IRS formation. As for VRS formation, the density-labeling data indicate that this specific and immediate derepression of IRS formation required an increased rate of de novo synthesis of this enzyme. These results are consistent with those of Nass and Neidhardt and our previous reports (6, 7) that generation of the repression control signal for IRS formation can be specifically affected by manipulation of the isoleucine supply to the cells, which is unlike the multivalent control of *ilv* regulation by isoleucine, valine, and leucine. Leucine restriction specifically derepressed the rate of LRS formation. Interestingly, the synthesis of LRS was derepressed at a lesser rate than that for IRS and VRS. Perhaps this observation is trivial; however, it does indicate that the rate of LRS formation is responsive to changes in the supply of leucine to the cells. That LRS formation is independent of the valine and isoleucine supply to the cells is similar to that for control of the leucine-specific biosynthetic enzymes (3). However, unlike *ilv* regulation, leucine restriction had no derepressive effect on VRS and IRS formation.

Since we have previously shown that there was parallel, noncoordinate repression control of formation of the branched-chain synthetases and the valine-, isoleucine-, and leucine-forming enzymes (6), the present study was designed to measure that initial rate of derepression of synthetase formation. This question was considered important in that the net change in specific activity of the synthetases is less than that observed for most of the *ilv* and *leu* biosynthetic enzymes. In fact, the results of the present study indicate that the rates of VRS, IRS, and

LRS formation can vary over a considerable range and that the derepression is dependent on active protein synthesis. In this respect, the two classes of enzymes (biosynthetic and synthetases) are controlled in a parallel manner. Does this parallel repression control suggest the existence of similar regulatory units other than the branched-chain amino acids? Several lines of evidence provide support for this possibility. We have recently obtained evidence that aminoacylation of $tRNA^{Val}$ and $tRNA^{Ile}$ is required for repression control of VRS and IRS formation (manuscript in preparation). Furthermore, Levinthal et al. (4) have reported that the *ilvA* gene product participates in regulation of *LeuS*, *IleS*, and *ValS* gene expressions as well as that of the *ilvADE* operon and the *ilvB* and *ilvC* structural genes. In addition, the altered synthesis of the branched-chain synthetases during pyridoxine restriction in the presence of excess amounts of the branched-chain amino acids suggest a common component for repression control of both classes of enzymes (9; unpublished data). Although several models are possible, our present consideration is that this repression control is mediated by a common regulatory element(s) and the specificity lies in differential responses of the unique repressor recognition elements (i.e., operator, promoter, initiator site) or is due to differential sensitivities of the effectors for the common regulatory element(s). Our preliminary data obtained with a variety of different regulatory mutants suggest that either of these considerations is reasonable.

ACKNOWLEDGMENTS

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