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Regulation of Proline Catabolism by Leucyl,Phenylalanyl-tRNA-Protein Transferase*

(*Escherichia coli*/proline oxidase/post-translational modification)

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ABSTRACT **A mutant of *Escherichia coli* lacking leucyl,phenylalanyl-tRNA-protein transferase (L-leucyl-tRNA:protein leucyltransferase, EC 2.3.2.6) exhibited several abnormal growth characteristics relative to the wild type or a revertant when grown with glycerol as a carbon source. All three strains were auxotrophic for proline. The mutant required higher levels of this amino acid than did the other strains to attain a normal growth yield and metabolized exogenous [^{14}C]proline more rapidly. The greater rate of proline utilization was associated with a 4-fold increase in specific activity of proline oxidase. When glucose rather than glycerol was employed as a carbon source, proline oxidase activity was reduced by catabolite repression and the growth characteristics of the mutant were similar to those of the parental and revertant strains. These results suggest that the mutant growth phenotype is due to an altered rate of proline catabolism and constitute evidence for regulation of a specific metabolic pathway by leucyl,phenylalanyl-tRNA-protein transferase.**

A number of enzymatic reactions have been described in which small molecules are added to proteins after the normal process of protein synthesis has been completed. Such reactions may play an important physiological role by altering activity of the acceptor proteins. Regulation of mammalian phosphorylase by phosphorylation (1) and of *Escherichia coli* glutamine synthetase by adenylylation (2) are well characterized examples of this control mechanism. Aminoacyl-tRNA-protein transferases (3) represent an enzymatic mechanism for the post-translational modification of specific polypeptide acceptors by NH_2-terminal addition of certain aminoacyl residues. The *E. coli* enzyme leucyl,phenylalanyl-tRNA-protein transferase (L-leucyl-tRNA:protein leucyltransferase, EC 2.3.2.6) catalyzes transfer of leucine or phenylalanine from tRNA into peptide linkage with basic NH_2-terminal residues of acceptor proteins (4) or peptides (5). This reaction accounts for the observation originally described by Kaji *et al.* (6) that these amino acids are incorporated into proteins by ribosome-free extracts of *E. coli*. The cellular acceptors and physiological function of leucyl,-phenylalanyl-tRNA-protein transferase are unknown. However, we have isolated a mutant lacking the enzyme and found that it possessed several abnormal growth characteristics which were absent in the parental strain and in a revertant that had regained enzymatic activity (7). In this report we present data indicating that these growth defects are due to an increased rate of proline catabolism, thus providing evidence for regulation of a specific metabolic pathway by an aminoacyl-tRNA-protein transferase.

* This is paper no. 12 in a series entitled "Enzymatic Modification of Proteins." The preceding paper in this series is ref. 7.

MATERIALS AND METHODS

Materials. L-[*U*-^{14}C]Proline (260 Ci/mol) was obtained from Schwarz/Mann.

Strains and Culture Conditions. The origins of *E. coli* strains W4977, MS845, and R18 have been described (7). MS845, which lacks leucyl,phenylalanyl-tRNA-protein transferase activity, was derived from W4977. R18 is a spontaneous revertant of MS845. All of the strains are proline auxotrophs, probably due to a deletion in *proA* or *proB*, since we have been unable to isolate any simple prototrophic revertants. Cultures were grown at 37° in a minimal salts medium (7), supplemented with a carbon source and L-proline as specified in each experiment.

Assays. Proline oxidase activity was measured in toluenized whole cells as described by Dendinger and Brill (8). Protein was determined by the method of Lowry *et al.* (9), using bovine serum albumin as the standard.

RESULTS

Growth Characteristics of MS845. We have reported (7) that when stationary phase cells of the parental strain (W4977) and the transferase-deficient mutant (MS845) were diluted into fresh minimal medium supplemented with 0.5% glycerol and 2 mM L-proline, two principal differences in the growth curves were apparent. The mutant exhibited a long lag which lasted up to 12 hr. It also ceased growth prematurely and the final yield of cells was about 40% that of W4977. In spite of these differences, the actual growth rate of MS845 during exponential phase was the same as that of W4977. A revertant (R18) exhibited the same growth characteristics as W4977.

Fig. 1 shows that the low yield observed in the transferase-less mutant reflected a requirement for unusually high exogenous levels of proline. While W4977 and R18 achieved the maximal yield in 1–2 mM proline, the mutant reached this level only if the proline concentration was increased to 10 mM. With suboptimal proline concentrations the yield of MS845 was low compared to that of the wild type, and directly proportional to the proline concentration of the medium.

To determine the relationship between the yield of cells and the growth lag shown by MS845, the mutant was grown to stationary phase either in 2 mM proline, which gave a low yield, or in 20 mM proline, which resulted in a normal yield. W4977 was also grown to stationary phase in 2 mM proline,

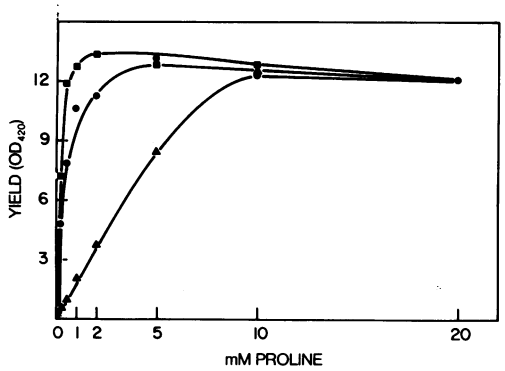

FIG. 1. Growth yields as a function of proline concentration with glycerol as a carbon source. Exponentially growing cells of W4977 (●), MS845 (▲), and R18 (■) were inoculated into fresh minimal medium containing 0.5% glycerol and the indicated concentrations of proline. The initial OD_{420} was approximately 0.05. Yields were determined as OD_{420} after 58 hr of growth.

which gave a normal yield, or in 0.2 mM proline, which resulted in a low yield similar to that originally described for MS845. Each culture was tested for growth in minimal medium containing 0.5% glycerol and 2 mM proline. Fig. 2 shows that the mutant cells grown in 20 mM proline resumed growth with little or no lag, just as did the W4977 cells grown in 2 mM proline. On the other hand, the parental strain grown in 0.2 mM proline showed a long lag similar to that exhibited by MS845 pregrown in 2 mM proline. The long lag is, therefore, a consequence of pregrowth in a suboptimal level of proline, and is not unique to the transferase-less mutant. MS845 requires higher levels of proline to prevent the premature cessation of growth, and this increased proline requirement is the basis of all of the abnormal growth characteristics originally described.

Proline Catabolism by MS845. A requirement for higher exogenous levels of proline might be due either to a decreased rate of proline uptake, or an increased rate of proline catabolism. If the growth defect in MS845 resulted from diminished proline transport, one would expect to find most of the

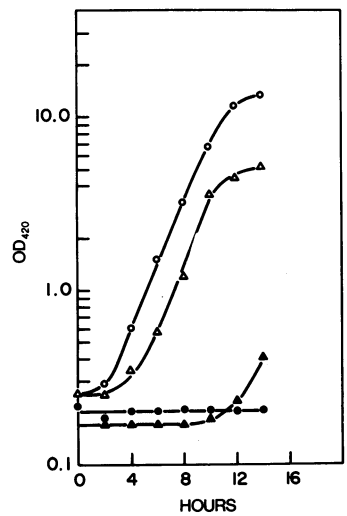

FIG. 2. Growth in 0.5% glycerol and 2 mM proline after pregrowth in different proline concentrations. W4977 was pregrown to stationary phase in 0.2 mM (●) or 2 mM (○) L-proline. MS845 was pregrown in 2 mM (▲) or 20 mM (△) proline.

TABLE 1. *Proline metabolism during growth in glycerol*

Time	Strain	Growth (OD_{420})	Radioactivity (cpm) Cells	Radioactivity (cpm) Medium	Radioactivity in medium as proline (%)	Residual proline in medium (%)
A	W4977	3.52	710	7654	92.9	80.2
	MS845	3.27	3913	1514	27.0	4.7
B	W4977	12.4	2642	5066	70.8	40.4
	MS845	4.48	4208	1586	25.0	4.5

Flasks containing 0.5% glycerol and 2 mM L-[^{14}C]proline (0.25 Ci/mol) were inoculated with exponentially growing W4977 or MS845 cells to give an OD_{420} of 0.08. Each culture initially contained 8800 cpm/10 μl. Time A (480 min) was the point where MS845 ceased growth; time B (720 min) was the point where W4977 entered stationary phase. Portions of each culture were centrifuged and the medium was saved. The cells were taken up in an equal volume of buffer and aliquots (10 μl) of the cell-free medium and the resuspended cells were analyzed for radioactivity on filter paper discs in a liquid scintillation spectrometer. Portions of the medium were subjected to descending paper chromatography on Whatman 3 MM filter paper strips in 1-propanol: NH_4OH (35:18). The strips were cut into 1-cm segments and the proportion of radioactivity as proline (R_F = 0.60) was determined. Residual proline in the medium was calculated relative to the concentration at the time of the original inoculation.

proline still in the medium under conditions where the culture ceased growth prematurely. If the defect were due to an increased rate of proline catabolism, there should be little or no proline in the medium at that point. To distinguish between these possibilities we grew W4977 and MS845 in minimal medium containing 0.5% glycerol and 2 mM L-[^{14}C]proline. The amount of radioactivity incorporated into the cells and the amount remaining in the medium were determined periodically. There was a rapid exponential decrease in radioactivity in the medium of the MS845 culture. By contrast, the level of radioactivity in the medium of the W4977 culture remained high until the cells entered stationary phase. Table 1 shows that less than 5% of the original proline remained in the medium of the MS845 culture at the point when it ceased growth. In contrast, about 40% of the proline was left when W4977 entered stationary phase. The growth defect in MS845 is therefore due to an increased rate of proline catabolism. More radioactivity was incorporated into cells of the mutant, probably because the [^{14}C]proline was degraded to give a variety of labeled intermediates. Moreover, whereas virtually all of the initial radioactivity could be accounted for in the wild-type culture at stationary phase, in the mutant culture almost 40% of the initial radioactivity was lost, presumably as CO_2.

Proline Oxidase Activity in MS845. Proline is degraded in *E. coli* to Δ^1-pyrroline-5-carboxylate, a precursor of glutamate (10). The activity of proline oxidase, the enzyme responsible for this reaction, was measured in toluenized whole cells of each strain. Proline oxidase is a membrane-bound activity in *E. coli* which is coupled to an electron transport chain (10). The specific activity in the mutant was found to be four to

TABLE 2. *Specific activities of proline oxidase*

Strain	Carbon source	Specific activity
W4977	Glycerol	2.15 ± 0.72
MS845	Glycerol	8.55 ± 0.59
R18	Glycerol	2.04 ± 0.54
W4977	Glucose	1.02 ± 0.12
MS845	Glucose	3.09 ± 0.58
R18	Glucose	0.99 ± 0.60

Cultures contained 0.5% of the carbon source indicated and 20 mM L-proline. Cells were harvested in mid-exponential phase and proline oxidase activity was determined. Specific activities are expressed as nanomoles of Δ^1-pyrroline-5-carboxylate generated per min/mg of protein at 37°. The data include the means and standard deviations for five experiments with each strain.

five times that of the parental strain or the revertant (Table 2). This increase in activity of the proline oxidative system seemed a reasonable explanation for the abnormal growth phenotype of the transferase-less mutant. To confirm this conclusion we took advantage of the fact that proline oxidase is subject to catabolite repression (8, 11). Activity was assayed in cultures grown with 0.5% glucose as the carbon source. The specific activity of the parental strain and the revertant were reduced about 50% from the level found in glycerol-grown cells (Table 2). The specific activity of MS845 was reduced about 65%; the resulting activity was comparable to that of wild-type cells grown in glycerol. With glucose as the carbon source the yields of MS845 with different proline concentrations were similar to those of W4977 and R18 (Fig. 3). Furthermore, stationary phase cells of the mutant grown in glucose and 2 mM proline resumed growth with little or no lag when diluted into fresh medium. The level of proline oxidase activity in MS845 was thus found to correlate with its growth characteristics in the presence of the different carbon sources.

DISCUSSION

These experiments suggest that proline catabolism in *E. coli* is regulated by leucyl,phenylalanyl-tRNA-protein transferase. The mutant lacking this enzyme shows an increased rate of proline catabolism associated with an increased specific activity of proline oxidase. This specific alteration acounts for the growth defects of the mutant. MS845 utilizes proline at a faster rate and, therefore, requires higher exogenous levels to attain a normal growth yield. If adequate proline is not provided, the cells undergo proline starvation and show a long lag before resuming growth in fresh medium. The mechanism responsible for the lag is not clear. However, it appears to be a consequence of proline deprivation rather than absence of leucyl,phenylalanyl-tRNA-protein transferase, since it was also observed in the parental strain grown under conditions where proline was growth-limiting.

The growth characteristics of MS845 are contingent on the fact that it is also a proline auxotroph. Proline oxidase activity is inducible by proline (8, 10), and, therefore, would not be present if the strain were prototrophic and grown in the absence of proline. The fact that a proline requirement was crucial to the identification of proline catabolism as a pathway controlled by leucyl,phenylalanyl-tRNA-protein transferase suggests that it may be useful to investigate the effect of the transferase mutation on strains with different genetic backgrounds. Previous evidence has indicated that there are a number of protein acceptor substrates of the enzyme *in vivo* (7) and the transferase, therefore, probably influences other metabolic pathways.

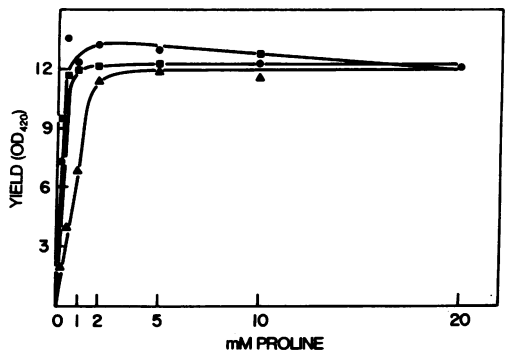

FIG. 3. Growth yields as a function of proline concentration with glucose as a carbon source. The experimental conditions were identical to those described in Fig. 1, except that 0.5% glucose was used in place of glycerol. W4977 (●); MS845 (▲); R18 (■).

Our results suggest that a component of the proline oxidase system is a protein whose activity is diminished by post-translational aminoacylation. We have attempted to obtain direct evidence for this conclusion by using purified leucyl,-phenylalanyl-tRNA-protein transferase to acylate a crude membrane fraction containing proline oxidase activity. This particulate fraction was found to accept approximately 0.5 nmol of phenylalanine per mg of protein. However, the experiments were technically unsatisfactory because proline oxidase activity was rapidly lost during incubation either in buffer alone or with the components required for addition of phenylalanine.

E. coli proline oxidase is membrane-bound and thought to be coupled to an electron transport chain using oxygen as the terminal acceptor (10). It is conceivable that any protein involved in this oxidation system may be the physiological substrate for leucyl,phenylalanyl-tRNA-protein transferase. However, since MS845 grows at a rate identical to that of the wild type during exponential phase, it is unlikely that an electron carrier involved in general oxidative metabolism is altered. Therefore, the most reasonable candidate as an acceptor would be the specific proline oxidase component. If this is the case, then this protein in MS845 should possess a basic NH_2-terminal residue (5). If acylation of this protein causes its complete inactivation, then heterogeneity of the NH_2-terminus might be anticipated in the wild-type protein. Leucine and/or phenylalanine should account for 80% of the residues and the basic amino acid for the remainder. Isolation and characterization of the specific proline oxidase protein from the mutant and wild-type strains should provide explicit evidence concerning these predictions.

The proline oxidative system appears to be subject to at least three different kinds of regulation: induction by proline, catabolite repression, and post-translational aminoacylation. The effect of acylation is to reduce proline oxidase activity and thus counteract the induction of this enzyme. On the surface, this would seem to be an unnecessary waste of protein-synthesizing capacity. However, this may be compensated for by a more efficient utilization of carbon and nitrogen sources. Under our growth conditions the cells are provided with a

carbon source (e.g., glycerol) and a nitrogen source (NH_4^+) in addition to the proline. Proline is, therefore, needed only for protein synthesis, and reduction of proline oxidase activity conserves the available proline and allows preferential utilization of simpler carbon and nitrogen compounds. It will be interesting to compare the growth and proline oxidase activities of W4977 and MS845 under conditions where proline must serve as the sole carbon or nitrogen source. It has been shown that during nitrogen-limited growth, proline oxidase is no longer subject to catabolite repression (10). Further analysis of proline oxidase activity should prove useful in understanding how different regulatory mechanisms interact and particularly in clarifying how leucyl,phenylalanyl-tRNA-protein transferase contributes to the control of metabolic pathways.

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1. Krebs, E. G., Kent, A. B. & Fisher, E. H. (1958) "The muscle phosphorylase b kinase reaction," *J. Biol. Chem.* **231**, 73–83.
2. Shapiro, B. M. & Stadtman, E. R. (1970) "The regulation of glutamine synthesis in microorganisms," *Annu. Rev. Microbiol.* **24**, 501–524.
3. Soffer, R. L. (1974) "Aminoacyl-tRNA transferases," *Advan. Enzymol.* **40**, 91–139.
4. Leibowitz, M. J. & Soffer, R. L. (1971) "Enzymatic modification of proteins. VII. Substrate specificity of leucyl,-phenylalanyl-transfer ribonucleic acid-protein transferase," *J. Biol. Chem.* **246**, 5207–5212.
5. Soffer, R. L. (1973) "Peptide acceptors in the leucine,-phenylalanine transfer reaction," *J. Biol. Chem.* **248**, 8424–8428.
6. Kaji, A., Kaji, H. & Novelli, G. D. (1965) "Soluble amino acid-incorporating system. I. Preparation of the system and nature of the reaction," *J. Biol. Chem.* **240**, 1185–1191.
7. Soffer, R. L. & Savage, M. (1974) "A mutant of *Escherichia coli* defective in leucyl,phenylalanyl-tRNA-protein transferase," *Proc. Nat. Acad. Sci. USA* **71**, 1004–1007.
8. Dendinger, S. & Brill, W. J. (1970) "Regulation of proline degradation in *Salmonella typhimurium*," *J. Bacteriol.* **103**, 144–152.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) "Protein measurement with the Folin-phenol reagent," *J. Biol. Chem.* **193**, 265–275.
10. Frank, L. & Ranhand, B. (1964) "Proline metabolism in *Eschericha coli*. III. The proline catabolic pathway," *Arch. Biochem. Biophys.* **107**, 325–331.
11. Prival, M. J. & Magasanik, B. (1971) "Resistance to catabolite repression of histidase and proline oxidase during nitrogen-limited growth of *Klebsiella aerogenes*," *J. Biol Chem.* **246**, 6288–6296.