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ERRONEOUS SYNTHESIS OF RIBOSOMAL PROTEINS IN AMINO ACID STARVED *E. COLI*

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SUMMARY : The effect of amino acid starvation on the accuracy of translation of ribosomal proteins was analyzed in a stringent (*relA*$^+$)/relaxed (*relA*) pair of *E. coli* strains. The degree of misreading was estimated from the amount of cysteine erroneously incorporated into individual proteins during arginine starvation of bacteria. Illegitimate incorporation of cysteine was found to occur to a significant extent in several proteins from both the small and the large subunits of ribosomes, in either type of strain.

When bacteria of stringent (*relA*$^+$) genotype are starved for any amino acid essential to growth, they initiate a program of metabolic adjustments termed the "stringent response". No such adaptation of physiological activity is however observed in mutant cells harboring the relaxed (*relA*) genotype (1,2). In either type of strain, amino acid starvation results in the same drastic reduction in the rate of protein synthesis to only a few percent of the value measured in normally growing cells (3). The persistent level of protein synthesis is then sustained by amino acid molecules arising from protein turnover (4).

From a qualitative point of view, a number of reports indicate that relaxed, but not stringent, bacteria respond to amino acid deprivation with an enhanced frequency of translational errors. For instance, β-galactosidase synthesized by starving *relA* mutants exhibits a reduced intrinsic specific activity per monomer and an increased thermolability (5,6). Also, arginine limitation of relaxed cells gives rise to the illegitimate incorporation of cysteine into flagellin, the protomeric subunit of bacterial flagella (7). Moreover, under starvation for certain amino acids, a pattern of charge heterogeneity in several proteins resolved by two-dimensional electrophoresis can be

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observed (8,9). Other results demonstrate that positive phenotypic suppression of frameshift and nonsense mutations occurs in *relA* strains during limited acylation of some tRNA species (10,11). In addition, amino acid shortage induces the accumulation of small polypeptides which represent prematurely released translation products due to the presence of out-of-phase terminating codons (8,12). Taken together these observations have led to the conclusion that the increased frequency of mistranslation in starving relaxed bacteria pertains to both missense and reading-frame errors (10,13).

The precise mechanism responsible for the differential effect of starvation on translational accuracy in *relA*$^+$/*relA* cells remains however to be elucidated. A variety of results suggest that the effect of the stringent response on the fidelity of translation is mediated by the guanosine tetraphosphate ppGpp which accumulates specifically in *relA*$^+$ cells upon starvation (2,8). This hypothesis is supported, in particular, by the fact that *relA* mutants are capable of normal enzyme synthesis during amino acid limitation if conditions independent of *relA* genotype produce an elevated ppGpp concentration (6,10). It is also favored by *in vitro* experiments showing that ppGpp reduces misincorporation in a starved system (14), possibly by interacting with the translation factor EF-Tu (15).

In the present work, the misreading effect of starvation was investigated further by analyzing the structural quality of the ribosome components synthesized by *relA*$^+$/*relA* bacteria during limited protein synthesis. More precisely, an attempt was made to estimate the degree of misincorporation of cysteine into ribosomal proteins during arginine deprivation of cells. Special attention was paid to those proteins which are naturally devoid of cysteine (16,17).

MATERIALS AND METHODS

The otherwise isogenic pair of *E. coli* strains CP78 (*relA*$^+$) and CP79 (*relA*) was used (18). Both strains require histidine, threonine, arginine, leucine and thiamin for growth. Bacteria were cultured at

37°C in a minimal Tris-glucose medium (19) containing the four essential amino acids (50 µg/ml each) and the vitamin (5 µg/ml). They were collected in mid-logarithmic phase by low-speed centrifugation and separated into two portions. The first portion was resuspended in the complete culture medium and the other in a starvation medium lacking arginine. In each case, cells were then double-labeled with ^{3}H-lysine (5-8 µCi/ml ; spec. act. 10-25 Ci/mmol) and ^{35}S-cysteine (2-4 µCi/ml ; spec. act. 800-1000 Ci/mmol). In order to minimize the labeling of methionine from radioactive cysteine by amino acid interconversion, an excess of unlabeled methionine (400 µg/ml) was simultaneously added. After 30-40 min, cells were pelleted and ribosomes were isolated as previously described (20). Ribosomal proteins were extracted by the acetic acid procedure (21), and analyzed by two-dimensional gel electrophoresis (22). Separation was carried out at pH 8.6 in 4 % acrylamide in the first dimension, and at pH 4.5 in 18 % acrylamide in the second dimension. After migration, proteins were visualized by Coomassie blue staining, then individual spots were cut out of the gel and dissolved in Soluene-350 (Packard Co.) at room temperature. Only well-resolved spots were analyzed. Radioactivity was counted in a Tri-Carb Packard spectrometer using the appropriate double-label setting, and the ratio $R = {}^{35}S/{}^{3}H$ was determined for each protein.

RESULTS AND DISCUSSION

The results presented in Table I show first, as a control, that proteins which are naturally devoid of cysteine (16,17), incorporate a relatively low amount of this aminoacid when *relA*$^{+}$/*relA* cells are normally grown in a complete culture medium. The only exception concerns protein S9, for unclear reasons. Under the same growth conditions, proteins containing one or several cysteine residues incorporate a much higher amount of ^{35}S-radioactivity, as expected (with again one exception concerning protein L31).

When bacteria are now subjected to arginine starvation, it can be observed that several proteins from both the small and the large subunits of ribosomes misincorporate cysteine with an enhanced frequency, in either type of strain. This applies namely for proteins which naturally do not contain cysteine, such as S3, S5, S7, S19, S20, L1, L3, L13, L15, L16, L18, L19, L21, L22, L23, L24, L25, L29 and L31 (16,17). Thus, for example, the amount of ^{35}S-radioactivity erroneously incorporated into proteins L16 and L18 is 11-12 times higher in *relA* starving cells than in normally growing cells. When comparing *relA*$^{+}$ to *relA* bacteria it appears that, in most instances, the illegitimate incorporation of cysteine into one given ribosomal protein occurs to a

comparable extent in the two strains (proteins S3, S5, S7, L1, L3, L15, L19, L21, L22, L23, L24, L25 and L31). However, for some proteins, it is more pronounced in the relaxed strain (proteins S20, L16, L18 and L29) and, at the opposite, it is sometimes higher in the stringent strain (proteins S19 and L13).

It can therefore be concluded that a significant number of ribosomal proteins, like several non-ribosomal proteins (5-9), are erroneously synthesized during amino acid starvation. The finding that, under our conditions, stringent cells are capable of mistranslation as well as relaxed cells seems to contradict some previous reports indicating that miscoding pertains specifically to *relA* strains (5,8). However, it has been shown that certain stringent strains also mistranslate to a high degree, almost as high as that observed in relaxed mutants(9,23). These latter results were obtained by using namely the *E. coli* CP78/CP79 strains which were also used in the present work. The possibility that such strains, and their derivatives, harbor a cryptic mutation which affects translational fidelity has been envisaged (23). Nevertheless, this could hardly explain why strain CP78 synthesizes fully active β-galactosidase during arginine deprivation while strain CP79 does not (5). Concerning this enzyme, controversial results yet indicate that, even in *relA*$^+$ cells, it is synthesized in an inactive form during starvation because of an incorrect, although complete, translation of the message (24).

Faulty ribosomal proteins were actually detected in the total ribosome population of the cells, including ribosomes actively engaged in protein synthesis as well as non-engaged ribosomes. No differentiation was therefore made between ribosomal proteins belonging to one class of ribosomes or the other, and further experiments would be required to check the quality of the protein content of the different classes of bacterial ribosomes. One can speculate that abnormal proteins are present only in those ribosomes which do not participate in polypeptide

TABLE I. Effect of arginine starvation on cysteine incorporation into ribosomal proteins.

Protein	Molar ratio Cys/Lys	$R = {}^{35}S/{}^{3}H \times 10^3$				$R_{starved}/R_{control}$	
		control		starved		CP78	CP79
		CP78	CP79	CP78	CP79		
S3	0:23	1.4	1.6	3.8	4.9	2.7	3.1
S4	1:20	22.0	21.9	20.7	14.3	0.9	0.7
S5	0:12	5.2	5.2	16.8	16.2	3.2	3.1
S7	0:14	3.0	3.3	18.1	15.9	6.0	4.8
S8	1:12	33.0	33.7	31.4	21.8	0.9	0.6
S9	0:10	37.6	43.7	39.1	26.4	1.0	0.6
S12	4:13	170.1	159.1	122.0	181.3	0.7	1.1
S13	1:11	37.9	41.3	27.0	18.7	0.7	0.5
S14	1:11	44.9	42.5	35.0	20.4	0.8	0.5
S15	0:6	25.0	26.9	30.3	24.3	1.2	0.9
S18	1:6	52.5	51.4	64.5	37.9	1.2	0.7
S19	0:13	2.4	2.5	5.9	3.8	2.5	1.5
S20	0:14	3.5	4.2	4.3	16.9	1.2	4.0
S21	1:7	50.1	47.0	43.8	34.7	0.9	0.7
L1	0:23	1.5	1.5	4.6	5.0	3.1	3.3
L2	2:25	51.3	47.4	35.2	21.8	0.7	0.5
L3	0:19	1.3	1.4	4.6	6.6	3.5	4.7
L5	1:16	27.9	27.1	27.4	19.5	1.0	0.7
L6	1:16	23.0	23.2	23.1	16.8	1.0	0.7
L11	1:13	27.0	27.7	23.3	17.9	0.9	0.6
L13	0:14	2.1	2.4	10.6	5.1	5.0	2.1
L14	1:11	68.2	68.6	54.8	33.5	0.8	0.5
L15	0:13	5.2	6.7	10.4	16.6	2.0	2.5
L16	0:16	9.7	9.8	17.1	122.5	1.8	12.5
L17	1:8	50.8	50.7	40.6	34.3	0.8	0.7
L18	0:8	1.3	1.4	6.7	15.5	5.1	11.1
L19	0:11	1.9	2.2	7.7	7.6	4.1	3.5
L21	0:10	2.2	2.1	15.2	15.7	6.9	7.5
L22	0:13	1.7	1.8	5.7	7.8	3.4	4.3
L23	0:14	1.2	1.3	4.9	4.8	4.1	3.7
L24	0:16	1.5	1.5	3.9	3.7	2.6	2.5
L25	0:11	3.2	3.2	7.8	6.0	2.4	1.9
L27	1:11	39.1	37.8	38.6	54.0	1.0	1.4
L28	1:7	57.7	78.3	52.8	46.6	0.9	0.6
L29	0:5	6.1	5.1	18.1	34.9	3.0	6.8
L31	4:7	23.7	32.8	46.1	77.0	1.9	2.3
L32	0:6	7.6	7.4	4.3	7.1	0.6	0.9
L33	0:12	10.5	8.2	4.9	5.0	0.5	0.6

formation. But, if this is not the case, it is then possible that the presence of erroneous proteins in translating ribosomes contributes to the observed increase in error frequency, especially if structural alterations occur in some specific ribosomal proteins crucially required for accurate protein synthesis. This could concern, for example, proteins L16 and L18 which are located in the same functional domain of ribosomes (17,25), and are essential for peptidyl-transferase activity and binding of aminoacyl- or peptidyl-tRNA (26,27). The qualitative modification of these two proteins might be responsible, at least in part, for the misfunctioning of ribosomes, particularly in relaxed cells (see Table 1). By contrast, it is possible that the presence within the ribosomes of some other proteins, although equally modified, would not interfere with the process of accurate translation if they are not critically involved in this process.

It must be noted that only undegraded faulty proteins, whose migration was identical to that of normal proteins in the electrophoretic system used, were analyzed under our experimental conditions. Since abnormal proteins are known to be rapidly degraded *in vivo* (28), it is likely that part of them escaped our analysis. Moreover, for the same technical reason, any polypeptide prematurely released because of the presence of out-of-phase terminating codons (12,13) could not be detected either. Therefore, the present investigation deals essen-

Table 1 (continued)

CP78 and CP79 cells were separately double-labeled for 30-40 min with ^{3}H-lysine and ^{35}S-cysteine during either exponential growth (control) or arginine deprivation (starved). Ribosomal proteins were extracted and analyzed by two-dimensional gel electrophoresis. For each of them, the respective amount of radioactive cysteine and lysine incorporated was measured, and the ratio R = $^{35}S/^{3}H$ was calculated. Average data from 4 experiments are given. Standard deviation of mean is ± 23 % of expressed values. For each strain, the R value in starving cells relative to exponentially growing cells was also calculated ($R_{starved}/R_{control}$). The statistical analysis of measurements indicated that any expressed relative value reflects a significant variation of cysteine incorporation induced by starvation when differing from 1.0 by more than ± 0.5. The Cys/Lys molar ratio for individual proteins was previously determined (16,17).

tially with the missense errors made by starving bacteria, that is amino acid substitutions without abortive protein synthesis. Concerning the specificity of these errors with regard to the genetic code, our data favor the concept that misreading occurs at the first codon position (7). Indeed, since bacteria are particularly prone to misread codons calling for amino acids which are in short supply (2,10), the misincorporation of cysteine into ribosomal proteins during arginine starvation indicates that the two arginine codons CGU and CGC are then confused with the cysteine codons UGU and UGC. Yet, it cannot be excluded that other types of amino acid replacement occur as well, due to misreading at the second or the third position, but they were not detectable by our experimental approach.

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