A Cycloheximide Sensitivity Factor from Yeast Required for *N*-Acetylphenylalanylpuromycin Formation†

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ABSTRACT: A protein (factor P) has been isolated from yeast, which was required for sensitivity to cycloheximide of a partially purified polyphenylalanine synthesis system. In the absence of factor P, 10^{-3} M cycloheximide was required for 50% inhibition of polyphenylalanine synthesis, while, in its presence, 10^{-6} M gave 50% inhibition. Coincident with cycloheximide sensitivity was an activity required for EF-2 dependent *N*-acetylphenylalanylpuromycin (*N*-AcPhePuro) formation. Transfer of *N*-AcPhe to puromycin from the tRNA bound in the presence of 26 mM $MgCl_2$ required factor P, as well as EF-2. Studies with antibody against EF-2 demonstrated that P factor was not required during the EF-2 translocation step but for some subsequent step.

Structure-function relationships in bacterial ribosomes have been developed in great detail (Wittmann, 1976). Eukaryotic ribosomes have not yet received the same attention and, consequently, much less is known about their structure. A useful approach in the attack on bacterial ribosomes was the study of how inhibitors of protein synthesis affect ribosome function. Presumably, a similar approach will provide information on eukaryotic ribosomes as well.

Cycloheximide is a well known inhibitor of protein synthesis which specifically affects cytoplasmic ribosomes from eukaryotes. Resistance to this inhibitor in yeast has been shown to result from alteration of the 60S ribosomal subunit (Cooper et al., 1967; Rao and Grollman, 1967). The specific site of inhibition has not been established.

While analyzing the properties of a highly purified polyphenylalanine synthetic system from yeast (Skogerson and Wakatama, 1976), we were surprised to find that sensitivity of the crude system to cycloheximide was greatly reduced after resolution of the elongation factors on DEAE[1]-Sephadex. These results suggested that a component necessary for cycloheximide sensitivity had been separated from the elongation factors. Analysis of the eluate from the DEAE-Sephadex column used to resolve the elongation factors revealed a fraction which restored cycloheximide sensitivity to the purified system.

Resolution of the elongation factors on DEAE-Sephadex resulted in an EF-2 fraction which had lost the capacity to promote *N*-acetylphenylalanylpuromycin synthesis. An activity which restored *N*-acetylphenylalanylpuromycin synthetic activity to EF-2 was detected in the DEAE-Sephadex eluate in a position coincident with the cycloheximide sensitivity factor.

The following report describes the isolation and initial characterization of these new yeast activities.

Materials and Methods

Biochemicals and reagents were obtained from standard sources as previously described (Skogerson et al., 1973; Skogerson and Wakatama, 1976). Ribosomes, elongation factors, and [^{3}H]Phe-tRNA (sp act., 7.79 Ci/mmol) were prepared as previously described (Skogerson et al., 1973; Skogerson and Wakatama, 1976).

Preparation of Antiserum Against EF-2. Elongation factor 2 was purified to apparent homogeneity by chromatography of the DEAE-Sephadex fraction (Skogerson and Wakatama, 1976) on DEAE-cellulose, followed by hydroxylapatite (Skogerson, in preparation). The criteria for homogeneity were single visible bands when 30-μg samples were subjected to sodium dodecyl sulfate (Weber and Osborn, 1969) or native (Gabriel, 1971) gel electrophoresis.

Each of two rabbits was injected in the toe pads with 400 μg of EF-2 in complete adjuvant. Subcutaneous boosters of 150 and 75 μg were administered at 6 and 8 weeks, respectively. Neither rabbit had an observable titer at 4 weeks. One rabbit had a weak response after the first booster but, again, nothing after the second one. Sera from the other rabbit gave a strong Ouchterlony (1964) test which peaked at about 10 days after the second booster. No response was detected after a third booster of 75 μg at 10 weeks.

DEAE-Sephadex Chromatography of Factor P. Conditions for chromatography and preparation of starting material were as described for resolution of elongation factors (Skogerson and Wakatama, 1976).

Preparation of N-Ac[^{3}H]Phe-tRNA. Acetylation of [^{3}H]Phe-tRNA was carried out as described by Haenni and Chapeville (1966). At the end of the reaction the *N*-Ac-[^{3}H]Phe-tRNA was desalted on Sephadex G-25 equilibrated with H_2O and was stored at -20 °C as the lyophilized powder. Chromatographic or electrophoretic analysis of the product of alkaline hydrolysis showed only *N*-Ac[^{3}H]Phe and no [^{3}H]Phe.

Polyphenylalanine Synthesis. Reaction mixtures of 50 μl contained 60 mM Tris-acetate, pH 7.0, 50 mM NH_4Cl, 12 mM $MgCl_2$, 20 mM DTT, 1 mM GTP, 8 μg of poly(U), 0.04 A_{260} unit of ribosomes, 10 μg of EF-1, 0.15 μg of EF-2, 0.2 μg of EF-3 and 10–15 pmol of [^{3}H]Phe-tRNA. After 5 min at 30 °C, the hot CCl_3CO_2H insoluble radioactivity was determined

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‡ Recipient of a Faculty Research Award from the American Cancer Society.

[1] Abbreviations used are: EF, elongation factor; EF-3, yeast ribosome-dependent GTPase described previously (Skogerson and Wakatama, 1976); *N*-AcPhePuro, *N*-acetylphenylalanylpuromycin; DEAE, diethylaminoethyl; DTT, dithiothreitol; GTP, guanosine triphosphate; ADP, adenosine diphosphate; poly(U), poly(uridylic acid).

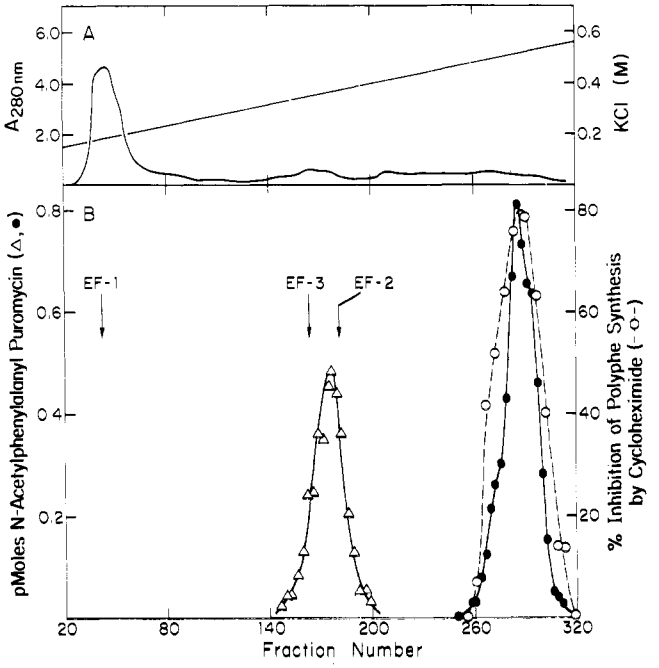

FIGURE 1: Separation of P factor from elongation factors on DEAE-Sephadex. (A) Protein profile and the applied KCl gradient. (B) Assay of the indicated fractions: (○) for the inhibition of polyphenylalanine synthesis by 10^{-4} M cycloheximide in the presence of 0.15 μg of EF-2, 0.2 μg of EF-3, 10 μg of EF-1, and 2 μl of column fractions; (●) *N*-AcPhePuro synthesis in the presence of 0.15 μg of EF-2, 0.2 μg of EF-3, and 1 μl of column fractions; (△) *N*-AcPhePuro synthesis in the presence of 0.3 μg of P factor. The reactions were carried out as described in the text.

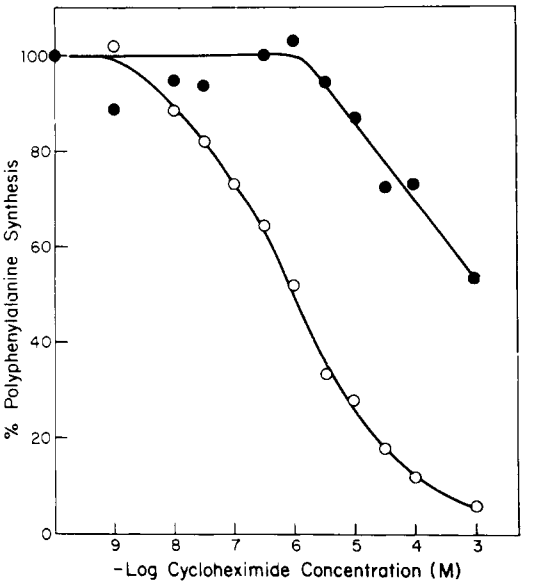

FIGURE 2: Cycloheximide concentration curves. Polyphenylalanine synthesis was measured at the indicated cycloheximide concentrations in the absence of P factor (●) or in the presence of 0.9 μg of P factor (○). Other factors are as indicated in Figure 1B. Uninhibited values were 1.45 pmol in the presence of P and 1.57 pmol in the absence of P.

as described (Skogerson et al., 1973).

N-Acetylphenylalanylpuromycin Synthesis. The reaction conditions were a modification of those reported by Siler and Moldave (1969). Reaction mixtures of 40 μl, containing 60 mM Tris-acetate, pH 7.0, 50 mM NH_4Cl, 26 mM $MgCl_2$, 8 μg of poly(U), 0.1 A_{260} unit of ribosomes, and 5 pmol of *N*-Ac[^{3}H]Phe-tRNA, were incubated for 20 min at 30 °C. Bound *N*-Ac[^{3}H]Phe-tRNA was determined by the nitrocellulose filter assay (Skogerson and Wakatama, 1976). In some cases, ten times more concentrated binding reactions were used.

For the transfer of *N*-Ac[^{3}H]Phe to puromycin, the binding reaction mixtures were diluted to 120 μl so that the final concentrations of salts were 60 mM Tris-acetate, pH 7.0, 50 mM NH_4Cl, $MgCl_2$ as indicated, 16 mM DTT, 0.8 mM GTP, 0.8 mM puromycin, and factors as indicated. After an appropriate time at 30 °C, the formation of *N*-Ac[^{3}H]PhePuro was stopped by the addition of 1 ml of 0.1 M Tris-Cl, pH 7.0, and the product was extracted into 1.5 ml of ethyl acetate as described by Leder and Bursztyn (1966). Radioactivity in 1 ml of the ethyl acetate phase was determined with 3 ml of Aquasol (New England Nuclear) at an efficiency of 18%. Results were corrected to 1.5 ml.

Results

Cycloheximide Sensitivity Factor. We previously reported the resolution by DEAE-Sephadex chromatography of three proteins required for poly(U)-directed polyphenylalanine synthesis (Skogerson and Wakatama, 1976). The strain of yeast used in the development of that system was sensitive to cycloheximide, as was the in vitro system using unresolved factors. We were surprised to find that polyphenylalanine synthesis, using factors resolved by DEAE-Sephadex chromotography, was insensitive to cycloheximide. These observations suggested that a component required for cycloheximide sensitivity had been separated from the elongation factors on the DEAE-Sephadex column. Accordingly, we assayed fractions from a DEAE-Sephadex column for their ability to restore cycloheximide sensitivity of polyphenylalanine synthesis with resolved factors. As seen in Figure 1, a fraction which eluted at about 0.5 M KCl greatly increased the sensitivity of polyphenylalanine synthesis to 10^{-4} M cycloheximide.

In order to characterize the inhibition observed in the presence of the sensitivity factor, the effect of cycloheximide concentration on inhibition in the presence and absence of the factor was determined. As seen in Figure 2, 50% inhibition was obtained at 10^{-3} M in the absence of the factor but 10^{-6} in the presence of factor. The presence of the sensitivity fraction decreased the concentration of cycloheximide required to produce a given amount of inhibition by about 1000-fold.

The above observation raised the question of whether the sensitivity factor might be involved in polyphenylalanine synthesis. As indicated in the legend to Figure 2, the uninhibited rates of polyphenylalanine synthesis were similar whether the sensitivity fraction was present or not. This result confirmed the observation that no activity required for polyphenylalanine synthesis had been detected in the region of the sensitivity factor. In order to substantiate this conclusion, we tested the effect of the sensitivity fraction on the rate and extent of polyphenylalanine synthesis, as well as its capacity to modulate an elongation factor requirement. These data all showed no detectable effect and are therefore not presented here.

N-AcPhePuro Factor. In order to identify the function of the three elongation factors separated by DEAE-Sephadex chromatography (Skogerson and Wakatama, 1976), we developed polymerization-independent assays for each. Translocation-dependent formation of *N*-AcPhePuro has been used as a convenient assay for EF-2 activity (Siler and Moldave, 1969; Tanaka et al., 1970; Gatica and Allende, 1971). When EF-2 containing fractions from the DEAE-Sephadex column were assayed for *N*-AcPhePuro formation by the method of Siler and Moldave (1969), no EF-2 dependent synthesis was observed. The unresolved fraction, prior to the DEAE-Sephadex step, had considerable activity in this reaction. These

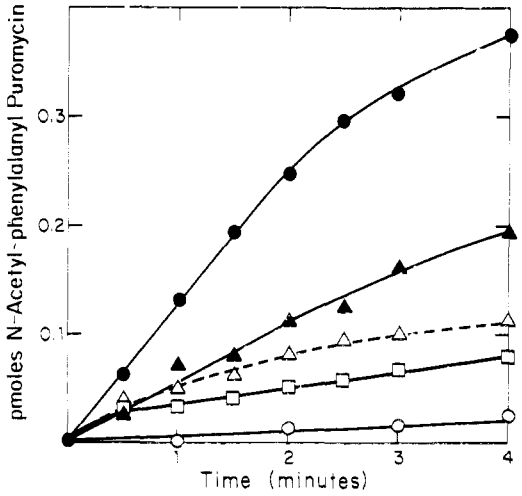

FIGURE 3: Factor requirement for *N*-AcPhePuro synthesis. The reaction was carried out as a time course in the presence of (●) 0.08 μg of EF-2, 0.2 μg of EF-3, and 0.3 μg of P factor; (▲) EF-2 and P factor; (△) EF-2 and EF-3. A background value of 0.08 pmol has been subtracted from all points.

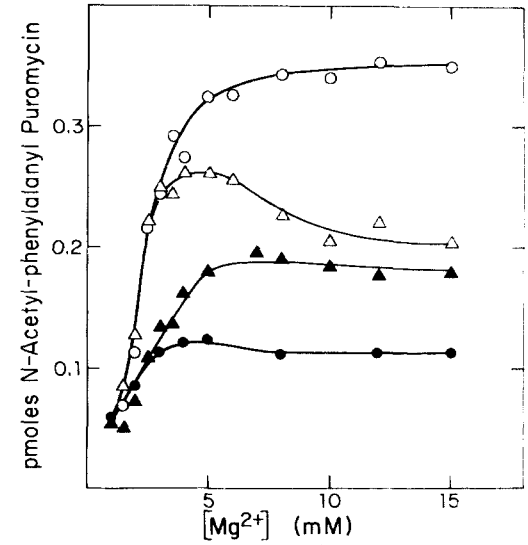

FIGURE 4: Effect of $MgCl_2$ concentration on the factor dependence of *N*-AcPhePuro synthesis. The reaction was carried out for 3 min at 30 °C in the presence of (○) 0.08 μg of EF-2, 0.2 μg of EF-3, and 0.3 μg of P factor; (△) EF-2 and P factor; (▲) EF-3 and P factors; (●) P factor. Activity in the absence of P factor was less than 0.1 pmol at all $MgCl_2$ concentrations.

results suggested that a component which was required in addition to EF-2 had been separated. Various fractions were assayed for *N*-AcPhePuro synthesis in the presence of EF-2 and EF-3. As seen in Figure 1, the additional factor required for this reaction coincided with the cycloheximide sensitivity fraction described above. We have temporarily designated the *N*-AcPhePuro activity factor P. The coincidence of the two activities strongly suggested that the same protein was required for both *N*-AcPhePuro synthesis and cycloheximide sensitivity. Final solution of this question depends on the purification to homogeneity of P factor. In preliminary attempts at purification, both cycloheximide sensitivity and *N*-AcPhePuro activities coincided after DEAE-cellulose and phosphocellulose chromatography.

Factor Requirements for N-AcPhePuro Synthesis. As mentioned above, the original purpose for developing the *N*-AcPhePuro synthetic system was to assay for EF-2 activity. In order to determine which elongation factor was complementary to factor P, we assayed various fractions from the DEAE-Sephadex column for *N*-AcPhePuro synthesis in the presence of factor P. As seen in Figure 1, the resulting activity was between the EF-3 and EF-2 peaks, determined by polyphenylalanine synthesis and diphtheria toxin catalyzed ADP ribosylation. This result suggested that both EF-2 and EF-3 were required along with factor P for *N*-AcPhePuro synthesis.

The results of an experiment to determine more directly the factor requirements for *N*-AcPhePuro synthesis are shown in Figure 3. Product was measured as a function of time with various combinations of the three factors. As discussed above, P factor was absolutely required and a small, but significant, reaction was observed in the presence of factor P alone. Some stimulation was obtained in the presence of factor P with either EF-2 or EF-3, while the greatest activity was obtained with all three factors. If the background activity in the presence of factor P alone was subtracted, a two- to threefold stimulation was obtained by EF-2 and EF-3 together over the activities with each separately.

One explanation for the relatively high background activities observed in this system could be some remaining factor contamination in the ribosomes. No factor contamination was detectable using polyphenylalanine synthesis as the assay.

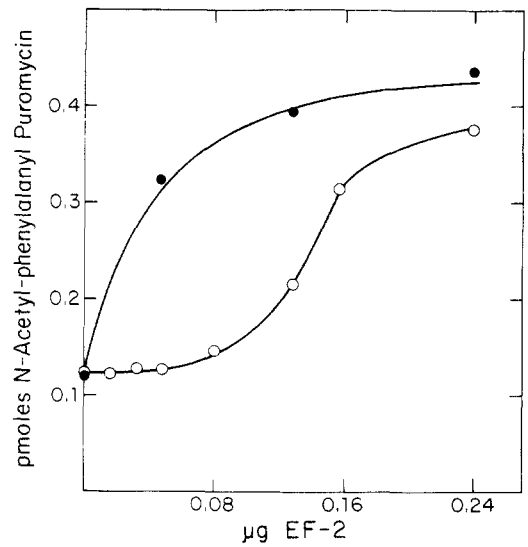

FIGURE 5: Inhibition of *N*-AcPhePuro synthesis by anti-EF-2. The reaction was carried out at 5 mM $MgCl_2$ in the presence of 0.3 μg of P factor and EF-2 as indicated by incubating at 30 °C for 3 min after an initial incubation at 0 °C for 3 min (○) in the presence or (●) in the absence of 2 μl at anti-EF-2 antiserum.

Since *N*-AcPhePuro synthesis only required a single translocation event, while polyphenylalanine synthesis required many, the single-step reaction might have been more sensitive to contamination. Further high-salt washes through either 0.5 or 1.0 M NH_4Cl did not lower the single factor activities. Either the single factor activities were not due to contamination or the contaminating factors were not accessible to the high-salt treatment.

In some ribosomal reactions, the factor requirements depend on the salt concentration. When *N*-AcPhePuro synthesis was determined as a function of NH_4Cl concentration, the reactions with P factor, EF-2, and EF-3 had the same optimum as with P and EF-2, or P and EF-3. The response of *N*-AcPhePuro synthesis to $MgCl_2$ concentration was dependent on the factors present, as shown in Figure 4. Below 4 mM, EF-3 had

TABLE I: Effect of P Factor on EF-2 Dependent Translocation.

Translocation Reaction[a]	Factors Added: Anti-EF-2 Step[b]	Factors Added: Peptidyl Transfer[c]	*N*-AcPhePuro (pmol Formed)[d]
1. EF-2, P	none	none	0.48
2. EF-2	none	P	0.41
3. EF-2, P	Anti EF-2	none	0.48
4. EF-2	Anti EF-2	P	0.45
5. EF-2, P, Anti EF-2	none	none	0.28
6. EF-2, Anti EF-2	none	P	0.24
7. EF-2, P, Anti EF-2	none	EF-2	0.44
8. EF-2, Anti EF-2	none	EF-2, P	0.42
9. P	none	none	0.26
10. EF-2	none	none	0.06

[a] Binding of *N*-AcPhe-tRNA was carried out as described in the text. Translocation was for 3 min at 30 °C at 5 mM $MgCl_2$ with 0.05 μg of EF-2, 0.3 μg of P factor, and 2 μl of Anti EF-2 antiserum as indicated. [b] All reaction mixtures were placed at 0 °C for 3 min and 2 μl of anti-EF-2 antiserum was added as indicated. [c] Peptidyl transfer was at 30 °C for 15 min, as described in the text, following addition of either 0.3 μg of P factor or 0.25 μg of EF-2 as indicated. [d] Formation of *N*-AcPhePuro was measured as indicated in the text. A background value of 0.25 pmol obtained in the absence of all factors was subtracted.

no effect on the EF-2 dependent reaction. Above 4 mM, the EF-2 dependent reaction appeared to be inhibited and the inhibition was reversed by EF-3. The previous experiments had been carried out at 9 mM $MgCl_2$, where the effect of EF-3 was maximal.

The $MgCl_2$ concentration effects further defined the properties of this system but did not reveal the origin of the single factor activities. One possible explanation is that different segments of the ribosome population active in this reaction might have different requirements for factors and salt. Another is that both factors are required and at low $MgCl_2$ concentrations contaminating EF-3 functions more efficiently than at the higher concentration. At the present time, we cannot offer a conclusive solution to this problem.

Detailed analyses of multicomponent systems, such as protein synthesis, are facilitated by the availability of specific inhibitors (Leder et al., 1969). We purified yeast EF-2 to electrophoretic homogeneity and used the purified protein to raise antibodies in rabbits. The resulting antiserum was a specific inhibitor of polyphenylalanine synthesis, which was reversed only by addition of excess EF-2 (Skogerson, unpublished results). The anti-EF-2 antiserum provided another way to test for possible EF-2 contamination of the ribosomal preparation. If the background activity obtained with factor P alone was due to contaminating EF-2 and EF-3, the antiserum might reduce this level. As seen in Figure 5, 2 μl of antiserum had no effect on the factor P alone reaction at 5 mM $MgCl_2$. This amount of antiserum was neutralized by about 0.1 μg of EF-2, which fully saturated the uninhibited reaction. If the background was due to contaminating factors, then the EF-2 was inaccessible to the antibody.

Function of P Factor. Elongation factor-2 dependent formation of *N*-AcPhePuro should require two distinct reactions, translocation and peptidyl transfer. In our system at 5 mM $MgCl_2$, the reaction depended on two factors, P and EF-2. The anti-EF-2 antiserum provided us with the means to ask whether P factor was required with EF-2 for translocation or was required for a subsequent step. As seen in Table I, the extent of *N*-AcPhePuro formation was similar, whether EF-2 and P factor were added together or whether P factor was added 6-min later (lines 1 and 2). After translocation had proceeded for 3 min, the antiserum had no effect, whether P was present during translocation or not (lines 3 and 4). When added before translocation had occurred, the antiserum reduced synthesis to the minus EF-2 level (lines 5, 6, and 9). The inhibition by the antiserum could be reversed by addition of excess EF-2, showing that inhibition did not result from release of the *N*-AcPhe-tRNA from the ribosomes (lines 7 and 8).

These results clearly demonstrated that P factor was not required during the translocation step initiated by EF-2, but was required for a subsequent reaction. Since the ribosomes used in these studies were active in polyphenylalanine synthesis in the absence of P factor, they must have had active peptidyl transferase. This fact made unlikely a direct requirement of P factor in peptidyl transfer and suggested its involvement in a reaction subsequent to EF-2 dependent translocation but prior to peptidyl transfer.

Discussion

Genetically determined sensitivity or resistance of yeast protein synthesis to cycloheximide has been shown to be a property of the 60S ribosomal subunits (Siegel and Sisler, 1963; Cooper et al., 1967; Rao and Grollman, 1967). The relationship of P factor to the genetic resistance mechanism has yet to be determined. Preliminary experiments suggested that P factor did not confer sensitivity to ribosomes from a cycloheximide-resistant mutant (Somasundaran, unpublished results). We are in the process of examining this question in detail with several different resistant mutants.

Under a variety of conditions, we could not detect an effect of P factor on polyphenylalanine synthesis. The simplest model to explain these results is that, although P factor is not required for polyphenylalanine synthesis, it is required for binding of cycloheximide to the ribosomes. In this case, mutant ribosomes might not bind P factor and, therefore, not bind cycloheximide. Another possibility is that other ribosomal proteins are required for binding of the inhibitor, in which case binding could be blocked by a mutational alteration in a protein other than P factor.

Somewhat analogous to the model just described is the mechanism for resistance to streptomycin in *Escherichia coli*. Mutational alteration of the ribosomal protein S-12 prevents binding of streptomycin and thus confers resistance (Cox et al., 1964; Davies, 1964; Ozaki et al., 1969). Three ribosomal

proteins, S-3, S-4, and S-5, are required for binding of streptomycin, which is, nevertheless, controlled by the phenotype of S-12 (Pongs et al., 1974).

Evidence presented above strongly suggested that P factor was not required for polyphenylalanine synthesis. Residual contaminating activity cannot be ruled out, but the large effect on cycloheximide sensitivity and the absolute requirement in *N*-AcPhePuro synthesis argue against that possibility. We have concluded, for the present, that P factor is not required for the elongation phase of protein synthesis.

If P factor is not required for the elongation phase of protein synthesis, then it is, presumably, not required for any of the individual elongation steps. Consistent with this idea are data presented above (Table I), which suggest that P factor is not directly required for translocation. Formation of *N*-AcPhePuro has been thought to depend on translocation and peptidyl transfer (Siler and Moldave, 1969; Tanaka et al., 1970; Gatica and Allende, 1971). The absolute requirement of this reaction for P factor suggests that another step besides translocation and peptidyl transfer might be required.

In systems from *E. coli*, binding of *N*-AcPhe-tRNA and subsequent *N*-AcPhePuro formation required initiation factors and was considered a model for initiation (Haenni and Chapeville, 1966; Lucas-Lenard and Lipmann, 1967; Economou and Nakamoto, 1967; Haenni and Lucas-Lenard, 1968; Klem and Nakamoto, 1968). Recently, a new factor, EF-P, was described which reduced the K_m of puromycin for this reaction (Glick and Ganoza, 1975). The experiments described above all employed a high concentration of puromycin (1 mM) and, presumably, P factor does not function in a manner similar to EF-P.

Experiments are in progress to complete the purification of P factor in order to define more precisely its role in *N*-AcPhePuro synthesis and protein synthesis.

Acknowledgments

We gratefully acknowledge the assistance of Mrs. Ruth DeBrunner in the preparation of this manuscript.

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