PEPTIDYL TRANSFERASE: A NEW METHOD FOR KINETIC STUDIES

R. Fico* and C. Coutsogeorgopoulos

Department of Biochemistry, Roswell Park Graduate Division, State University of New York at Buffalo and Department of Experimental Therapeutics, Roswell Park Memorial Institute, Buffalo, New York

Received April 10, 1972

Summary: The reaction of N-Acetyl-phenylalanyl-t-RNA with E. coli ribosomes in the presence of poly Uridylic acid, guanosine 5'-triphosphate, and initiation factors (ribosomal wash), leads to the formation of N-Acetyl-phenylalanyl-RNA-ribosome-poly Uridylic acid complex, which can be isolated on a cellulose nitrate filter. The isolated complex retains its ability to react with puromycin producing N-Acetyl-phenylalanyl-puromycin. The formation of N-Acetyl-phenylalanyl-puromycin follows simple enzyme kinetics with respect to puromycin giving linear Lineweaver-Burk plots. A number of inhibitors of peptidyl transferase inhibit this reaction. The reaction can also be used to study the nature of the complex formed and the factors or conditions which modify its formation.

Protein biosynthesis involves a number of enzymic steps, such as the initiation of peptide chains, the binding of amino acyl-t-RNAs, peptide bond formation per se, and the translocation of peptidyl-RNA (1). The formation of N-Ac-phe-pu** from N-Ac-phe-RNA and puromycin (1) has been conveniently used as a model system for investigating certain aspects of protein synthesis. Many of the cell-free systems developed for the study of N-Ac-phe-pu formation include enzymic steps other than peptide bond formation per se. Such systems are not suitable for studying the kinetics of a single enzymic step, namely the formation of peptide bonds by the ribosomal enzyme peptidyl transferase (2,3). Direct kinetic data for peptidyl transferase would be helpful not only for examining the intrinsic nature of this enzymic reaction, but also useful for pinpointing the locus and mechanism of action of certain inhibitors of protein synthesis. The form-

*The material in this paper forms part of a dissertation to be submitted to the Graduate School, State University of New York at Buffalo, in partial fulfillment of the requirements for the Ph.D. degree.

**Abbreviations used: Poly U, poly Uridylic acid; FWR, factors washable from ribosomes; N-Ac-phe-RNA, N-Acetyl-phenylalanyl-t-RNA; N-Ac-phe-pu, N-Acetyl-phenylalanyl-puromycin, N-Ac-phe, N-Acetyl-phenylalanine; GTP, guanosine 5'-triphosphate; GMPPCP, 5'-guanylyl-methylene diphosphonate; t-complex, N-Ac-phe-RNA-ribosome-poly U complex.

ation of complexes containing ribosomes, polynucleotides and amino acyl-t-RNAs can be conveniently followed after adsorption on cellulose nitrate filters (4). In this paper we describe an assay system which arises because of the ability of cellulose nitrate filters to adsorb the N-Ac-phe-RNA-ribosome-poly U complex (t-complex) in a state reactive towards puromycin. The so isolated t-complex readily reacts with puromycin to form N-Ac-phe-pu and this reaction gives linear Lineweaver-Burk plots with respect to puromycin. Known inhibitors of peptidyl transferase (5) also inhibit this reaction, while several inhibitors which have been reported to act at other steps in protein synthesis (6), have no effect. Since the reaction which leads to the complex formation (binding reaction) is separate from the peptide bond forming step, the assay can also be used to study factors governing the formation of the t-complex itself.

Materials and Methods: "Washed ribosomes" were prepared from frozen E. coli B cells as described previously (7). Fraction FWR (Factors washable from ribosomes) was used as a source of initiation factors. It was prepared from the first NH_4Cl ribosomal wash which was brought to 80% saturation with ammonium sulfate while maintaining the pH at 7.2 with 1 N NaOH. The resulting precipitate was dissolved in "standard buffer" (10 mM Tris-HCl pH 7.8, 10 mM $MgAc_2$, 60 mM KCl, 6 mM β-mercaptoethanol), dialyzed overnight at 4°C against this buffer, and stored in small aliquots at -70°C. Protein content of the FWR fraction was determined according to Lowry et al. (8). L-C^{14}-phe-RNA was prepared as previously described (9) and then converted to N-Ac-C^{14}-phe-RNA according to Haenni and Chapeville (10). The source of inhibitors was as stated previously (7). GMPPCP was purchased from Miles Lab. Inc. Cellulose nitrate filters, type HA, 25 mm diameter, 0.45 micron pore size, were purchased from the Millipore Corporation.

The assay is divided into two steps. The first step (binding reaction) involves the formation of the t-complex and its isolation by adsorption on a cellulose nitrate filter. The second step, peptide bond formation, involves the incubation at 25°C of the isolated t-complex on the filter with puromycin. The reaction is started by the addition of the filter to the reaction mixture and stopped by the addition of an equal volume of 1 N NaOH. The alkaline mixture is then incubated at 25°C to assure complete hydrolysis of the

TABLE I

EFFECTS OF INHIBITORS OF PROTEIN SYNTHESIS ON THE REACTION BETWEEN THE DISC-ADSORBED COMPLEX AND PUROMYCIN

	Inhibitor	Conc (M)	% Inhibition
A)	Chloramphenicol	1.2×10^{-6}	64
	Sparsomycin	1.0×10^{-7}	60
	Blasticidin S	1.0×10^{-6}	80
	Amicetin	1.0×10^{-5}	54
	Gougerotin	1.0×10^{-5}	30
B)	Tetracycline	1.0×10^{-4}	5
	Fusidic Acid	2.5×10^{-5}	3
	GMP-PCP	1.0×10^{-4}	0
	GTP	1.0×10^{-4}	2

Legend to Table I

A) Binding reaction: the "standard reaction mixture" (4.5 ml) contained 100 mM tris-HCl (pH 7.2), 100 mM NH_4Cl (pH 7.6), 12 mM Mg Acetate, 6 mM β-mercaptoethanol, 0.8 mM GTP, 360μg of Poly U, 144 A_{260} units of washed ribosomes, 1.02 mg (protein) FWR fraction, 28.8 A_{260} units of N-Ac-C^{14}-phe-RNA (10,233 cpm/A_{260}; 500 cpm/pmole C^{14}phe). The reaction was started by adding the N-Ac-C^{14}-phe-RNA and incubating at 25° for 8 min. The reaction mixture was then cooled in ice and 0.4 ml aliquots were diluted with 5.0 ml cold buffer A (0.1 M Tris-HCl pH 7.2, 0.1 M NH_4Cl adjusted to pH 7.6 with NH_4OH, 0.012 M $MgAc_2$, 0.006 M β-mercaptoethanol). The diluted samples were left standing in ice for 30 min., after which time the solution was passed through a cellulose nitrate filter. The isolated t-complex, on the filter, was washed three times with 5.0 ml portions of cold buffer A. At no time was air passed through the filter. After the last wash, the disc was placed on a glass plate and cut with a razor into quarters which were left immersed in cold buffer A. Under these conditions, there was no loss of N-Ac-C^{14}-phe-RNA from the quarter discs even after standing for two hours. Quarters were chosen because of their ease of handling and in order to minimize the volume of the reaction which will follow. The exact size of the quarters is not critical for the assay; half or whole discs can also be used.

B) Peptide bond formation: The reaction mixture (1.0 ml) contained 100 mM tris-HCl (pH 7.2), 100 mM NH_4Cl (pH 7.6), 12 mM Mg Acetate, 6 mM β-mercaptoethanol, 0.04 mM puromycin and, where mentioned, inhibitors at the concentration levels listed. The reaction was started by the addition of the quarter disc, bearing the t-complex, to this reaction mixture. After incubation at 25° for 2 min the reaction was stopped by the addition of 1.0 ml 1 N NaOH and the mixture was left incubating for 20 min. At the end of this time, a 0.8 ml aliquot of the reaction mixture was removed, 0.5 ml of 1.0 N HCl, and Bray's phosphor (14) were added, and the total amount of N-Ac-C^{14}-phe-RNA bound to the quarter disc was determined by radioactivity counting in a Packard liquid scintillation spectrometer equipped with external standardization which was used in correcting for quenching. A duplicate aliquot (0.8 ml) of the reaction mixture was extracted three times with twice the volume of ethyl acetate, adapting the method of Leder and Bursztyn (15). To the extract, Bray's phosphor was added and the sample was counted in order to determine the total amount of N-Ac-C^{14}-phe-pu formed. The per cent of the ribosome-bound N-Ac-phe-RNA that reacted to form N-Ac-phe-pu is taken to be the ratio, of the total N-Ac-C^{14}-phe-pu formed, to the total amount of N-Ac-C^{14}-phe-RNA bound to the quarter disc, multiplied by 100. These values were corrected by subtracting corresponding values obtained from control incubation mixtures containing no puromycin.

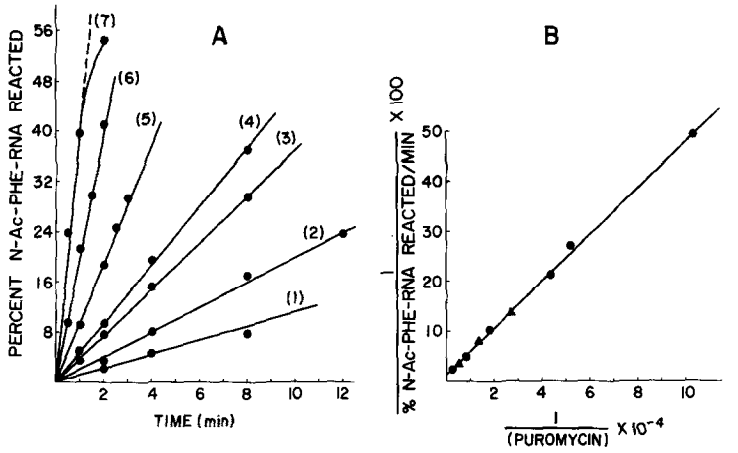
unreacted N-Ac-phe-RNA, to N-Ac-phe and t-RNA. The total amount of N-Ac-phe-RNA originally held on the filter is determined by measuring the radioactivity in an aliquot of the reaction mixture. The amount of product formed (N-Ac-phe-pu) is determined by extracting an equal aliquot of the reaction mixture with ethyl acetate. Under these conditions only the reaction product is extracted. Details are given in the legend of Table I.

Results and Discussion: In order to examine the nature of the reaction between the disc-adsorbed t-complex and puromycin, the effect of a number of compounds, which are known to inhibit protein synthesis, was studied. The results, shown in Table I, distinguish these compounds into two groups. The compounds of group A inhibit the reaction, whereas those of group B do not. The activity of the compounds in group A, which are known to be inhibitors of peptidyl transferase (5), strongly suggests that the reaction under study is indeed catalyzed by peptidyl transferase. Group B is composed of compounds which are known to affect steps other than peptide bond formation per se (6) and their lack of effect suggests that these other steps of protein synthesis do not occur during the reaction between the disc-adsorbed t-complex and puromycin.

We next examined the kinetics of this reaction. Fig. 1A illustrates the time course of the reaction obtained at various concentrations of puromycin. Reproducible results are obtained over a wide range of concentrations (0.5×10^{-5} - 40×10^{-5} M) and this becomes possible mainly for two reasons. First, a distinct advantage of this assay, is that one has precise control over the incubation time permitting, if necessary, incubations at half minute intervals. Second, an accurate measurement can be made of both the initial starting material and the amount of product formed.

A Lineweaver-Burk plot (12) of initial velocities (obtained from the slopes at zero time of the curves of Fig. 1A), at various puromycin concentrations is shown in Fig. 1B. The values obtained fall on a straight line indicating that the reaction follows simple enzyme kinetics with respect to puromycin. The K_m for puromycin, 3.13×10^{-4} M, is calculated from the 1/S intercept, and is in agreement with the reported K_m value of 1.8×10^{-4} M (11).

It should be pointed out that in the present system each enzyme molecule catalyzes the formation of only one molecule of N-Ac-phe-pu. Consequently, the reaction should



Legend to Fig. 1

A. Time course of N-Ac-phe-pu formation at various concentrations of puromycin. The binding reaction was carried out as described in the legend of Table I. "Peptide bond formation" was also carried out as described in the legend of Table I, with the difference that the concentration of puromycin (puro) was varied. Curve (1) with 5.3μM puro; (2) with 9.6μM puro; (3) with 19.3μM puro; (4) with 22.7μM puro; (5) with 55.8μM puro; (6) with 122.5μM puro; (7) with 418.8μM puro.

B. Lineweaver-Burk plot of initial velocities of N-Ac-phe-pu formation: ●——●——● Data from Fig. 1A; ▲——▲——▲ additional data from similar experiments (not shown).

---

follow the rate expression $v = (E_o - E_i)\ k/(1 + K_m/s)$ where k, s, and K_m have the usual meaning for single substrate kinetics (12). E_o is the original enzyme concentration and E_i is the fraction of E_o that has reacted to form N-Ac-phe-pu. This rate expression does not represent a straight line. However, at zero time, E_i equals zero, reducing the above rate expression to $v = (E_o)\ k/(1 + K_m/s)$, the familiar linear rate expression for single substrate reactions (12). A similar kinetic treatment has been applied to the kinetics of reactivation of diethylphosphoryl acetylcholinesterase (13).

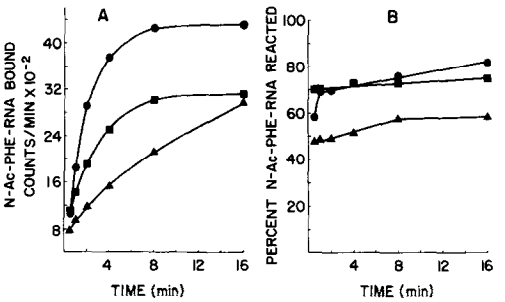
Presently we are investigating the kinetics of this reaction in the presence of a number of inhibitors of peptidyl transferase, and the results of these studies form the subject of a separate communication.

The assay described in this communication was developed to study the kinetics of peptide bond formation. However, in the presence of excess puromycin, it can also be used for determining the total amount of the bound N-Ac-phe-RNA that is reactive towards puromycin i.e. at the "donor" ribosomal site (1). In this way, the assay can

be used to study factors which influence the formation of the t-complex itself.

Figure 2A represents a time study of t-complex formation. In the presence of ribosomes and poly U at 12 mM Mg^{++} there is a significant binding of N-Ac-phe-RNA. Addition of the FWR fraction and GTP stimulates not only the rate, but also the extent of binding of N-Ac-phe-RNA to the ribosomes. Tetracycline, a known inhibitor of the binding reaction (6), inhibits both the rate and the extent of binding.

Fig. 2B is the same time study shown in Fig. 2A, with the difference that, by using excess puromycin and carrying the reaction to completion, we now determine the total amount of N-Ac-phe-RNA which, at the various time intervals (shown on the abscissa), became bound to the "donor" site. The lines obtained (Fig. 2B) show a small but posi-



Legend to Fig. 2

A. Time course of the binding reaction. The reaction was carried out as described in the legend of Table I except that 0.1 ml aliquots were removed from the reaction mixture at the times indicated on the abscissa and the reaction was stopped by adding 5.0 ml of ice-cold buffer A. The amount of N-Ac-C^{14}-phe-RNA bound to the t-complex was determined by carrying out the "peptide bond formation" reaction in the presence of 0.4 mM puromycin for 8 min at 25°C as described in the legend of Table I. After determining the total amount of N-Ac-C^{14}-phe-RNA bound to the quarter disc, the values obtained for the four quarters, derived from the same millipore disc, were added and the sum is given on the ordinate. A blank value of 104 cpm, found when the ribosomes were omitted from the reaction mixture, was subtracted. ●——●——●, standard reaction mixture; ■——■——■ standard reaction mixture including 8 x 10^{-4} M tetracycline; ▲——▲——▲ standard reaction mixture without the FWR fraction.

B. Reactivity, towards puromycin, of N-Ac-C^{14}-phe-RNA bound under the conditions described in the legend of Fig. 2A. The percent N-Ac-phe-RNA reacted, given on the ordinate, corresponds to the "peptide bond formation" reaction carried out at 25° for 8 min in the presence of 0.4 mM puromycin, and was determined as described in the legend of Table I. The reaction time of 8 min corresponds to an end-point of the reaction, after which time no further formation of N-Ac-phe-pu was observed. The time intervals given on the abscissa correspond to the duration of the "binding reaction" carried out as described in the legend of Fig. 2A. ●——●——●, standard reaction mixture; ■——■——■ standard reaction mixture including 8 x 10^{-4} M tetracycline; ▲——▲——▲ standard reaction mixture without the FWR fraction.

tive slope which indicates that, as the binding reaction progresses, a higher proportion of N-Ac-phe-RNA becomes bound to the "donor" site. This proportion depends on the conditions used in the binding reaction. In the absence of the FWR fraction, 48-58% of the bound N-Ac-phe-RNA is in the "donor" site. When the FWR fraction is included this proportion becomes 68-82%. This increase in binding to the "donor" site, caused by the FWR fraction, is in agreement with the proposed mechanism of action of the initiation factors (1) which are contained in the FWR fraction. When tetracycline is also included, the proportion of N-Ac-phe-RNA bound to the "donor" site is only slightly affected (70-75%), despite the fact that the overall binding of N-Ac-phe-RNA is inhibited by 30% (Fig. 2A). This inhibition by tetracycline apparently affects equally all binding sites for N-Ac-phe-RNA.

Acknowledgements: This investigation was supported in part by grant GM-12276 and by Institutional Grant RR-05648 from the U.S.P.H.S. R.Fico is a New York State Predoctoral Fellow. We are indebted to Dr. W.C. Werkheiser for helpful discussions.

References

1. Lucas-Lenard, J., and Lipmann, F., Ann. Review Biochem. 40, 409 (1971).
2. Maden, B.E.H., Traut, R.R., and Monro, R.E., J. Mol. Biol. 35, 333 (1968).
3. Maden, B.E.H., and Monro, R.E., Europ. J. Biochem. 6, 309 (1968).
4. Nirenberg, M.W., and Leder, P., Science 145, 1399 (1964).
5. Monro, R.E., and Vazquez, D., J. Mol. Biol. 28, 161 (1967).
6. Pestka, S., Ann. Review Microbiol. 25, 487 (1971).
7. Coutsogeorgopoulos, C., Biochim. Biophys. Acta 240, 137 (1971).
8. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. Biol. Chem. 193, 265 (1951).
9. Coutsogeorgopoulos, C., Biochemistry 6, 1704 (1967).
10. Haenni, A.L., and Chapeville, F., Biochim. Biophys. Acta 114, 135 (1966).
11. Fahnestock, S., Neumann, H., Shashoua, V., and Rich, A., Biochemistry 9, 2477 (1970).
12. Dixon, M., and Webb, E.C., Enzymes, Academic Press, New York, 1964.
13. Kitz, R.J., Ginsburg, S., and Wilson, I.B., Biochem. Pharm. 14, 1471 (1965).
14. Bray, G.A., Anal. Biochem. 1, 279 (1960).
15. Leder, P., and Bursztyn, H., Biochem. Biophys. Res. Comm. 25, 233 (1966).