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Role of the 30S Ribosomal Subunit, Initiation Factors, and Specific Ion Concentration in Barotolerant Protein Synthesis in *Pseudomonas bathycetes*

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Washed (1 M NH_4Cl) ribosomes from *Pseudomonas bathycetes*, *Pseudomonas fluorescens*, and *Escherichia coli* were tested for their ability to synthesize protein or polypeptide at high pressure when used as such, when recombined with homologous initiation factors, and when recombined with heterologous initiation factors. The responses of natural messenger ribonucleic acid (MS-2)-directed systems to pressure were independent of the source of initiation factors and paralleled those of the washed ribosomes in polyuridylate-directed systems. In all cases, the responses to pressure were parallel to those obtained when unwashed ribosomes were utilized; therefore, we concluded that the initiation factors were interchangeable among these organisms, and that these factors did not play a critical role in determining the pressure responses of the protein-synthesizing systems. *P. bathycetes* ribosomal subunits were isolated under a variety of ionic conditions. These were tested for their ability to synthesize protein and polyphenylalanine at a variety of pressures when used in reconstituted *P. bathycetes* homologous systems and in hybrid systems with ribosomal subunits from *E. coli* and *P. fluorescens*. *P. bathycetes* 30S subunits, isolated in a buffer solution containing 0 mM NaCl and 0 mM KCl, were nonfunctional at any pressure; those isolated in the presence of 150 mM NaCl and 0 mM KCl were functional at 1 atmosphere but barosensitive, and those isolated in the presence of 0 mM NaCl and 150 mM KCl retained the ion-mediated barotolerance characteristic of crude *P. bathycetes* ribosome preparations. The 50S subunit remained functional regardless of the method of isolation, and it had no effect on pressure sensitivity.

Previous reports from our laboratory have demonstrated that protein synthesis in both whole cells and cell extracts of *Escherichia coli* is relatively sensitive to inhibition by increased hydrostatic pressure, whereas that in whole cells or cell extracts of *Pseudomonas fluorescens* is relatively resistant (10, 13, 14). The results of experiments utilizing hybrid protein-synthesizing systems (composed of ribosomes isolated from either *E. coli* or *P. fluorescens* and supernatant fractions from the other organism) and hybrid ribosomal particles (30S subunits isolated from one organism and 50S subunits from the other as well as S-100 from either of the organisms) showed that pressure sensitivity or resistance was a function of the 30S ribosomal subunit (10, 11). This finding supports the hypothesis of Schwarz and Landau (13, 14), which suggests that the primary inhibitory effect of increased hydrostatic pressure is due to the disruption of a ribosomal function, possibly translocation and/or prevention of aminoacyl-transfer ribonucleic acid (RNA) from binding to the ribosome. Recent studies utilizing *Pseudomonas bathycetes* cell extracts indicate that the barotolerant characteristic associated with the ribosomes of this organism is ion dependent (12). Although protein synthesis in whole cells of *P. bathycetes* was relatively resistant to increased pressures, polypeptide synthesis by cell extract preparations was found to be quite sensitive to increased pressures when tested in reaction mixtures containing low concentrations of specific ions (16 mM Mg^{2+}, 0 mM K^+, 0 mM Na^+). Increasing the concentrations of Mg^{2+} and Na^+ in the cell extracts was particularly effective in moderating the inhibitory effects of increased pressure. Synthesis under these conditions was, in fact, more resistant to increased pressure than it was in whole cells (10, 12).

Initial attempts at isolation of functional ribosome subunits from *P. bathycetes* for delineation of the pressure and/or ion-sensitive sites

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were unsuccessful. Washed and isolated ribosome subunits of this organism could not achieve polypeptide synthesis upon reconstitution when our previous methods were used. We report here the results of experiments done to determine the effect(s) of washing procedures and the removal and interchange of initiation factors (IF) of *P. bathycetes*, *P. fluorescens*, and *E. coli* on pressure response. We also report the specific conditions under which *P. bathycetes* ribosomal subunits can be isolated and ribosomes reconstituted for successful peptide synthesis and subsequent determination of the pressure-sensitive site.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *P. fluorescens*, *P. bathycetes*, and *E. coli* strains used in this study and the conditions for the growth and maintenance of these organisms have been described previously (10).

Preparation of cell extracts. S-30, S-100, and unwashed ribosome preparations from *E. coli*, *P. bathycetes*, and *P. fluorescens* were prepared by the method of Modolell (9), with the modifications previously reported (10). Washed ribosomes, which are IF-free (3), were prepared as follows: crude ribosomes were suspended in buffer 1 [10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, 60 mM NH_4Cl, 10 mM magnesium acetate, 6 mM β-mercaptoethanol], made 1 M with respect to NH_4Cl, and kept at 0°C overnight. The suspension was then centrifuged at 100,000 × *g* for 2 h. The supernatant was aspirated and used for the preparation of crude IFs. The pellet (washed ribosomes) was suspended in buffer 1 and dialyzed for 12 h at 4°C against 4,000 volumes of the same buffer. After dialysis, the ribosomes were diluted to a concentration of 10 mg/ml with buffer 1, as determined spectrophotometrically, and either used immediately or stored at −70°C for a maximum of 1 week. Immediately preceding use, the ribosomes were activated by incubation at 40°C for 11 min.

Preparation of crude IFs. Crude IFs were prepared by a method similar to that of Gottlieb and Davis (3). To the supernatant from NH_4Cl-washed ribosomes, dry $(NH_4)_2SO_4$ was added to saturation. The mixture was then centrifuged for 10 min at 30,000 × *g*, and the pellet was suspended again in buffer 1 to a concentration of 10 mg of protein per ml, as determined by the method of Lowry et al. (8). The samples were dialyzed as described above. Immediately preceding use in polypeptide-synthesizing systems, approximately 1 mg of ribosomes (in 0.1 ml) was incubated with 0.25 mg of IF protein (in 0.01 ml) at 37°C for 11 min.

Preparation of ribosomal subunits. Ribosomal subunits from *E. coli* and *P. fluorescens* were prepared by centrifugation in a sucrose gradient containing 1 mM Mg^{2+}, as described previously (11). Subunits from *P. bathycetes* were isolated by three different methods, all similar to the methods developed for use with *E. coli* and *P. fluorescens*. For method I, crude ribosomes were suspended in buffer 2 (10 mM Tris-hydrochloride, 60 mM NH_4Cl, 1 mM magnesium acetate) and dialyzed overnight at 4°C against the same buffer. Approximately 5 mg of ribosomes (in 0.5 ml) was layered on a 30-ml linear sucrose gradient, 15 to 30%, made up in buffer 2 and centrifuged at 25,000 rpm for 15 h in a Beckman SW 25.1 rotor. The gradient tubes were pierced at the bottom, and the absorbance of the outflowing gradient solution was monitored at 260 nm with Beckman flow-cell and Gilford 2000 recording spectrophotometers. Sixty 0.5-ml fractions were collected from each gradient. Two components absorbing at 260 nm were observed, corresponding to the 30*S* and 50*S* subunits. Fractions containing these two components were separately pooled and diluted with buffer 2, and their Mg^{2+} concentration was raised to 10 mM. The separately pooled fractions were centrifuged for 6 h at 150,000 × *g*; the sedimented subunits were suspended in buffer 1, to a concentration of 10 mg of ribosomal protein per ml, and either used immediately or stored at −70°C for not more than 1 week. Prior to use in a protein-synthesizing system, the subunits were activated by incubation at 40°C for 11 min.

Isolation of the subunits by methods II and III was similar to that described for method I, with the following modifications. In method II, the crude ribosomes were suspended in buffer 2, containing 150 mM Na^+ (as NaCl), for separation into subunits. In method III, the buffer 2 solution contained 150 mM K^+ (as KCl). During gradient separation and collection of the subunits, the stipulated ion concentrations were maintained. After collection of the 30*S* and 50*S* fractions, they were suspended in standard buffer 1 (10 mM Mg^{2+}) and dialyzed overnight at 4°C against 4,000 volumes of the same buffer. The subunits were then sedimented and stored as in method I.

Determination of cell-free protein synthesis. Incorporation of ^{14}C-labeled amino acids into protein and of [^{14}C]phenylalanine into polyphenylalanine was determined by methods described in earlier publications (10–12).

Chemicals. ^{14}C-labeled amino acids (0.07 mg/μCi) and [^{14}C]phenylalanine (0.36 mg/mCi) were obtained from New England Nuclear Corp., Boston, Mass. MS-2 viral RNA was obtained from Miles Laboratory, Elkhart, Ind. Polyuridylate, pyruvate kinase (type III), adenosine 5′-triphosphate (Tris salt), guanosine 5′-triphosphate (Tris salt), and phosphoenolpyruvate (Tris salt) were purchased from Sigma Chemical Co., St. Louis, Mo. Ultrapure sucrose was obtained from Schwarz/Mann, Orangeburg, N.Y. All other chemicals used were of reagent grade.

Pressure apparatus. Details of the pressure apparatus used have been described in a previous publication (14).

RESULTS

A series of experiments involving the interchange of washed (IF-free) ribosomes and IFs from *E. coli*, *P. bathycetes*, and *P. fluorescens* in polyuridylate and MS-2 viral messenger

RNA-directed synthesizing systems was performed. Table 1 summarizes the results obtained when protein-synthesizing systems containing either crude ribosomes, washed ribosomes, washed ribosomes plus homospecific IFs, or washed ribosomes plus heterospecific IFs were tested at 1 and 680 atmospheres (atm) at 25°C in reaction mixtures containing either high (60 mM Mg^{2+} and 150 mM Na^{+}) or low (16 mM Mg^{2+} and 0 mM Na^{+}) ion concentrations. The rates of synthesis at high and low ion concentrations at 680 atm are expressed relative to the rates of the controls at low ion concentration and 1 atm.

The pressure response of systems utilizing washed *E. coli* or washed *P. bathycetes* ribosomes was identical to the response of each respective system utilizing unwashed preparations (Table 1). Systems utilizing washed *E. coli* ribosomes appeared very sensitive to inhibition by increased pressure (680 atm), whereas washed *P. bathycetes* ribosomes were barosensitive in low and barotolerant in high ionic conditions. Addition of homospecific or heterospecific IFs to either *E. coli* or *P. bathycetes* washed ribosomes had no modifying effects on the pressure response of these systems. However, washed *P. bathycetes* ribosomes with or without added IFs were approximately twice as active, at 1 atm and 25°C, as the crude preparations. It has been reported that NH_4Cl washes will increase the activity of various ribosomal preparations by removing ribonuclease activity associated with the ribosomes (9), but the twofold stimulation of synthesis such as seen here has not been reported. Systems utilizing washed ribosomes from *P. fluorescens* showed a much higher degree of barotolerance at 680 atm than previously observed with crude preparations. Since this increased level of barotolerance was not modified by either the addition of homospecific or heterospecific IFs or by the absence of IFs in polyuridylate-directed systems, the modification of the pressure response could be due to some alteration of ribosomal conformation by the washing process itself or the removal of specific ribosomal proteins. Although the data shown in Table 1 are for 1 and 680 atm only, pressures at increments from 1 to 800 atm were applied, and the results indicate that, with either *E. coli* or *P. bathycetes* systems, at each pressure the same respective level of inhibition was exhibited whether washed or crude ribosomes were utilized. Washed ribosomes from *P. fluorescens*, on the other hand, were more tolerant than crude ribosomes at all pressures tested.

Experiments were performed to determine

TABLE 1. *Rates of protein and polyphenylalanine synthesis by cell extracts of E. coli, P. fluorescens, and P. bathycetes at 1 and 680 atm and 25°C*

Source of S-100	Source of ribosomes	Source of IFs	Type of messenger RNA	Rate at 1 atm and low ion concentration[a] (dpm/min per mg of ribosomes) (± 6%)	Relative rate[b] at 680 atm and low ion concentration	Relative rate at 680 atm and high ion concentration[c]
E. coli	*E. coli* (crude)	None	MS-2	860	0%	0%
	E. coli (washed)	None	Poly(U)[d]	840	0%	0%
	E. coli (washed)	None	MS-2	0	–[e]	–
	E. coli (washed)	*E. coli*	MS-2	880	0%	0%
	E. coli (washed)	*P. bathycetes*	MS-2	680	0%	0%
	E. coli (washed)	*P. fluorescens*	MS-2	820	0%	0%
P. fluorescens	*P. fluorescens* (crude)	None	MS-2	1000	35%	65%
	P. fluorescens (washed)	None	Poly(U)	960	80%	80%
	P. fluorescens (washed)	None	MS-2	0	–	–
	P. fluorescens (washed)	*E. coli*	MS-2	940	80%	80%
	P. fluorescens (washed)	*P. bathycetes*	MS-2	920	80%	80%
	P. fluorescens (washed)	*P. fluorescens*	MS-2	760	80%	80%
P. bathycetes	*P. bathycetes* (crude)	None	MS-2	400	12%	65%
	P. bathycetes (washed)	None	Poly(U)	860	12%	65%
	P. bathycetes (washed)	None	MS-2	0	–	–
	P. bathycetes (washed)	*E. coli*	MS-2	840	12%	67%
	P. bathycetes (washed)	*P. bathycetes*	MS-2	800	12%	63%
	P. bathycetes (washed)	*P. fluorescens*	MS-2	800	12%	64%

[a] Low ion concentration is 16 mM Mg^{2+}.
[b] Relative rate expressed as percentage of 1 atm rate at low ion concentration.
[c] High ion concentration is 60 mM Mg^{2+}, 150 mM Na^{+}.
[d] Poly(U), Polyuridylic acid.
[e] –, Indicates no data.

the pressure response and activity at 1 atm of protein-synthesizing systems composed of reconstituted *P. bathycetes* ribosomes prepared from subunits isolated by the three methods described in Materials and Methods and *P. bathycetes* S-100 (see Fig. 1). When subunits were isolated by method I, particles were obtained that were unable to synthesize polypeptide in either low or high ion concentrations. The data for these preparations are, therefore, not shown in Fig. 1. Subsequent to isolation, manipulations such as heat activation, or resuspension in buffers with high Mg^{2+}, Na^{+}, or K^{+} concentrations, or combinations thereof failed to activate these particles. Data to be reported elsewhere indicate that these particles were modified, possibly by a loss of some component, and were deficient in their ability to bind [^{3}H]polyuridylate under conditions in which initiation of protein synthesis should occur. Ribosomes prepared from *P. bathycetes* ribosomal subunits isolated by either method II or III were approximately 60% as active in protein synthesis, at 1 atm and 25°C, as ribosomes that had not been dissociated. However, with the utilization of method II, the ion-mediated barotolerant characteristic found with the undissociated ribosomes had been lost (Fig. 1). These particles were barosensitive in reaction mixtures of either high or low ion concentration. Reconstituted ribosomes formed from subunits isolated by method III displayed the same pressure response as undissociated ribosomes; i.e., synthesis was barosensitive at low, and barotolerant at high, specific ionic conditions (Fig. 1). The stimulation of protein synthesis at lower pressures has been previously reported (10, 12) and is under investigation.

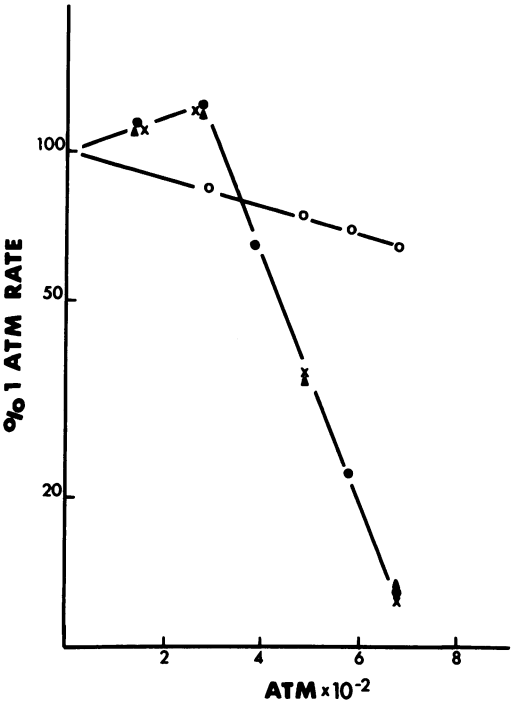

FIG. 1. *Effect of increased hydrostatic pressure on polyphenylalanine synthesis by cell extracts of P. bathycetes at 25°C. Symbols: ▲, 30S and 50S subunits isolated by method II with reaction mixture containing low specific ion concentration; ×, 30S and 50S subunits isolated by method II with reaction mixture containing high specific ion concentration; ●, 30S and 50S subunits isolated by method III with reaction mixture containing low specific ion concentration; ○, 30S and 50S subunits isolated by method III with reaction mixture containing high specific ion concentration.*

Table 2 summarizes the results of experiments done to determine the pressure response of hybrid protein-synthesizing systems utilizing S-100 fractions, from *P. bathycetes* or *P. fluorescens*, and hybrid ribosomes, composed of subunits from *E. coli*, *P. bathycetes*, and *P. fluorescens*. Each result represents the average value from the data of at least five individual experiments. Systems utilizing a 30S subunit from *P. bathycetes*, isolated by method I, were inactive in polypeptide synthesis, regardless of the origin of the 50S subunit or the concentration of salts in the reaction mixture. The *P. bathycetes* 50S subunit isolated by method I supported active synthesis when used in combination with a 30S subunit of different origin. *P. bathycetes* 30S subunits, isolated by method II or III, were active in polypeptide synthesis with either homologous or heterologous 50S subunits. Furthermore, systems utilizing 30S subunits from *P. bathycetes* prepared by method II were barosensitive in both low and high ionic conditions, regardless of the origin of the 50S subunit, but systems utilizing *P. bathycetes* 30S subunits prepared by method III displayed the ion-mediated barotolerant characteristic, regardless of the origin of the 50S subunit used. Systems utilizing the 50S subunit from *P. bathycetes* isolated by method I, II, or III were active in polypeptide synthesis, and the origin and method of isolation of the 50S subunit seemed to have no effect on the pressure response of the system. Although the results in Table 2 are for 1 and 680 atm only, data not shown indicate that the pressure response of systems utilizing hybrid ribosomes is identical to the pressure response of each respective homologous system, from which the 30S subunits were obtained, over the range from 1 to 1,000 atm.

TABLE 2. *Effect of specific ion concentrations on the relative rates of polyphenylalanine incorporation by systems utilizing homologous and hybrid reconstituted ribosomes at 1 and 680 atm and 25°C*

Source of 30S ribosomal subunit	Source of 50S ribosomal subunit	Rate at 1 atm and low ion concentration[a] (dpm/min per mg of ribosomes) (± 6%)	Relative rate[b] at 680 atm and low ion concentration	Relative rate at 680 atm and high ion concentration[c]
P. bathycetes I[d]	*E. coli* I	0	–[e]	–
P. bathycetes I	*P. fluorescens* I	0	–	–
P. bathycetes I	*P. bathycetes* I, II, III	0	–	–
E. coli I	*P. bathycetes* I, II, III	230	6%	6%
P. bathycetes II	*E. coli* I	220	11%	13%
P. bathycetes II	*P. fluorescens* I	230	11%	13%
P. bathycetes II	*P. bathycetes* I, II, III	220	11%	13%
P. fluorescens I	*P. bathycetes* I, II, III	280	36%	65%
P. bathycetes III	*E. coli* I	300	12%	66%
P. bathycetes III	*P. fluorescens* I	240	11%	67%
P. bathycetes III	*P. bathycetes* I, II, III	270	12%	65%

[a] Low ion concentration is 16 mM Mg^{2+}.
[b] Relative rates expressed as percent of the 1 atm rate at low ion concentration.
[c] High ion concentration is 60 mM Mg^{2+}, 150 mM Na^{+}.
[d] I, II, and III refer to methods of isolation as stipulated in Materials and Methods.
[e] –, Indicates no data.

DISCUSSION

The data clearly indicate that removal of IFs by washing the ribosomes in 1 M NH_4Cl did not affect the pressure response of *E. coli* or *P. bathycetes* protein-synthesizing systems. Ribosomes of *P. fluorescens*, when washed in 1 M NH_4Cl, showed an increased barotolerant capacity. Protein synthesis in this organism has been shown to be barotolerant when compared with *E. coli* under reaction mixture conditions of low ion concentration, and resistance to inhibition by pressure is further increased at high ion concentration (12). It is possible that the high NH_4Cl concentration of the wash helped to maintain a ribosome configuration that would allow for a minimum activation volume change during synthesis, which, therefore, resulted in a maximum barotolerance.

That the IFs were fully interchangeable in the protein-synthesizing systems of the three organisms tested suggests that some type of homology of these factors may exist in quite diverse groups of bacteria. Overall, the pressure response of these synthesizing systems was identical, whether homospecific or heterospecific IFs were utilized. Furthermore, the response was identical when IFs were omitted and synthetic messenger was utilized in the test system. All of this suggests that the functional roles of these factors in protein synthesis were not affected by increased pressure and, therefore, did not involve marked volume changes.

The ion-mediated barotolerant characteristic of protein synthesis in *P. bathycetes* was clearly shown to be a property of the 30S subunit. The 50S subunit was functional under all test conditions and was not a factor in barotolerance. This finding agrees with our previous report on *E. coli* and *P. fluorescens* (11). The isolation of *P. bathycetes* subunits under the different ionic conditions of methods I, II, and III resulted in 30S particles displaying distinctly altered properties with respect to synthesis. Such results closely parallel the findings of Bayley and coworkers (1, 2), who reported that isolation of ribosomal subunits from *Halobacterium cutirubrum* under different salt conditions resulted in ribosomal particles of varying protein composition being obtained. These same investigators, as well as Lanyi (5), reported that lowering the concentration of Na^{+}, K^{+}, or Mg^{2+}, in the isolation buffer they used, selectively removed specific ribosomal proteins. Their findings, as well as the results reported here, would suggest that, in certain groups of bacteria, specific cations are responsible for maintaining the structural integrity of the ribosomal subunit. For a marine organism such as *P. bathycetes*, in vivo, high intracellular ion concentrations may be a major factor in maintaining a specific ribosome configuration such that barotolerant protein synthesis can occur. Preparation of cell extracts for protein synthesis by dialysis resulted in removal of most of these ions, and therefore specific ions must be added to the isolation and reaction mixtures to compensate for this loss.

Only under such conditions is a 30S subunit configuration preserved that is capable of supporting barotolerant protein synthesis.

Isolation of the subunits by method I resulted in particles which our preliminary results indicate were unable to bind to messenger RNA. We believe, as mentioned previously, that this inability may be due to the removal of specific acidic ribosomal proteins during centrifugation. These proteins seem to require either Na^+ or K^+ ions to permit them to bind to ribosomes. Isolation of *P. bathycetes* 30S ribosomal subunits by method II resulted in particles that were functional in protein synthesis at 1 atm, but were incapable of supporting barotolerant protein synthesis under any of the ionic conditions utilized in these experiments. Isolation of ribosomal subunits by method III yielded normal *P. bathycetes* ribosomal particles that were barosensitive in conditions of low specific ion concentration and barotolerant at the higher ion concentration.

It is important to note that the Na^+ and K^+ effects were evoked only upon separation and isolation of the subunits; i.e., it is during the separation of the ribosome into subunits by centrifugation that the concentration of specific ions must be maintained to permit reconstitution of functional ribosomes. Once the 30S subunits had been isolated, the Na^+ or K^+ ions were again removed by dialysis. This dialysis procedure was performed on the isolated particles in the presence of 10 mM Mg^{2+} (buffer 1). Possibly, the alteration of the subunits during isolation by sucrose gradient centrifugation was a result of a combination of factors, including a low concentration of Mg^{2+} (1 mM), absence of either Na^+ or K^+ or both, as well as hydrostatic pressures generated by prolonged ultracentrifugation (4).

The isolation of the 30S subunit in Na^+ may allow the retention of subunit components necessary for barosensitive synthesis while losing components necessary for barotolerant synthesis. Isolation in K^+, under this hypothesis, would then prevent the loss of any such components. There is also the distinct possibility that no components were lost in either Na^+ or K^+ isolation procedures, and that the ions acted directly on the subunit structure to maintain a specific conformational shape. The physical differences between the Na^+ and K^+ ions, and their respective hydration layers, could account for distinct spatial and conformational relationships among the subunit components. Low and Somero (6, 7) have reported that the molecular volume changes accompanying the formation of activated enzyme-substrate complexes may undergo a considerable degree of modification as a result of changes in the concentrations and types of ions present in the reaction mixture. Such a system may be considered analogous to the formation of activated messenger RNA-ribosome-transfer RNA complexes during the process of polypeptide synthesis.

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