Protein Synthesis in a Cell-Free Extract from *Staphylococcus aureus*

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Cell-free *Staphylococcus aureus* extracts have been prepared which actively incorporate amino acids into protein. The requirements for amino acid incorporation of this preparation were strongly suggestive of de novo protein synthesis, since it showed an absolute requirement for ribosomes, $105,000 \times g$ supernatant fluid, energy source, and magnesium ion. The stability of these extracts was greatly improved by use of dithiothreitol instead of mercaptoethanol as a sulfhydryl protecting reagent. Data were presented to show that the binding of aminoacyl-soluble ribonucleic acid to ribosomes did not require guanosine triphosphate and supernatant enzyme. The major characteristic which distinguishes this system from other cell-free systems is the much higher magnesium concentration required to maintain ribosomes intact and to obtain the maximal incorporation of amino acids. Addition of polyuridylic acid, polyadenylic acid, or polycytidylic acid caused about 60-fold, 30-fold, or 4-fold stimulation of the incorporation of phenylalanine, lysine, or proline, respectively. Studies by density gradient sedimentation indicated that radioactive polyuridylic acid or polyadenylic acid was associated with the monosomes. This complex can actively synthesize polypeptides. On the other hand, the nascent protein synthesized under the direction of endogenous messenger ribonucleic acid was associated with both polysomes and monosomes.

Gale and Folkes (5) reported that disrupted cells of *Staphylococcus aureus* which contained cell membranes and cell walls were able to incorporate amino acids. Later, Gale et al. (6) found that a substantial amount of amino acids was incorporated into cell wall peptides. The results obtained with this crude fraction are complicated by cell wall synthesis, making it difficult to interpret the mechanism of protein synthesis. Since these reports, no subcellular amino acid-incorporating system from *S. aureus* has been reported. Young and Barker (20) examined ribosomes from *S. aureus* and found that at a magnesium ion concentration of 10 mM, which is adequate to maintain *Escherichia coli* ribosomes intact (19), *S. aureus* ribosomes existed mainly as subunits which are not active for protein synthesis.

The present communication describes a method to obtain highly active, cell-free extracts from *S. aureus* which contain large amounts of polysomes and monosomes, and reports the characteristics of this system.

MATERIALS AND METHODS

Materials. Uniformly ^{14}C -labeled L-amino acid mixture ($1.5 \mu\text{C}/\text{mg}$), L-phenylalanine ($375 \text{ mc}/\text{mmole}$),

L-lysine ($240 \text{ mc}/\text{mmole}$), and L-proline ($200 \text{ mc}/\text{mmole}$) were purchased from New England Nuclear Corp., Boston, Mass. Triphosphates of adenosine, cytidine, guanosine and uridine (ATP, CTP, GTP and UTP, respectively), phosphoenolpyruvate, pyruvate kinase, yeast ribonucleic acid (RNA), ribonuclease, bovine albumin, and dithiothreitol were obtained from Calbiochem, Los Angeles, Calif. Polyuridylic acid (poly-U), polyadenylic acid (poly-A), polycytidylic acid (poly-C), and tritium-labeled poly-U ($29.9 \text{ mc}/\text{mmole}$) and poly-A ($64.6 \text{ mc}/\text{mmole}$) were products of Miles Laboratories, Inc., Elkhart, Ind. ^{14}C -phenylalanyl-soluble RNA (sRNA; 5,240 counts per min per A_{260} unit) and ^{14}C -lysyl-sRNA (1,060 counts per min per A_{260} unit) were prepared by the procedure of Nathans and Lipmann (12).

Growth of microorganisms. *S. aureus* 209P was grown aerobically in an Erlenmeyer flask overnight at 37°C in 3.7% Brain Heart Infusion (Difco), passed to a second flask containing 20 volumes of fresh medium, and grown for 3 hr; the contents of the flask were then transferred to a fermentor containing 12.5 volumes of fresh medium (it is important to use heavy inoculum, 8%, to obtain active preparations) and incubated with agitation and forced aeration until the absorbance of the culture had increased above its initial reading about 0.5 to 0.7, as measured at $420 \text{ m}\mu$ in a 1-cm cuvette (about 2 hr). The cells, harvested by

centrifugation after rapid chilling, were washed twice with a 0.01 M tris(hydroxymethyl)aminomethane (Tris)-acetate buffer (pH 7.6) containing 16 mM magnesium acetate, 50 mM ammonium chloride, and 0.1 mM dithiothreitol (medium A).

Preparation of cell fractions. All isolation procedures were carried out at 0 to 5 C. The washed cells were disrupted by hand grinding with two times their weight of alumina (Alcoa-305) for 3 to 5 min. Medium A equivalent to two times the wet weight of cells was added to the paste and centrifuged at $30,000 \times g$ for 1 hr. Centrifugation was repeated twice to ensure complete removal of intact cells from the final supernatant fluid (S-30). The S-30 fraction was centrifuged at $105,000 \times g$ for 2 hr to collect the ribosomes. The supernatant fluid (S-100) was aspirated. The ribosomes were washed twice by resuspension in medium A and centrifugation at $105,000 \times g$ for 2 hr, and were suspended in medium A. In some cases, S-30 was preincubated at 37 C for 15 min (Inc-S-30) to reduce endogenous synthesis (14). All fractions were dialyzed at 4 C for 17 hr against medium A and then stored in small portions at -24 C until needed. The ribosomal subunits were obtained by dialyzing the ribosomes in a low-magnesium medium which had a composition similar to that of medium A, except that magnesium acetate was reduced to 0.1 mM (medium B).

Assay for amino acid incorporation. The standard reaction mixture consisted of 1 mM ATP, 0.05 mM GTP, CTP and UTP, 5 mM phosphoenolpyruvate, 0.02 mg of pyruvate kinase, 0.25 μ C of 14 C-labeled amino acid, and 0.06 mM each of the remaining amino acids in medium A (pH 7.4). The assay usually was started by adding 0.1 ml of the S-30 fraction and was carried out at 37 C for 30 min in a total volume of 0.5 ml. In the studies of 14 C-phenylalanine, -lysine, or proline incorporation, 50 μ g of poly-U, 100 μ g poly-A, or 150 μ g poly-C was added, respectively. Poly-A and poly-C were heated at 90 C for 5 min to destroy secondary structures, and then were chilled in an ice bath just before being added to tubes. Protein synthesis was measured by the incorporation of 14 C-labeled amino acid into hot trichloroacetic acid-precipitable material, isolated and washed on membrane filters (HA, 25 mm; Millipore Corp., Bedford, Mass.) Polyllysine formation was assayed as described by Gardner et al. (7). Radioactivity was measured by the scintillation method.

Assay for aminoacyl-sRNA binding to ribosomes. The assay was performed as described by Nirenberg and Leder (13). The complete system, in a volume of 0.25 ml, contained 4.5 A_{260} units of ribosomes, 1.3 A_{260} units of 14 C-phenylalanyl-sRNA (6,812 counts/min) or 2.6 A_{260} units of 14 C-lysyl-sRNA (2,756 counts/min), and 50 μ g of poly-U or 100 μ g of poly-A. The reaction mixtures were incubated in medium A (pH 7.2), for 30 min at 37 C.

Ultracentrifugation. Linear sucrose gradients of 4.8 ml containing 5 to 20% (w/v) in medium A (for intact ribosomes) or medium B (for dissociated ribosomes) were prepared. A sample of 0.2 ml was layered over each gradient, and the tubes were centrifuged at 4 C for 120 min (intact ribosomes) or 210 min (dissociated ribosomes) at 30,000 rev/min in the SW-39

rotor of a Spinco model L ultracentrifuge. Fractions of 15 drops were collected, 0.5 ml of water was added to each fraction, and the absorbancy at 260 m μ was measured. The samples were recovered from the cuvettes; the radioactive macromolecules were precipitated with trichloroacetic acid, and were collected on membrane filters, washed, dried, and counted.

Sedimentation coefficients of ribosomal particles were determined by use of the Spinco model E analytical ultracentrifuge. The sedimentation coefficients were corrected to water and 20 C.

Determination of protein and RNA. Protein was measured by use of the biuret reagent (11) with bovine serum albumin as a standard. RNA was determined by the orcinol method (15) with yeast RNA as a standard. It was found that 1 mg of ribosomes equals 19 A_{260} units.

RESULTS

Stability of cell-free extracts. It appears that mercaptoethanol (6 mM) is not very effective in protecting the cell-free preparations of *S. aureus*. The preparations become inactive after 1 week at -24 C. It was found that if dithiothreitol (0.1 mM) replaces mercaptoethanol in the buffer, the storage life of all fractions is prolonged to at least 1 month. However, the synthetic activity directed by endogenous messenger RNA (mRNA) disappeared in 2 to 3 days at -24 C.

Requirements for incorporation of amino acid into protein. As shown in Table 1, the incorporation of amino acids was dependent upon ATP, supernatant fluid, and ribosomes. Omission of GTP or CTP and UTP resulted in about 30% inhibition. The incorporation was completely inhibited by ribonuclease, and was partially inhibited by erythromycin or chloramphenicol.

Response to synthetic polynucleotides. Both the nonpreincubated and preincubated S-30 can be

TABLE 1. Requirements for incorporation of 14 C-labeled amino acid mixture into protein by the cell-free system from *Staphylococcus aureus*

Experimental conditions	Amino acid incorporation (counts per min per mg of protein)
Complete system.....	6,293
-S-100.....	35
-ribosomes.....	138
-ATP.....	30
-GTP.....	4,578
+4 $\times$ GTP.....	6,474
-CTP and UTP.....	4,258
+ribonuclease, 10 μ g.....	32
+chloramphenicol, 10 μ g.....	3,145
+erythromycin, 10 μ g.....	3,672
+erythromycin, 1 μ g.....	4,213
+erythromycin, 0.1 μ g.....	5,344

stimulated by addition of synthetic polynucleotides. The optimal time for preincubation is 15 min at 37 C. Preincubation reduced the total incorporation primarily because endogenous incorporation was substantially lowered. Thus, the relative stimulation increased after incubation. The stimulation of phenylalanine incorporation by poly-U (100 μ g/ml), the stimulation of lysine incorporation by poly-A (200 μ g/ml), and proline by poly-C (300 μ g/ml) usually was approximately 60-fold, 30-fold, and 4-fold, respectively. Figure 1 shows the effect of increasing polynucleotide concentration on the incorporation of the appropriate amino acid.

Time course of protein synthesis. Figure 2 illustrates the time courses of the incorporation directed by poly-U, poly-A, and endogenous mRNA. The rates of incorporation were constant up to 40 min for phenylalanine, and to 15 min for lysine and the mixture of amino acids. The shortness of the period of incorporation for lysine and the amino acid mixture probably is due to lack of mRNA. Although poly-A was added in large amounts, it forms a secondary structure and becomes inactive as mRNA (16) during the incubation. That mRNA is the limiting factor was demonstrated in the following experiment. A complete reaction mixture (minus poly-U) was incubated at 37 C for 15 min, and then poly-U was added. Incorporation of phenylalanine

FIG. 1. Effect of synthetic polynucleotides on the incorporation of amino acids. Symbols: ●, poly-U stimulated incorporation of phenylalanine; ○, poly-A stimulated incorporation of lysine; △, poly-C stimulated incorporation of proline.

FIG. 2. Time course of amino acid incorporation. Symbols: ●, incorporation of phenylalanine, 100 μ g of poly-U added at zero time; △, incorporation of phenylalanine, 100 μ g of poly-U added after 15 min; □, incorporation of lysine, 200 μ g of poly-A added at zero time; ○, endogenous mRNA directed incorporation of amino acid mixture.

occurred immediately, and the rate was linear for at least 45 min. These results suggested that S-30 and cofactors were stable for at least 15 min at 37 C.

Effect of pH on the incorporation of amino acids. Figure 3 shows that the optimal pH for incorporation of phenylalanine, lysine, or the amino acid mixture was in the range of 7.2 to 7.4.

Effect of magnesium ion concentration. Figure 4 shows that the optimal magnesium concentration for the incorporation of amino acid mixture directed by endogenous mRNA was about 16 to 18 mM. The poly-U directed incorporation of phenylalanine required higher concentrations, about 22 mM.

Properties of *S. aureus* ribosomes. The twice-washed ribosomes were 65% RNA and 35% protein, which is quite similar to *E. coli* ribosomes (19). The proportion of protein and RNA in 50S and 30S subunits of *S. aureus* is identical to the whole ribosomes.

Figure 5a shows that ribosomes in 0.1 mM magnesium gave two peaks with approximate $S_{20,w}^0$ values of 51 and 31. Figure 5b shows sedimentation patterns of *S. aureus* ribosomes in 16 mM magnesium. Three peaks with approximate $S_{20,w}^0$ values of 107, 73, and 57 were observed. These values will be referred to as 100S, 70S, 50S, and 30S. The presence of four types of particles

in ribosomal preparation was also confirmed by sucrose density gradient centrifugation (Fig. 6).

Nature of the ribosome complex. Sucrose density gradient patterns of the mRNA-ribosome-polypeptide complex are shown in Fig. 7. It can be seen that polynucleotide and polypeptide were

associated with 70S particles. Figure 6b shows that without addition of artificial mRNA the radioactive polypeptide was bound to both 100S and 70S particles.

Binding of aminoacyl-sRNA to ribosomes. Table 2 summarizes the requirements for aminoacyl-sRNA binding to ribosomes. Magnesium and a specific polynucleotide to serve as mRNA are

FIG. 3. Effect of pH on amino acid incorporation. Symbols: ●, poly-U stimulated incorporation of phenylalanine; ○, poly-A stimulated incorporation of lysine; △, endogenous mRNA directed incorporation of amino acid mixture.

FIG. 4. Effect of magnesium concentration on amino acid incorporation. Symbols: ○, poly-U stimulated incorporation of phenylalanine; △, endogenous mRNA directed incorporation of amino acid mixture.

FIG. 5. Ultracentrifugal schlieren patterns of *Staphylococcus aureus* ribosomes. Photographs were taken at 32 min after attaining speed of 25,980 rev/min. (a) The 50S and 30S subunits of ribosomes. (b) The 100S and 70S ribosomes.

FIG. 6. Sucrose gradient analysis of ribosomal particles of *Staphylococcus aureus*. (a) The 50S and 30S subunits of ribosomes. (b) The 100S and 70S ribosomes. Symbols: ●, absorbance at 260 mμ; △, incorporated ¹⁴C-labeled amino acid mixture.

required. These results are similar to those obtained with *E. coli* (13).

DISCUSSION

The cell-free preparations from *S. aureus* seem less stable than those from *E. coli*. However, dithiothreitol, which is much more effective in protecting sulfhydryl groups (3), can increase the storage life of *S. aureus* extracts significantly. It is suggested that protein synthesis extracts from other sources may be benefited by using dithiothreitol instead of mercaptoethanol or glutathione.

There are several important differences between the *S. aureus* and the *E. coli* cell-free systems. The magnesium ion concentration required for maximal incorporation in endogenous protein synthesis is much higher (16 to 18 mM) than in *E. coli* (7 to 11 mM; 18). The poly-U stimulated incorporation of phenylalanine required even higher magnesium concentrations (22 mM).

The higher magnesium concentration was also required to maintain *S. aureus* ribosomes intact or in polysomal form. Tissières et al. (19) have shown that 5 mM magnesium was enough to maintain *E. coli* ribosomes intact. It was found that, in 5 mM magnesium, *S. aureus* ribosomes dissociated to 50S and 30S particles. Young and

Barker (20) found that even in 10 mM magnesium *S. aureus* (strain Duncan) ribosomes were dissociated to subunits. Hignett (10) found only a trace of polysomes in 10 mM magnesium with ribosomes isolated from the same strain. They postulated that lack of monosomes and polysomes was due to the contamination of ribosomes with teichoic acid. The work reported here with 16 mM magnesium showed 50% of the ribosomes in 100S particles and 45% in 70S particles (Fig. 7). The ratio between 100S and 70S particles may change in different preparations, but 100S and 70S always comprised more than 90% of the ribosomes. These results suggest that the failure of earlier workers to obtain large amounts of polysomes and monosomes was probably due to the low magnesium concentration employed. This is consistent with the observation that optimal magnesium concentrations for protein synthesis in the *S. aureus* system are higher than in *E. coli*.

Various investigators have shown that, upon the addition of artificial mRNA to ribosomes, polysomes were readily formed and that amino acid incorporation is primarily associated with polysomes (1, 9). In the present experiments, poly-U or poly-A did not cause aggregation of *S. aureus* monosomes to polysomes. This is probably due to the high ratio of polynucleotides to ribo-

FIG. 7. Sucrose gradient analysis of mRNA-ribosome-polypeptide complex. (a) The reaction mixture contained 8.9 A_{260} units of ribosomes, 0.8 mg of S-100 protein, 1 μC of ^3H -poly-U, and 0.25 μC of ^{14}C -phenylalanine in 0.5 ml of the complete system. (b) The reaction mixture contained 8.2 A_{260} units of ribosomes, 0.8 mg of S-100 protein, 1 μC of ^3H -poly-A, and 0.25 μC of ^{14}C lysine in 0.5 ml of the complete system. Symbols: ●, absorbance at 260 mμ; △, incorporated phenylalanine or lysine; ×, poly-U or poly-A.

TABLE 2. Requirements for aminoacyl-sRNA binding to ribosomes

Experimental conditions	Phenylalanyl-sRNA bound to ribosomes	Lysyl-sRNA bound to ribosomes
	counts/min	counts/min
Complete.....	1,130	406
—poly-U.....	285	—
—poly-A.....	—	134
—ribosomes.....	72	26
—poly-U + poly-A.....	280	—
—poly-A + poly-U.....	—	101
— Mg^{++}	278	104

somes employed in the incubation mixture, which would not be particularly favorable to polysome formation (17).

Figure 7 shows that poly-U and poly-A were associated mainly with 70S particles. (Probably the 100S particles had already bound a piece of natural mRNA.) It is reasonable to expect, as shown by the data in Fig. 7, that the newly synthesized polyphenylalanine and polylysine would be associated with 70S particles. It is not likely that these monosomes were quantitatively con-

verted from polysomes at the end of the incubation. The synthesis of polypeptide by monosomes from reticulocyte (8), yeast (4), and tobacco leaves (2) has been reported. That monosomes are active in synthesis of polypeptide is further illustrated in Figure 6b, which shows that an amino acid mixture was incorporated into both monosomes and polysomes under the direction of the endogenous mRNA.

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