PROTEIN SYNTHESIS IN RABBIT RETICULOCYTES:

CHARACTERISTICS OF PEPTIDE CHAIN INITIATION FACTORS

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Summary

In a cell-free amino acid incorporating system using preincubated reticulocyte ribosomes, the transfer of methionine from [^{35}S] Met-tRNA$_f^{Met}$ in response to poly r((U-G) messenger (in the presence of unlabeled valine, cysteine and crude tRNA) was dependent on addition of the 0.5M KCl wash of crude reticulocyte ribosomes. The optimum Mg^{++} concentration for this transfer reaction was 3mM.

The ribosomal salt wash preparation was fractionated by DEAE-cellulose column chromatography. At least three factors (I_1, I_2 and I_3) that eluted from the column stimulated poly r(U-G) directed methionine transfer from Met-tRNA$_f^{Met}$. All three factors were necessary to obtain maximum transfer of methionine.

Preincubated reticulocyte ribosomes actively catalyzed the incorporation of [^{14}C] phenylalanine in response to poly r(U) and the addition of the ribosomal salt wash or the above three factors (I_1, I_2 and I_3), had no significant effect on [^{14}C] phenylalanine incorporation.

Miller and Schweet first demonstrated that the protein fraction (I fraction) obtained by washing crude reticulocyte ribosomes with 0.5M KCl was necessary for *in vitro* hemoglobin synthesis directed by salt washed or preincubated reticulocyte ribosomes (1). Shafritz *et al.* separated two factors (M_1 and M_2) from the 0.5M KCl ribosomal wash by column chromatographic procedures and demonstrated the requirement of these two factors for poly r(U) directed polyphenylalanine synthesis at low Mg^{++} (2-3). One of these factors (M_1) also stimulated binding of f-Met-tRNA$_f^{Met}$ to reticulocyte ribosomes in response to AUG triplet codon, whereas both factors (M_1 and M_2) were necessary for binding of Met-tRNA$_f^{Met}$ to reticulocyte ribosomes in

response to the same codon (4). These authors found that another factor (M_3), also present in the 0.5M KCl ribosomal wash, was necessary in addition to M_1 and M_2 for in vitro translation of endogenous hemoglobin messenger (5). Several other laboratories have reported the presence of protein factors in the salt wash of mammalian ribosomes which are required for protein synthesis directed by physiological messengers (6-8), poly r(U) directed polyphenylalanine synthesis at low Mg^{+2} (9), and for poly r(U) directed binding of Phe-tRNA (10,11) or N-acetyl phe-tRNA (11) to 40s ribosomal subunits. However, it is not clear how these ribosomal salt wash factors specifically recognize the initiator tRNA, which is now known to be Met-$tRNA_f^{Met}$ (12-15), and how peptide chains are initiated in response to the initiator codons (AUG or GUG) (12-14) in mammalian protein synthesis.

Recently, we described a peptide chain initiation assay using preincubated reticulocyte ribosomes and demonstrated the requirement for the 0.5M KCl wash of crude reticulocyte ribosomes for the transfer of methionine from Met-$tRNA_f^{Met}$ in response to poly r(U-G) messenger (16-17). The optimum Mg^{++} concentration for this transfer reaction was about 3mM, and the transferred methionine was located entirely at the terminal positions of the synthesized polypeptide chains (16-17). In this paper, we describe a partial fractionation of the 0.5M KCl ribosomal wash fraction by DEAE-cellulose column chromatography. At least three factors (I_1, I_2 and I_3) stimulated poly r(U-G) directed methionine transfer from [^{35}S] Met-$tRNA_f^{Met}$ when assayed using preincubated ribosomes. All three factors were necessary to obtain maximum stimulation of the methionine transfer reaction. As described previously (18), preincubated reticulocyte ribosomes actively catalyze the incorporation of phenylalanine in response to poly r(U) messenger. We now present evidence that the addition of the unfractionated ribosomal salt wash or the above three factors (I_1, I_2 and I_3) do not have any significant effect on poly r(U) directed (^{14}C)-phenylalanine incorporation when catalyzed by the preincubated ribosomes.

Materials and Methods

Washed, twice pelleted, reticulocyte ribosomes used in these experiments were prepared from anemic rabbit blood by the procedure described previously (18). The assay for poly r(U) and poly r(U-G) directed polypeptide synthesis using preincubated ribosomes was performed as previously described (17,18). The 0.5M KCl reticulocyte ribosomal wash fraction was prepared by modification of the procedures of Miller and Schweet (1) and has been described in detail (17).

Rabbit reticulocyte tRNA preparations used in these experiments were prepared as described previously (18). Crude reticulocyte tRNA preparation was charged with [^{35}S] methionine using E. coli synthetase (12), which charges only the $tRNA_f^{Met}$ species.

Other materials and methods used in these experiments were the same as previously described (12,17,18).

Results

As mentioned previously, preincubated reticulocyte ribosomes retain most of the enzymic activities necessary for artificial polyribonucleotide messenger directed amino acid incorporation (18). We studied in detail the nature of poly r(U) directed (^{14}C) phenylalanine incorporation using several preincubated ribosome preparations (fresh, aged, repeatedly frozen and thawed) and the effect of the addition of ribosomal salt wash on phenylalanine incorporation. With most ribosomal preparations tested, the addition of ribosomal salt wash or S_{100} supernatant enzyme preparation did not have any significant effect on the nature of (^{14}C) phenylalanine incorporation.

The Mg^{++} optimum for poly r(U) directed (^{14}C) phenylalanine incorporation using preincubated ribosomes was 5-6mM, both in the presence and absence of ribosomal salt wash (Fig. 1). It should be noted that this Mg^{++} concentration (5mM) was also optimum for poly r(U) directed polyphenylalanine synthesis with salt washed ribosomes supplemented with M_1 and M_2 as described

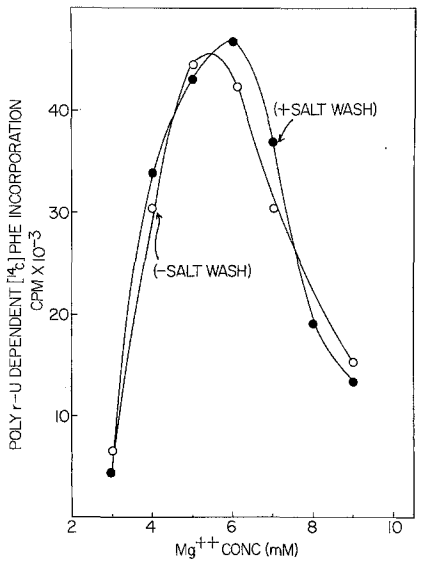

Fig. 1. Effect of the addition of ribosomal salt wash on poly r(U) directed [^{14}C] phenylalanine incorporation as catalyzed by preincubated ribosomes at different Mg^{+2} ion concentrations. Experimental conditions were as previously described (18). Standard reaction mixtures contained (in a total volume of 0.075 ml), in addition to the usual protein synthesizing components, 0.7 A_{260} units preincubated ribosomes, 0.4 A_{260} units unfractionated reticulocyte tRNA, 25 µg poly r(U), 0.25 nmoles [^{14}C] phenylalanine (550 cpm per pmole), and where indicated, approximately 0.3mg of unfractionated ribosomal salt wash. The reaction mixtures were incubated at 37° for 15 minutes and a 0.05 ml aliquot was assayed for hot trichloroacetic acid (5%) insoluble radioactivity by the filter paper disc assay method (18).

by Shafritz et al. (2-4). Apparently, the factors required for the Mg^{++} shift for poly r(U) directed polyphenylalanine synthesis are already present in the preincubated ribosomes.

In a few cases (15%), using preincubated ribosomes, poly r(U) directed (^{14}C) phenylalanine incorporation showed a significant lag for 2-3 minutes. During this short period of incorporation, addition of ribosomal salt wash partially relieved this lag. After the initial lag period, the rate of phenylalanine incorporation was unaffected by addition of the ribosomal salt wash.

However, the same preincubated ribosome preparation was inactive in poly r(U-G) directed methionine transfer from Met-$tRNA_f^{Met}$ into polypeptides and the addition of the ribosomal salt wash was necessary for this transfer

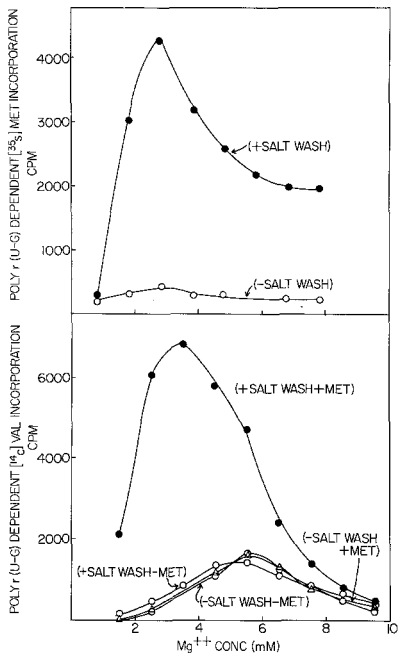

Fig. 2. Effect of the addition of ribosomal salt wash on poly r(U-G) directed methionine (upper curve) and valine (lower curve) incorporation as catalyzed by preincubated ribosomes at different Mg^{++} ion concentrations. Upper curve: Standard reaction mixtures contained (in a total volume of 0.075 ml) in addition to the usual protein synthesizing components, 0.7 A_{260} units of preincubated ribosomes, 0.4 A_{260} units of crude reticulocyte tRNA containing 6.5 pmoles [^{35}S] Met-$tRNA_f^{Met}$ (150,000 cpm), 0.9 nmoles of base residues as poly r(U-G), 10 nmoles each of unlabelled methionine valine and cysteine, Mg^{++} ion concentration as indicated and where mentioned 0.3 mg unfractionated ribosomal salt wash. The reaction mixtures were incubated at 37° for 15 minutes and a 0.05 ml aliquot of the reaction was used for amino acid incorporation assay. Lower curve: The reaction conditions were the same as above except the amino acids used were 0.45 nmoles (^{14}C) valine (350 cpm per pmole), 10 nmole unlabelled cysteine and where indicated 10 nmoles unlabelled methionine.

reaction. The Mg^{++} optimum for this transfer reaction was 3mM (Fig. 2, upper curve). Under these conditions, poly r(U-G) messenger presumably synthesized Met-$(Cys\text{-}Val)_n$ polypeptides. With preincubated ribosomes alone, poly r(U-G) also directed (^{14}C) valine incorporation in the presence of crude tRNA and unlabelled cysteine. The Mg^{++} optimum for (^{14}C) valine incorporation was 6-7 mM. However, in the presence of both unlabelled methionine and ribosomal salt wash, the Mg^{++} optimum for (^{14}C) valine incorporation was shifted to 3mM (Fig. 2, lower curve). This system, there-

fore, represents polypeptide synthesis at low Mg^{++} (3mM) which is dependent on the N-terminal methionine incorporation in response to an initiator codon (GUG). These experiments were done using crude reticulocyte tRNA and the results obtained were similar to those described previously using crude liver tRNA (17).

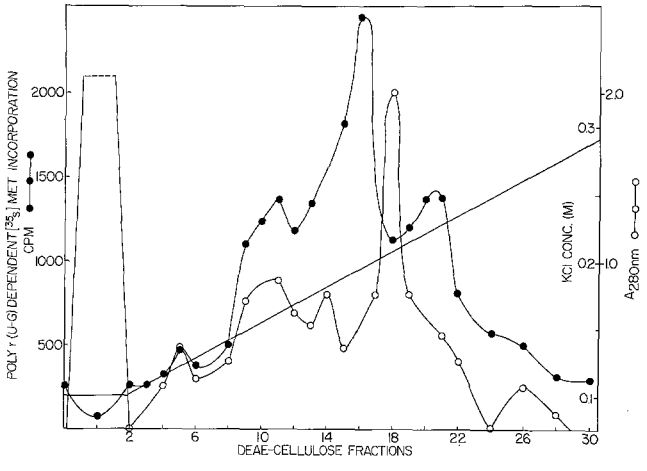

Fig. 3. DEAE-cellulose column chromatography of the 0.5M KCl wash of crude reticulocyte ribosomes. The preparation of ribosomal salt wash was as previously described (17). Approximately 10 ml of the ribosomal salt wash containing 25 mg protein per ml, was thoroughly dialyzed against Buffer C (5mM Tris-HCl, pH 7.5, 0.1M KCl, 1mM dithiothreitol and 50μM EDTA). The solution was applied to a 1.5x15cm column of DEAE-cellulose previously equilibrated with Buffer C at the rate of 0.7 ml/min. The column was thoroughly washed with Buffer C, then eluted with a 0.1-0.35M KCl linear gradient in Buffer C, (total volume 150 ml). Approximately 4 ml fractions were collected in 5 minutes. The fractions were concentrated by dialysis against Buffer C containing 15% (w/v) polyethylene glycol, then thoroughly dialyzed against Buffer C. The 280nm absorbance of the fractions was monitored with an Isco recorder and continuous flow cell, and then determined with a Zeiss spectrophotometer after concentration. A 15μl aliquot of each fraction was assayed for its ability to stimulate poly r(U-G) directed [^{35}S] methionine incorporation as described in Figure 2. The Mg^{++} concentration used in these transfer reactions was 3mM.

Figure 3 shows the results of a typical DEAE-cellulose fractionation of the crude ribosomal salt wash. At least three factors stimulated poly r(U-G) directed (^{35}S) methionine transfer from (^{35}S) Met-$tRNA_f^{Met}$ when assayed using preincubated ribosomes. None of these factors gave significant stimulation of poly r(U) directed (^{14}C) phenylalanine incorporation.

Table 1 describes the effect of these factors, added singly and in

TABLE I

Effects of the Additions of Ribosomal Salt Wash Fractions on Poly r(U-G) Directed [^{35}S] Methionine Transfer and Poly r(U) Directed [^{14}C] Phenylalanine Incorporation

Fractions Added	Poly r(U-G) Directed [^{35}S] Methionine Transfer	Poly r(U) Directed [^{14}C] Phenylalanine Incorporation
	cpm	cpm
None	550	10,870
Unfractionated Salt Wash (0.3mg)	3,030	12,590
I_1 (4μg)	600	12,530
(8μg)	700	10,360
(12μg)	850	11,900
I_2 (3μg)	780	10,260
(6μg)	1,200	8,540
(9μg)	1,390	6,700
I_3 (8μg)	960	10,670
(16μg)	1,050	9,830
(24μg)	1,730	8,400
I_1 (4μg) + I_2 (3μg)	1,230	9,145
I_1 (4μg) + I_3 (8μg)	2,220	10,620
I_2 (3μg) + I_3 (8μg)	2,210	8,500
I_1 (4μg) + I_2 (3μg) + I_3 (8μg)	4,790	9,075

The reaction conditions were the same as described in Figs. 1 and 2. The Mg^{++} concentrations used in these experiments were 3mM for poly r(U-G) directed [^{35}S] methionine transfer and 6mM for poly r(U) directed [^{14}C] phenylalanine incorporation. Amounts of crude ribosomal salt wash fractions or DEAE-cellulose fractions added are shown in the parenthesis. DEAE-cellulose fractions 10, 16, and 20 were used as representatives of I_1, I_2, and I_3 respectively. The incubations were at 37° for 5 minutes and 0.050 ml aliquots were used for amino acid incorporation assay.

combinations on poly r(U-G) directed methionine transfer and poly r(U) directed (^{14}C) phenylalanine incorporation. DEAE-cellulose fractions 10, 16 and 20 were used as representatives of factors I_1, I_2 and I_3, respectively.

All three factors were necessary to obtain maximum stimulation of methionine transfer. The reconstitution described in Table 1 is more than that observed with unfractionated ribosomal salt wash. This is partly because the DEAE-cellulose fractions used here were concentrated approximately 20 fold to obtain better reconstitution. As seen in the right hand columns, none of these factors were necessary for poly r(U) directed (^{14}C) phenylalanine incorporation. Only with the I_1 preparation was slight stimulation (10-20%) of [^{14}C] phenylalanine incorporation observed.

Discussion

The advantage of using preincubated reticulocyte ribosomes in the present studies, instead of salt washed ribosomes, is that preincubated ribosome preparations retain most of the enzymic activities for amino acid incorporation at nearly saturating levels, including amino acyl tRNA synthetase and peptidyl transferase activities. However, these ribosomes lack at least three protein factors necessary for peptide chain initiation, and the addition of the ribosomal salt wash to the preincubated ribosomes reconstituted efficient peptide chain initiation. The salt washed ribosomes, as prepared by the procedure described by Shafritz et al. (3), were found deficient in several other factors necessary for protein synthesis. Furthermore, the reconstitution of poly r(U-G) directed methionine transfer using salt washed ribosomes with the I_1, I_2 and I_3 was very poor. In agreement with the results obtained by Shafritz et al. (2-3), we also observed that the salt washed ribosomal preparation required the addition of the ribosomal salt wash for poly r(U) directed [^{14}C] phenylalanine incorporation, and at least three factors which stimulated poly r(U) directed phenylalanine incorporation could be separated by DEAE-cellulose chromatography of the ribosomal salt wash. The elution pattern of at least one of these factors was identical to M_1 as described by Shafritz et al. (3). This factor (Fraction 1, Figure 2) was not absorbed by the DEAE-cellulose column and was eluted with the 0.1M KCl wash of the column. When assayed using pre-

incubated reticulocyte ribosomes, this factor did not stimulate either poly r(U) directed phenylalanine incorporation or poly r(U-G) directed methionine transfer. Apparently, this factor is present in the preincubated ribosome preparation. It is not clear at present whether this factor is necessary for poly r(U-G) directed methionine transfer.

The three factors described here, which are required for poly r(U-G) directed methionine transfer, eluted from the DEAE-cellulose column at salt concentrations 0.16M, 0.19M, and 0.22M KCl, respectively. M_3 and M_2 factors as described by Shafritz et al. eluted from the DEAE-cellulose column at 0.17M and 0.26M KCl respectively (3-5). Judged from the DEAE-cellulose chromatographic elution pattern, it is possible that one of the factors described here (I_1) may be identical to M_3. The other two factors are clearly necessary for poly r(U-G) directed methionine transfer and are not required for poly r(U) directed polyphenylalanine synthesis when assayed using preincubated ribosomes.

Further work is in progress to determine the roles of each factor in the overall process of protein synthesis, and their specificities when protein synthesis is directed by poly r(U-G) messenger, where only the terminal GUG codon is involved in initiation (17), and poly r(A-U-G) messenger in which both terminal and internal initiations are presumably possible (17,19).

Acknowledgments

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References

1. Miller, R. L., and Schweet, R., Arch. Biochem. Biophys., 125, 632 (1968).
2. Shafritz, D. A., Prichard, P. M., Gilbert, J. M., and Anderson, W. F., Biochem. Biophys. Res. Commun., 38, 721 (1970).
3. Shafritz, D. A., and Anderson, W. F., J. Biol. Chem., 245, 5553 (1970).
4. Shafritz, D. A., and Anderson, W. F., Nature, 227, 918 (1970).
5. Prichard, P. M., Gilbert, J. M., Shafritz, D. A., and Anderson, W. F.,

Nature, 226, 511 (1970).
6. Heywood, S. M., Cold Spr. Harb. Symp. Quant. Biol., 34, 799 (1969).
7. Gee-Clough, J. P., and Arnstein, H. R. V., Eur. J. Biochem., 19, 539 (1971).
8. Ascione, R., and Vande Wonde, G. F., Biochem. Biophys. Res. Commun., 45, 14 (1971).
9. Grummt, F., and Bielka, H., Eur. J. Biochem., 21, 210 (1971).
10. Leader, D. P., Wool, I. G., and Castles, J. J., Proc. Natl. Acad. Sci., 67, 523 (1970).
11. Moldave, K., and Gasior, E., Fed. Proc., 30, 1290 (1971).
12. Gupta, N. K., Chatterjee, N. K., Bose, K. K., Bhaduri, S., and Cheung, A., J. Mol. Biol., 54, 145 (1970).
13. Bhaduri, S., Chatterjee, N. K., Bose, K. K., and Gupta, N. K., Biochem. Biophys. Res. Commun., 40, 402 (1970).
14. Smith, A. E., and Marcker, K., Nature, 226, 607 (1970).
15. Brown, J. C., and Smith, A. E., Nature, 226, 610 (1970).
16. Chatterjee, N. K., Woodley, C. L., Bose, K. K., and Gupta, N. K., Fed. Proc., 30, 1236 (1971).
17. Gupta, N. K., Chatterjee, N. K., Woodley, C. L., and Bose, K. K., J. Biol. Chem. (in press).
18. Gupta, N. K., J. Biol. Chem., 249, 4959 (1968).
19. Chatterjee, N. K., Bose, K. K., Woodley, C. L., and Gupta, N. K., Biochem. Biophys. Res. Commun., 43, 771 (1971).