Transfer of Serine into Polypeptides and Myosin by Chromatographic Species of Seryl-Transfer Ribonucleic Acid

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The efficiencies of two chromatographic species of [^3H]seryl-tRNA, namely peaks I and II, in cell-free amino acid incorporation were investigated. The maximum yield of polypeptide seems to be the same for the reaction mixtures containing either peak I or peak II, suggesting that the efficiency of both peaks in total protein synthesis is the same. The efficiency of transfer of serine into myosin heavy subunit (myosin H) by peaks I and II was also investigated. Peak II of [^3H]seryl-tRNA transfers three times as much serine into myosin H as peak I.

A direct causal relationship between transfer RNA and specific cell differentiation has not been incontrovertibly demonstrated (see Sueoka & Kano-Sueoka, 1970). In the present study, I have attempted to demonstrate differential utilization of seryl-tRNA iso-accepting species in specific myosin synthesis as a first step towards elucidating the role of tRNA iso-accepting species in muscle differentiation. I chose to study seryl-tRNA because serine is coded by two families of unrelated codons, U-C-X (where X is U, C, A or G) and A-G-Y (where Y is a pyrimidine). Since different mRNA species may contain one or the other of the two families of codons, or may contain both families but in different proportions, there is a wide scope for the control of translation by the matching isoaccepting species of seryl-tRNA. Thus the rate of insertion of serine into a protein, for example myosin, in response to A-G-Y codons would depend on the availability of the corresponding tRNA species (anticodons) even though large quantities of other tRNA species responding to U-C(X) codons may be present.

A report from this laboratory (Nwagwu & Lianga, 1974) showed that during muscle development in the chick embryo, (a) the combination of tRNA and aminoacyl-tRNA synthetase from the 14-day embryo is more efficient in seryl-tRNA formation than similar combinations from 11- and 17-day embryos, (b) seryl-tRNA from 11-, 14- and 17-day embryos is fractionated into three peaks on benzoylated cellulose columns and (c) in 14-day chick embryonic muscle, the amount of material eluted in peak II of seryl-tRNA is two times that eluted in peak I, unlike the patterns at 11 and 17 days, in which the relative amounts of peaks I and II are the same.

The results obtained from a calculation of the data presented by Herrmann *et al.* (1970) (see Table III, p. 197) suggest that the fourteenth day of chick embryonic development seems to represent a transition from a period of a comparatively rapid

rate of myosin accumulation to a period when the rate seems to fall or level off. The observed increase in peak II of seryl-tRNA during development from 11 to 14 days may therefore be significant, especially if the tRNA concentration is rate-limiting in protein synthesis. Thus there may be a greater requirement for peak II of seryl-tRNA than for peak I in muscle protein synthesis. I have therefore investigated whether or not there is a differential utilization of peaks I and II of seryl-tRNA in the cell-free synthesis of total muscle protein and of myosin. Our results demonstrate that similar amounts of serine are transferred from seryl-tRNA peaks I and II into hot-trichloroacetic acid-precipitable polypeptide. By contrast, peak II transfers a greater amount of serine into the myosin heavy subunit (mol.wt. 200000) than does peak I, suggesting that peak II of seryl-tRNA responds to myosin mRNA more efficiently than does peak I.

Methods and Materials

The leg skeletal muscle of 14-day chick embryos was used throughout this study. All solutions were prepared with doubly distilled water. Dialysis tubing was thoroughly cleaned as described by McPhie (1971).

Preparation of tRNA

tRNA was prepared by a modification of the method described by Ehrenstein (1967). After the DEAE-cellulose chromatography step, the RNA was fractionated on a column (100 cm $\times$ 2.5 cm) of Sephadex G-75 equilibrated and eluted with 200 mM-NaCl–10 mM-MgCl₂–10 mM-Tris-HCl (pH 7.0). The tRNA peak was pooled, dialysed against 2 litres of 1 mM-NaCl–1 mM-MgCl₂–1 mM-Tris-HCl (pH 7.0) for 12–18 h and stored at -20°C .

Preparation of aminoacyl-tRNA synthetases

Aminoacyl-tRNA synthetases were prepared by a modification of the method described by Muench & Berg (1966). The fractions eluted with 250mM-KCl-1mM-MgCl₂-20mM-KH₂PO₄ (pH6.5)-10% glycerol-20mM-2-mercaptoethanol were devoid of RNAase* activity, as determined by their ability to degrade poly(U). The synthetase was dialysed against 10mM-Tris-HCl (pH7.4)-10% glycerol-20mM-2-mercaptoethanol for 12-18h and stored at -20°C.

Seryl-tRNA formation

Seryl-tRNA formation was assayed with saturating amounts of enzyme under conditions in which the tRNA concentration was limiting and both the initial rate of reaction and the maximum amount of product formed at plateau were directly proportional to the amount of tRNA. These conditions were determined to be: 2.5-10 µg of tRNA, 500 µg of aminoacyl-tRNA synthetase, 5 µmol of ATP, 12.5 µmol of MgCl₂, 100 µmol of Tris-HCl (pH7.4) 2.5 µmol of dithiothreitol and 4-8 nmol of [³H]serine in a final volume of 0.5ml. Incubation was at 37°C for 45min, after which time addition of enzyme or ATP did not produce an increase in seryl-tRNA formation.

Benzoylated DEAE (BD)-cellulose chromatography

[³H]Seryl-tRNA formed in the aminoacylation reaction mixture was recovered free from all other components, especially unbound [³H]serine and aminoacyl-tRNA synthetase, by layering the mixture on a DEAE-cellulose column (0.9cm×5cm) equilibrated with 250mM-NaCl-10mM-MgCl₂-10mM-sodium acetate (pH4.4) at room temperature, washing the column with the 250mM-NaCl buffer until no radioactivity was contained in the eluate, and then eluting [³H]seryl-tRNA with 700mM-NaCl-10mM-MgCl₂-10mM-sodium acetate (pH4.4). [³H]Seryl-tRNA was eluted in the first 50ml; it was stored at -20°C. Before being layered on a BD-cellulose column it was diluted with 10mM-MgCl₂-10mM-sodium acetate (pH4.4) to give a final NaCl concentration of 400mM.

The BD-cellulose column was prepared for chromatography by washing it with 1 litre of 2M-NaCl, at a flow rate of 20-30ml/h, and then with 1 litre of 200mM-NaCl-10mM-MgCl₂-10mM-sodium acetate (pH4.4) at the same flow rate.

[³H]Seryl-tRNA was layered on the column at a flow rate of 20-30ml/h, at room temperature. The column was then eluted with a linear gradient of 400mM-1M-NaCl. Other specific conditions are described in the legend to Fig. 1.

* Abbreviation: RNAase, ribonuclease.

Recovery of [³H]seryl-tRNA after BD-cellulose chromatography

After BD-cellulose chromatography, peaks I and II of [³H]seryl-tRNA were separately pooled and the approximate NaCl concentration of each peak was determined from the position of elution on the linear NaCl gradient (the NaCl concentration of peak I was calculated as approx. 550mM and that of peak II as approx. 850mM). The NaCl concentration of each peak was diluted to 200mM with 10mM-MgCl₂-10mM-sodium acetate (pH4.4). All subsequent operations were carried out in a cold-room at 4°C. The solution containing [³H]seryl-tRNA (500-1600ml) was then layered on a DEAE-cellulose column (2cm×12cm) equilibrated with 200mM-NaCl-10mM-MgCl₂-10mM-sodium acetate (pH4.4) at a flow rate of 70ml/h. The eluate was collected in 10ml fractions. The radioactivity content of every third tube was determined and was shown to be the same as the blank value of about 100d.p.m. [³H]-Seryl-tRNA was eluted with 700mM-NaCl-10mM-MgCl₂-10mM-sodium acetate (pH4.4). Fractions (8-10ml) were collected and the peak of radioactivity eluted in tubes 3-5 was pooled, dialysed against 2 litres of 1mM-MgCl₂-1mM-sodium acetate (pH4.4) for 15-18h and freeze-dried. The sample was dissolved in 0.6-1ml of doubly distilled water and stored at -20°C. By this technique, approx. 75% of the total radioactivity of each peak was recovered in the sample after the freeze-drying step.

Preparation of tRNA-free S-240 fraction

Muscle (15g) was homogenized (2g/ml) in 150mM-KCl-10mM-MgCl₂-6mM-2-mercaptoethanol-10% glycerol-10mM-Tris-HCl (pH7.4) with a loose-fitting Dounce homogenizer. The homogenate was centrifuged at 17000g for 20min and the resulting supernatant further centrifuged at 240000g for 1h. Then 10ml of the final supernatant (S-240) was layered on a column (100cm×2.5cm) of Sephadex G-100 equilibrated and eluted with the above 150mM-KCl buffer at 4°C. Fractions (5ml) were collected and the major absorbance peak (*E*₂₈₀) eluted in 125-130ml was pooled. The *E*₂₈₀/*E*₂₆₀ ratio of the peak was 1.75, indicating very little RNA contamination. Under the chromatographic conditions, tRNA is eluted in 250-300ml. After being pooled, the peak was concentrated (to 5.4mg of protein/ml) with an Amicon Ultrafiltration TCF 10 system on an XM 50 filter. The S-240 fraction was then stored at -20°C and used within 1 week.

Preparation of polyribosomes

Polyribosomes were prepared by homogenizing 56g of muscle in portions of 10g in 5ml of 250mM-KCl-10mM-MgCl₂-10mM-Tris-HCl (pH7.4) with a

loose-fitting Dounce homogenizer. The homogenate was centrifuged at 10000g for 15 min and 4.3 ml of the supernatant was layered on 4 ml of 40% sucrose in 250 mM-KCl-10 mM-MgCl₂-10 mM-Tris-HCl (pH 7.4). The sample was centrifuged at 240000g for 30 min and the polyribosome pellet was rinsed with 150 mM-KCl-5 mM-MgCl₂-10 mM-Tris-HCl (pH 7.4)-6 mM-2-mercaptoethanol. Cell-free amino acid incorporation was carried out in the same tubes containing the polyribosome pellet after resuspending the pellet in 0.4 ml (1.35 mg of protein) of the tRNA-free S-240 fraction.

Standard components for cell-free myosin synthesis (Rourke & Heywood, 1972)

The following components were added to the S-10 supernatant fraction in a final volume of 0.5 ml: 2 μ mol of ATP, 0.5 μ mol of GTP, 7.5 μ mol of creatine phosphate, 100 μ g of creatine phosphokinase, 0.1 nmol of each of the 20 natural L-amino acids and seryl-tRNA, peak I or peak II containing 20000-40000 d.p.m./0.1 ml (100000 d.p.m./E₂₆₀ unit). The transfer of serine from seryl-tRNA peaks I and II was also assayed in a reaction mixture containing polyribosomes (200-400 μ g) resuspended in tRNA-free S-240 fraction (1.35 mg of protein) and the other standard components.

Myosin

Myosin used as a standard in electrophoresis was prepared as described by Paterson & Strohmman (1970) for their myosin-II sample.

Protein determination

Protein concentration was determined by the procedure of Lowry *et al.* (1951) with bovine serum albumin as standard.

Radioactivity detection

Radioactivity was measured in a Tri-Carb liquid-scintillation spectrometer, model 3310, with a ³H-counting efficiency of 30% for solutions and 25% for polyacrylamide gels.

Materials

DEAE-cellulose (DE-52, pre-swollen), a Whatman product, was obtained from Mandel Scientific Co., Montreal, Que., Canada. Benzoylated DEAE-cellulose (20-50 mesh) was a product of Schwarz/Mann, Orangeburg, N.Y., U.S.A. Sephadex G-75 was obtained from Pharmacia, Montreal, Que., Canada. All chemicals were of analytical grade. The chemicals for cell-free amino acid incorporation were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. [³H]Serine (specific radioactivity 6.3 Ci/mmol) and NCS and PCS scintillation solutions were obtained from Amersham-Searle, Toronto, Ont., Canada.

Results and Discussion

[³H]Seryl-tRNA, prepared with components from 14-day embryonic chick leg muscle, was fractionated into three peaks on a BD-cellulose column; peak II was twice as large as peak I (Fig. 1). In an attempt to

Fig. 1. BD-cellulose chromatography of [³H]seryl-tRNA

[³H]Seryl-tRNA (containing 100000-200000 d.p.m.) prepared as described in the Methods and Materials section was fractionated on BD-cellulose columns as previously described (Nwagwu & Lianga, 1974). Peaks I and II were separately pooled, dialysed against 400 mM-NaCl-10 mM-MgCl₂-10 mM-sodium acetate (pH 4.4) and rechromatographed separately on two new BD-cellulose columns. Peak III is the material eluted with 25% ethanol-2 M-NaCl-10 mM-sodium acetate (pH 4.4) (addition of which is shown by the arrow). The gradient of NaCl is shown as a straight line running through the chromatogram. —, [³H]Seryl-tRNA (d.p.m.); ----, E₂₅₄.

Fig. 2. Time-course of transfer of serine by peaks I and II of [^3H]seryl-tRNA into polypeptides and effect of aurintricarboxylic acid on this transfer

Muscle (6.5g) was homogenized in 3.2ml of 150mM-KCl-5mM-MgCl₂-10mM-Tris-HCl (pH7.4) and the homogenate centrifuged at 10000g for 10min. The cell-free system contained S-10 supernatant fraction (600 μg of RNA and 2mg of protein), 0.1ml of [^3H]seryl-tRNA (40000 d.p.m.; 100000 d.p.m./ E_{260} unit) and the standard components given in the Methods and Materials section in a total volume of 0.5ml. Aurintricarboxylic acid (100mM) was added to another reaction mixture containing [^3H]seryl-tRNA (40000 d.p.m.), peak I or peak II, and the standard components; similar results were obtained with peak I and peak II. A large amount of unlabelled serine (0.01%) was added to dilute the radioactive serine liberated from [^3H]seryl-tRNA (see Saito *et al.*, 1971). The mixture was incubated at 37°C and protein synthesis was assayed as described by McCarthy (1968). The samples were resuspended in 10ml of PCS-xylene (3:1, v/v) scintillation mixture and the radioactivity was determined. Each point on the graph is the average result of two separate experiments each of which was carried out in two parallel duplicates. ○, Peak I; ●, peak II; △, in the presence of aurintricarboxylic acid.

assay the efficiency of seryl-tRNA peaks I and II in the transfer of serine into polypeptides, the time-course of amino acid incorporation was followed in reaction mixtures containing the seryl-tRNA peaks. The results presented in Fig. 2 show that at the plateau of amino acid incorporation, 10 and 12.5% of the serine radioactivity of peaks I and II respectively is transferred into polypeptide, suggesting that the peaks have similar efficiencies in the synthesis of total protein.

We have considered the possibility that only chains with radioactive serine close to the C-terminal are released during cell-free amino acid incorporation (see Woodward & Herbert, 1972). If this were so, then the differences observed in Fig. 2 may be due to the fact that a greater proportion of the available mRNA sequences contain more serine codons

recognized by peak II of seryl-tRNA than the codons recognized by peak I. In other words, the differences would only reflect differences in the ability of peaks I and II to translate only a portion of the mRNA sequences and not of the whole mRNA sequences. However, from the result presented in Fig. 2, and from the criteria described by Woodward & Herbert (1972), I calculate that the cell-free system is very efficient in polypeptide synthesis, incorporating 1–2mol of serine per mol of ribosomes under the following conditions: 14-day embryonic chick leg muscle contains 16.28 E_{260} units/g fresh weight and 11.2 E_{260} units of ribosomes, weight 1mg (T'so & Vinograd, 1961).

The result presented in Fig. 2 shows that addition of aurintricarboxylic acid to the cell-free system markedly inhibits the transfer of serine from peaks I and II into polypeptides by as much as 75 and 80% respectively. Since aurintricarboxylic acid inhibits further binding or rebinding of ribosomes to mRNA initiator sites (Webster & Zinder, 1969; Hoerz & McCarty, 1971) but allows the ribosomes already bound to complete translocation (Grollman & Stewart, 1968; Webster & Zinder, 1969) its effect observed in this study suggests that cell-free protein synthesis is dependent on continued proper attachment of ribosomes to initiator regions of mRNA. The possibility of translation of only a part of the mRNA is therefore unlikely.

The main thrust of this paper is aimed at a determination of the efficiency with which serine is transferred from seryl-tRNA peaks I and II into myosin. As shown in Table 1, the amount of radioactivity in the hot-trichloroacetic acid-precipitable product is directly proportional to the amount of [^3H]seryl-tRNA added, and also the amount of radioactivity in myosin H is directly proportional to the amount of radioactivity layered. Thus an increase in the amount of peak I of [^3H]seryl-tRNA by a factor of 1.7 results also in a corresponding increase in the radioactivity of the hot-trichloroacetic acid-precipitable product by a factor of 1.7 and, also, in an increase in the radioactivity content of myosin H, by a factor of 1.4. Similarly an increase in the amount of peak II [^3H]seryl-tRNA by 1.6 times gives rise to a similar increase in the radioactivity content of the hot-trichloroacetic acid-precipitable product and myosin H. These results show that the extent of amino acid incorporation is proportional to the amount of tRNA added. The results given in Table 1 also show that about 11% of the radioactive serine of peaks I and II of [^3H]seryl-tRNA is transferred to hot-trichloroacetic acid-precipitable material, in general agreement with the findings described in Fig. 2.

The most important finding of this paper is that approximately three times more serine is transferred from seryl-tRNA peak II into myosin heavy subunit (myosin H) than from seryl-tRNA peak I (Table 1).

Table 1. *Transfer of serine from peaks I and II of seryl-tRNA into myosin H subunit*

Peaks I and II of [^3H]seryl-tRNA (20000–40000 d.p.m./0.1 ml) were each incubated in the standard cell-free system for myosin synthesis, as described in the Methods and Materials section, except that the volume of the S-10 fraction was 0.1 ml (containing 200 μg of rRNA and 600 μg of protein) and the total reaction volume was 0.2 ml. All other components were scaled down accordingly. After incubation of the cell-free system at 37°C for 10 min, 4M-KCl was added to the reaction mixture to give a final concentration of 500 mM. The mixtures were then centrifuged at 240000g for 1 h at 4°C and the supernatant (S-240) was dialysed against 1 litre of 1% sodium dodecyl sulphate–1% mercaptoethanol for 15 h. Electrophoresis of the S-240 fractions and a myosin standard was performed as described in the legend to Fig. 3. The destained sample gels were matched with the gel of myosin standard; the band of myosin heavy subunit (myosin H) was identified and sliced into 2 mm slices with a razor. The gel slices were placed in vials containing 1.5 ml of NCS–water mixture (9:1, v/v) and incubated at 50°C for 4 h. After cooling, 10 ml of PCS–xylene (3:1, v/v) was added to the vials and the radioactivity determined. The gels were counted for radioactivity at 25% efficiency. The radioactivity content of the myosin H band of the myosin standard gel (60–100 d.p.m.) was used as the blank value and was subtracted from all sample values.

	[^3H]Seryl-tRNA (d.p.m.)*	Layered (d.p.m.)†	Myosin H (d.p.m.)	Myosin H (d.p.m.)	Myosin H (d.p.m.)
				Layered (d.p.m.)	[^3H]Seryl-tRNA (d.p.m.)
				(%)	(%)
Peak I	13 820	1520	162	10.65	1.17
	24 355	2660	227	8.53	0.9
	20 250‡	2237	206	9.20	1.01
Peak II	17 100	1809	640	33.86	3.7
	27 850	3080	976	31.68	3.5
	22 750‡	2532	780	30.85	3.4

* Amount of radioactivity added to the cell-free system.

† Amount of radioactivity in the hot-trichloroacetic acid-precipitable product of cell-free amino acid incorporation.

‡ The cell-free system contained tRNA-free S-240 fraction (1 mg), polyribosomes (300 μg of RNA) and other components of the standard cell-free system, described in the Methods and Materials section.

The possibility that the efficiency of transfer of serine into myosin may be affected by components of the S-10 supernatant fraction, particularly tRNA, was considered. Thus the efficiency of transfer of serine from seryl-tRNA peaks into myosin heavy subunit was determined with a cell-free system lacking in tRNA. As shown in Table 1, both peaks I and II transferred equivalent amounts (approx. 11%) of serine into hot-trichloroacetic acid-precipitable polypeptide. However, the amount of serine transferred by peak II into myosin heavy subunit is approximately three times that transferred by peak I. These results corroborate those obtained with S-10 supernatant fraction and indicate that the presence of the corresponding chromatographic peaks in the S-10 supernatant did not alter the specific radioactivity of seryl-tRNA peaks I and II.

Other components of the cell-free system may inhibit the activity of peak I. For example, peak I of seryl-tRNA may be more easily degraded by RNAase than peak II, resulting in transfer of a smaller amount of serine into myosin. However, other results from this laboratory make this possibility highly unlikely; (a) the RNAase activity of muscle S-10 fraction is quite low in the 14-day embryo; (b) on incubation of peaks I and II with the same amount of the S-10 fraction at 37°C for 5–30 min, both the rate of loss of label and the final residual amount of [^3H]seryl-tRNA were similar for both samples.

The chromatographic peaks of seryl-tRNA prepared in this study contain other tRNA species. If the tRNA species contaminating either peak I or peak II, or both, serve as 'modulators' (Ames & Hartman, 1963) of protein synthesis, then the results obtained in this study may not be directly attributable to the specific radioactivity of peaks I and II in translation but to the effect of the modulating tRNA species. Use of highly purified seryl-tRNA isoaccepting species [obtained by chromatography on RPC-5 and RPC-6 columns (Pearson *et al.*, 1971)] would help resolve this problem.

The increase in seryl-tRNA peak II in the muscle of 14-day chick embryo may be related to the increased myosin synthesis which occurs at this age. In the silk-worm, it has been suggested that the early increase in the amounts of glycine, serine, alanine and tyrosine is required for the subsequent decoding of fibroin mRNA (Garel *et al.*, 1970; Garel, 1974). It has also been found that the concentration of tRNA^{Leu} and tRNA^{Glu} relative to tRNA^{Gly} increased five and seven times respectively in cow mammary gland at the inception of lactation (Elska *et al.*, 1971).

One interpretation of our results is that there are three times as many serine codons in myosin mRNA to which seryl-tRNA peak II responds as there are codons which match peak I. The availability of seryl-tRNA peak II would therefore be rate-limiting in myosin synthesis.

Fig. 3. Gel electrophoresis of myosin standard (a) and the S-240 supernatant fraction of the cell-free system for myosin synthesis (b) showing the position of the myosin heavy subunit (H)

After incubating the components of the cell-free myosin-synthesizing system with [^3H]seryl-tRNA at 37°C for 10 min (as given in Table 1 and the Methods and Materials section), 4M-KCl was added to the reaction mixture to give a final concentration of 500mM. The mixture was centrifuged at 240000g for 1 h and the resulting S-240 fraction and the myosin standard were separately dialysed against 1 litre of 1% sodium dodecyl sulphate and 1% mercaptoethanol. Gel electrophoresis was carried out as described by Paterson & Strohman (1970, 1972). The band patterns are reproduced in the Figure. The bands shown by the supernatant fractions of the myosin-synthesizing cell-free systems containing peaks I and II of [^3H]seryl-tRNA were similar. The myosin H band was identified from its position in the gel as described by Weber & Osborn (1969) by comparison with the relative band positions of bovine serum albumin (fraction V), RNAase, pyruvate kinase and creatine kinase.

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