Evidence for Defective Transfer Ribonucleic Acid in Polymyopathic Hamsters and its Inhibitory Effect on Protein Synthesis

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(Received 31 July 1972)

1. Different reaction steps involved in protein synthesis were studied in skeletal muscles from control and myopathic hamsters. 2. There was no difference between partially purified aminoacyl-tRNA synthetases from myopathic and control animals in yield or catalytic activity, as tested with exogenous deacylated tRNA. 3. However, isolated deacylated tRNA from myopathic muscle was aminoacylated by these synthetases to a lesser extent than that derived from control muscle. 4. Addition of deacylated tRNA isolated from control muscle improved the performance of pH5 enzymes from myopathic muscle in polypeptide synthesis on homologous polyribosomes; tRNA isolated from myopathic animals did not. 5. Preparation of extracts from both types of animals in the presence of the ribonuclease-absorbent bentonite led to an increased capacity of endogenous tRNA to accept amino acids in pH5 enzymes prepared from normal and abnormal tissue, but the difference between the two systems remained the same. 6. Total tRNA nucleotidyltransferase activity, tested with twice-pyrophosphorolysed rat liver tRNA, was identical in both extracts. 7. Added tRNA nucleotidyltransferase incorporated more AMP and CMP into endogenous tRNA with the pH5 enzyme from myopathic muscle than with that from control muscle. 8. Preincubation of deacylated tRNA from myopathic muscle with ATP, CTP and tRNA nucleotidyltransferase more than doubled its subsequent aminoacyl-acceptor activity, and halved the extent of the defect relative to aminoacylation of control tRNA similarly treated. Endogenous tRNA in pH5 enzyme preparations behaved likewise. 9. It is suggested that a 3'-exonuclease in myopathic muscles attacks tRNA molecules in such a way that some of them remain substrates for tRNA nucleotidyltransferase, which may incorporate into RNA not only AMP and CMP, but also GMP. 10. Cell-free protein synthesis in preparations from myopathic hamster muscles is limited by the supply of intact tRNA molecules.

In the accompanying paper (Bester & Gevers, 1973), defective protein synthesis *in vitro* in myopathic hamsters is described. It has previously been generally believed that the genetic defect in these animals is restricted in its expression to striated muscles (Bajusz, 1969). However, we have found that the impairment in cell-free protein synthesis is not tissue-specific, but affects all types of muscle and also other metabolically active organs, such as liver and brain.

In recent years considerable attention has been paid to the enzymic mechanisms involved in polypeptide synthesis, with regard to both reaction pathways and control aspects. tRNA molecules have been especially considered for their possible role in the regulation of protein synthesis (Sueoka & Kano-Sueoka, 1964; Kano-Sueoka & Sueoka, 1966; Fangman & Neidhardt, 1964; Nass & Neidhardt, 1966). All tRNA molecules studied to date carry the trinucleotide sequence -pCpCpA at their 3'-end. The terminal adenosine residues, which form activated ester bonds with amino acids, undergo rapid turnover

(Cannon, 1966; Rosset & Monier, 1963), but the amino acid-acceptor capacity of tRNA depends on intact 3'-trinucleotide end-sequences. Thus removal of these terminal nucleotides, in part or whole, caused complete inability to accept amino acids (Hecht *et al.*, 1958; Cannon, 1964, 1965; Herrington & Hawtrey, 1970*b*; Carré *et al.*, 1970) or to bind to ribosomes (Cannon *et al.*, 1963). As found by Rosset & Monier (1963) and by Cannon (1966) in *Escherichia coli* preparations, and by Herrington & Hawtrey (1970*a,b*) in bovine mammary glands, it is possible that ribonuclease II, or an exonuclease with a similar mechanism, hydrolytically removes the terminal adenylate group from tRNA molecules, causing inactivation. However, once tRNA molecules are linked to amino acids, they become resistant to 3'-exonucleolytic attack catalysed by these enzymes (Rosset & Monier, 1963).

In the present paper we present evidence for defective tRNA in muscle tissues from myopathic hamsters, probably caused by the excessive activity

of an exonuclease which overcomes the efforts of a normally active repair process catalysed by tRNA nucleotidyltransferase. The relation of these findings to the primary genetic lesion, with its autosomal recessive pattern of inheritance, is not clear, but the defect is already present in very young myopathic hamsters

Materials and Methods

Animals and chemicals

These were as described by Bester & Gevers (1973) except that CTP (disodium salt) was obtained from C.F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, $(\text{NH}_4)_2\text{SO}_4$ (special enzyme grade) from Miles-Seravac, Cape Town, South Africa, and streptomycin sulphate and protamine sulphate from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. $[^{14}\text{C}]\text{ATP}$ (450 mCi/mmol), $[^{14}\text{C}]\text{CTP}$ (430 mCi/mmol), $[^3\text{H}]\text{CTP}$ (20 Ci/mmol), $[^{14}\text{C}]\text{GTP}$ (36 mCi/mmol), $[^{14}\text{C}]\text{UTP}$ (471 mCi/mmol) and $\text{U-}^{14}\text{C}$ -labelled protein hydrolysate (57 mCi/mg-atom of C) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Preparation of subcellular fractions

After decapitation of hamsters, the hearts and skeletal muscles (limbs and pectoral area) were isolated and dissected free of connective tissue. Homogenates were prepared in medium A, containing (final concns.): 0.25 M-sucrose, 10 mM- MgCl_2 , 80 mM-KCl and 50 mM-Tris-HCl buffer, pH 7.8. All procedures were carried out at 0–4°C.

(1) *Polyribosomes and pH5 enzyme fractions.* These were prepared as described in the accompanying paper (Bester & Gevers, 1973).

(2) *Deacylated tRNA.* tRNA was isolated from pH5 enzyme fractions by the method of Herrington & Hawtrey (1970a), before being deacylated by incubation with 1.8 M-Tris-HCl buffer, pH 8.0, at 37°C for 30 min (Sarin & Zamecnik, 1964). The deacylated tRNA was reprecipitated twice with ethanol and 0.1 vol. of 20% (w/v) potassium acetate before being dissolved in twice-distilled water and dialysed against the latter for 6–8 h. The concentration of RNA was determined spectrophotometrically at 260 nm ($E_{1\%}^{1\text{cm}}$ 170) (Hawtrey *et al.*, 1966).

(3) *Twice-pyrophosphorolysed rat liver tRNA.* This was prepared from rat liver pH5 enzyme by the method of Daniel & Littauer (1963).

(4) *Aminoacyl-tRNA ligases.* These enzymes were prepared from pH5 enzyme fractions from skeletal muscle by the methods of Herrington & Hawtrey (1970a) and Hele (1961). Most of the RNA was removed from pH5 enzyme fraction by adding to it, with constant stirring over a period of 5 min, a freshly prepared solution of protamine sulphate

(10 mg/ml) in the proportion of 3 mg of protamine to each 100 mg of pH5 enzyme protein. The precipitate was removed by centrifugation at 25000g for 20 min in a Sorvall refrigerated centrifuge. To each 10 ml of supernatant was added 0.5 ml of cold 10 M-potassium acetate in 20 mM-EDTA (sodium salt) and 2.8 g of solid $(\text{NH}_4)_2\text{SO}_4$. The resulting precipitate was collected by centrifugation and dissolved in medium A (crude enzyme fraction), to give a protein concentration of 10 mg/ml. The crude enzyme fraction was further purified by treatment with calcium phosphate gel (Keilin & Hartree, 1938). To every 15 mg of protein was added 1 mg dry wt. of calcium phosphate gel. The gel was stirred in very rapidly and the precipitate was removed by centrifugation at 15000g for 10 min immediately. This preliminary treatment was found to remove an inhibitor. The precipitate was discarded and the supernatant was treated with a further quantity of gel in the proportion of 1 mg dry wt. of gel/1.5 mg of starting amount of protein (Keilin & Hartree, 1938). The mixture was again centrifuged as described above and the precipitate was eluted with a mixture amounting to 0.75 vol. of the original crude enzyme fraction, and containing 3 parts of 0.1 M-sodium phosphate buffer, pH 8.0, and 1 part of saturated $(\text{NH}_4)_2\text{SO}_4$, pH 6.5. The gel was collected by centrifugation at 15000g for 10 min, eluted with 0.5 vol. of the same elution mixture, and the eluates were subsequently combined. Then 10 M-potassium acetate was added, equivalent to 0.025 vol. of the combined eluates, followed by saturated $(\text{NH}_4)_2\text{SO}_4$ (0.25 vol. of the combined eluates). The final $(\text{NH}_4)_2\text{SO}_4$ saturation was approx. 40%. After 5 min the precipitate was collected by centrifugation (15000g for 10 min), dissolved in a small volume of medium A and dialysed extensively against medium A for 8–10 h until free of sulphate. The enzyme was stored at –180°C until used.

(5) *tRNA nucleotidyltransferase.* Two stages were used for the preparation of the skeletal muscle tRNA nucleotide-incorporating enzyme [RNA adenylate (cytidylate) pyrophosphorylase; EC 2.7.7.-]. pH5 fractions were used as starting material. The method of Daniel & Littauer (1963) was used for the extraction and purification except that the calcium phosphate-gel-adsorption step was omitted from the procedure. The enzyme fraction obtained at 100% $(\text{NH}_4)_2\text{SO}_4$ saturation was dissolved in medium A and dialysed against the latter until free of sulphate. The enzyme was stored at –180°C until used.

Measurement of incorporation of labelled amino acids into protein

The reaction conditions are given in the legends to the Tables. The reaction was terminated by adsorption on to Whatman 3MM filter-paper discs, which were placed in cold 10% (w/v) trichloroacetic acid

for 15 min and then further processed by the method of Mans & Novelli (1961).

Aminoacylation of endogenous tRNA

The reaction mixture contained in 0.25 ml: pH 5 enzyme (0.4 mg of RNA), 1.6 mM-ATP, 5 mM-phosphoenolpyruvate, 20 μ g of pyruvate kinase, 10 mM-2-mercaptoethanol, 14 C-labelled protein hydrolysate (1.25 μ Ci), 40 mM-Tris-HCl buffer, pH 7.8, 18 mM-KCl, 6 mM-MgCl₂ and 0.2 M-sucrose. Incubations were carried out at 37°C and terminated at selected times by adsorption on to Whatman 3MM filter papers, which were treated as described above, except for omission of the heating stage. In another experiment, the aminoacylation of endogenous tRNA was studied by using individual labelled amino acids (1.0 μ Ci each).

Measurement of aminoacyl-tRNA synthetase activity

Deacylated tRNA (50 μ g of RNA) was incubated for selected time-periods with 1.6 mM-ATP, 10 mM-mercaptoethanol, 5 mM-phosphoenolpyruvate, 15 μ g of pyruvate kinase, 14 C-labelled protein hydrolysate (1.25 μ Ci), purified aminoacyl-tRNA synthetases (25 μ g of protein), 0.2 M-sucrose, 40 mM-Tris-HCl buffer, pH 7.8, 18 mM-KCl and 6 mM-MgCl₂, in a total volume of 0.25 ml at 37°C. The reactions were terminated by adsorption on to Whatman 3MM filter-paper discs, which were then treated as described above, except for the omission of the heating step.

Aminoacylation of tRNA (3'-end-group enriched) from myopathic and control skeletal muscles

Deacylated tRNA from myopathic or control muscle (50 μ g of each) was incubated for 60 min at 37°C in a medium containing tRNA nucleotidyl-transferase from control muscle (20 μ g of protein), 1 mM-ATP, 1 mM-CTP, 70 mM-glycine buffer, pH 9.0, 5 mM-MgCl₂, 5 mM-phosphoenolpyruvate, 5 μ g of pyruvate kinase, 16 mM-KCl and 0.1 M-sucrose, in a total incubation volume of 0.1 ml. At the end of the incubation period the pH was lowered to 7.8 and a complete assay mixture for aminoacylation of the intact tRNA was added (see above) to give a total incubation volume of 0.25 ml. A further incubation was carried out at 37°C for 15 min and then was terminated as described above.

Formation of 14 C-labelled (pC-C-A-OH)-tRNA

[14 C]Cytosine and [14 C]adenine nucleotides were added to the 3'-terminal ends of deacylated tRNA molecules lacking them, as follows. The incubation medium contained 70 mM-glycine buffer, pH 9.0,

5 mM-MgCl₂, 5 mM-phosphoenolpyruvate, 20 μ g of pyruvate kinase, 16 mM-KCl, 0.1 M-sucrose, 1.25 μ Ci of [14 C]ATP, 2.5 μ Ci of [14 C]CTP, 100 μ g of deacylated tRNA and tRNA nucleotidyltransferase (20 μ g of protein), in a total volume of 0.25 ml. Incubations were carried out at 37°C for 60 min. The reactions were terminated by adsorption on to Whatman 3MM filter-paper discs, which were placed in a large excess of cold 10% (w/v) trichloroacetic acid, then 5% (w/v) trichloroacetic acid (twice), and finally into ethanol-diethyl ether (1:1, v/v) and ether, before being dried and counted for radioactivity in a liquid-scintillation counter.

Micro-radiometric assay for the measurement of ribonuclease activity in partially purified tRNA nucleotidyl-transferase

Control isolated deacylated tRNA was labelled with [14 C]CTP and [14 C]ATP, by using tRNA nucleotidyltransferase from both myopathic and control muscles, as described above. The total incubation volume was 0.5 ml, and incubations were for 30 min at 37°C. The reaction was stopped by cooling on ice and passage through a column (1 cm $\times$ 30 cm; void volume 5.5 ml) of Sephadex G-25 (fine grade) equilibrated with medium A. Elution was performed with medium A and 0.2 ml fractions were collected. The radioactivity of each fraction was determined and an elution profile was drawn to separate 14 C-labelled tRNA from free [14 C]ATP and [14 C]CTP. The 14 C-labelled (pC-C-A-OH)-tRNA fractions (containing associated tRNA nucleotidyl-transferase) were pooled (1.2 ml), and incubated at 37°C. At selected times, 100 μ l was adsorbed on to Whatman 3MM filter-paper discs and placed in 10% (w/v) trichloroacetic acid before being washed as described above. Residual 14 C-labelled (pC-C-A-OH)-tRNA was determined by counting for radioactivity in a liquid-scintillation spectrometer.

Measurement of ribonuclease activity in pH 5 enzyme preparations

Deacylated tRNA was labelled with [3 H]CTP and [14 C]ATP, and the free labelled nucleotides were separated from the labelled tRNA by filtration on a Sephadex G-25 column as described above. At zero time, pH 5 enzyme fractions were added to equivalent amounts (in c.p.m.) of labelled tRNA, and incubated at 37°C. At selected times, fractions were adsorbed on to Whatman 3MM filter-paper discs, which were placed in 10% (w/v) trichloroacetic acid and further treated as described above, enabling the remaining labelled tRNA to be determined.

Preliminary experiments, in which the two radioactive labels were used individually, were carried out according to the full procedure described above, to

prepare filters containing only one of the two labels. These filters were then used to establish optimum gain and window settings for the separation of ^{14}C and ^3H counts by the channels-ratio method. The counting efficiency for ^{14}C was 67%, and that for ^3H was 28%.

Determination of RNA and protein

Protein in all preparations was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. The concentration of tRNA was measured spectrophotometrically at 260nm ($E_{1\text{cm}}^{1\%}$ 170) (Hawtrey *et al.*, 1966). The RNA concentrations of pH5 enzyme fractions were determined by the orcinol method (Mejbaum, 1939) with hydrolysed yeast RNA as standard.

Results

Aminoacylation by purified synthetases of exogenous deacylated tRNA from control and myopathic skeletal muscles

To determine whether the defect in the pH5 enzyme of myopathic muscle was due to lowered activity of the individual synthetases or to defective endogenous tRNA, the synthetases were partially purified and separated from tRNA, with pH5 enzyme fractions from myopathic and control muscles as starting material. The yields of aminoacyl-tRNA synthetases, in terms of total protein, were identical. These enzymes were tested by using purified deacylated tRNA from pH5 enzyme preparations from myopathic and control muscles. In both cases, there was no difference in the catalytic activity of the different synthetases, but deacylated tRNA from myopathic muscle was much less effective as aminoacyl acceptor than was control tRNA (Fig. 1).

Effect of added deacylated tRNA on the defect of myopathic muscles in polypeptide synthesis

The ability of isolated deacylated tRNA (from control and myopathic preparations) to correct the defect of myopathic muscles in cell-free polypeptide synthesis was tested by adding exogenous deacylated tRNA, at different concentrations, to complete reaction mixtures for polypeptide synthesis (Table 1). The pH5 enzyme from myopathic muscle was markedly less active in supporting polypeptide synthesis on both types of polyribosomes, as described in the accompanying paper (Bester & Gevers, 1973). Addition of exogenous deacylated control tRNA to a complete control polypeptide-synthesizing system increased the incorporation of labelled amino acids into polypeptides to some extent, whereas deacylated tRNA from myopathic muscle exerted no effect in

this system. Added deacylated tRNA from control muscle in a system containing control polyribosomes but with pH5 enzyme from myopathic muscle stimulated the incorporation to a value near that of the unstimulated control system, whereas tRNA from myopathic muscle caused no improvement.

It is known that addition of tRNA can lead to inhibition of the incorporation of amino acids in protein-synthesizing systems (Herrington & Hawtrey, 1970b). A significant feature here, however, is that tRNA isolated from the pH5 enzyme fraction from myopathic muscle had no stimulatory effect in both systems studied (Table 1), although it could be aminoacylated to a certain extent (Fig. 1). Herrington & Hawtrey (1970b) showed that bovine tRNA, lacking the terminal -pC-C-A-OH trinucleotide group, competitively inhibits the aminoacylation of rat liver tRNA. On the other hand, when the terminal -pC-C-A-OH group has been added the material stimulates the synthesis of rat liver aminoacyl-tRNA.

In an attempt to find out whether tRNA isolated from pH5 enzyme fractions from myopathic muscle lacked the complete terminal trinucleotide sequence or only the terminal adenosine, and whether the tRNA nucleotidyltransferase believed to be responsible for addition of these terminal nucleotides was defective, nucleotide incorporation into both

Fig. 1. Aminoacylation of isolated deacylated tRNA from myopathic and control muscle by partially purified aminoacyl-tRNA synthetases

Aminoacyl-tRNA formation was measured as described in the Materials and Methods section, by using isolated deacylated tRNA from myopathic muscle with synthetases from myopathic (○) or control (□) muscles, and exogenous tRNA from control muscles with synthetases from control (●) or myopathic (■) muscle.

Table 1. *Effects of addition of deacylated tRNA to polypeptide-synthesizing systems when using control polyribosomes*

Reaction mixtures contained: pH5 enzymes (0.4mg of RNA), control polyribosomes (0.6mg of RNA), 1.6mM-ATP, 0.6mM-GTP, 5mM-phosphoenolpyruvate, 20 μ g of pyruvate kinase, 10mM-mercaptoethanol, 6mM-MgCl₂, 18mM-KCl, 40mM-Tris-HCl buffer, pH7.8, 0.2M-sucrose, ¹⁴C-labelled protein hydrolysate (1.25 μ Ci) and increasing amounts of deacylated tRNA prepared from control or myopathic skeletal muscles. The final reaction volume was 0.25ml. Incubations were carried out at 37°C for 30min.

Incubation system		Radioactivity incorporated (c.p.m.)
pH 5 enzyme	tRNA	
Control		2080
	Control (100 μ g)	2496
	Control (200 μ g)	2750
	Control (300 μ g)	2816
	Myopathic (100 μ g)	2096
	Myopathic (200 μ g)	2117
	Myopathic (300 μ g)	2146
Myopathic		1470
	Control (100 μ g)	1696
	Control (200 μ g)	1810
	Control (300 μ g)	2096
	Myopathic (100 μ g)	1413
	Myopathic (200 μ g)	1500
	Myopathic (300 μ g)	1511

Fig. 2. *Time-course of incorporation of [¹⁴C]ATP and [¹⁴C]CTP into twice-pyrophosphorolysed rat liver tRNA by tRNA nucleotidyltransferases, prepared in parallel from myopathic (●) and control (○) hamster skeletal muscles*

Preparative procedures and reaction conditions were all as described in the Materials and Methods section.

types of tRNA was studied, as catalysed by endogenous enzymes and also by added partially purified nucleotide-incorporating enzyme.

Activity of nucleotide-incorporating enzymes from myopathic and control hamster skeletal muscles

tRNA nucleotidyltransferase was partially purified, by using pH5 enzyme fractions from myopathic and control muscles as starting material, and generally following the procedures of Herrington & Hawtrey (1970b). Twice-pyrophosphorolysed rat liver tRNA (lacking -pC-C-A-OH) was used as substrate. There was no significant difference between the incorporation of labelled ATP (or CTP) catalysed by enzymes from myopathic and control muscle prepared in parallel and containing the same concentrations of protein (Fig. 2). This suggests that the defect in aminoacyl-acceptor activity exhibited by tRNA from myopathic muscle in pH5 enzyme preparations is not the result of a decreased activity of nucleotide-incorporating enzyme in intact muscle cells. The aminoacyl-acceptor status of tRNA isolated from pH5 enzyme from myopathic and control muscle was assessed by measuring its activity before and after partial restitution of -pC-C-A-OH end groups during a preincubation. Deacylated tRNA prepared from both sources, and partially purified aminoacyl-tRNA synthetases and nucleotide-incorporating enzymes from control hamster skeletal muscles, were used as follows. The two tRNA preparations were treated with control nucleotide-incorporating enzyme, CTP and ATP before general aminoacylation was tested with control aminoacyl-tRNA synthetases.

Table 2. *Effect of preincubation with ATP, CTP and tRNA nucleotidyltransferase on the aminoacyl-acceptor activity of tRNA from control and myopathic muscle*

Deacylated tRNA from control and myopathic muscle (50 μ g) was incubated in a total volume of 0.1 ml containing (final concns.): 20 μ g of control partially purified nucleotide-incorporating enzyme, 1 mM-ATP, 1 mM-CTP, 70 mM-glycine buffer, pH 9.0, 5 mM-MgCl₂, 5 mM-phosphoenolpyruvate, 15 μ g of pyruvate kinase, 16 mM-KCl and 0.1 M-sucrose, at 37°C. After 60 min the pH was adjusted to 7.8 and complete mixtures for aminoacylation (described, together with preparative procedures, in the Materials and Methods section) were added to final total volumes of 0.25 ml; each also contained 25 μ g of aminoacyl-tRNA synthetases from control or myopathic muscle as indicated. These were incubated for 15 min at 37°C.

Incubation system		Radioactivity incorporated (c.p.m.)	Inhibition (%)
tRNA	Aminoacyl-tRNA synthetase		
Control*	Control	8486	
Myopathic*	Control	5489	36
Control*	Myopathic	8730	
Myopathic*	Myopathic	5463	36
Control	Control	14012	
Myopathic	Control	11177	18
Control	Myopathic	14937	
Myopathic	Myopathic	11619	18

* Deacylated tRNA incubated for 60 min in the absence only of nucleotide-incorporating enzyme and CTP.

Without preincubation, tRNA from myopathic muscle was about 36% less active than tRNA from control muscle in aminoacyl-acceptor activity (Table 2). However, when 3'-terminal -pC-C-A-OH groups had been added enzymically during preincubation, the impairment in aminoacyl-tRNA formation was only about 18%. Since it is known that the complete -pC-C-A-OH terminus is essential for aminoacylation, the results indicate that some of the tRNA molecules from myopathic muscle are defective in this respect. The finding that the control system also benefited from the preincubation (Table 2) suggests that a substantial, but smaller, deficiency of aminoacyl-acceptor tRNA termini also existed in control pH5 enzyme preparations.

Ribonuclease activity in tRNA nucleotidyltransferase and pH5 enzyme preparations

To detect the presence of nucleases attacking tRNA in the tRNA nucleotidyltransferase-rich fractions from myopathic and control muscles, deacylated tRNA from control muscles was pre-labelled with [¹⁴C]CTP and [¹⁴C]ATP, by using either nucleotide-incorporating enzymes from control or myopathic muscle in separate systems. After removal of the unincorporated radioactive nucleotides, the samples were further incubated to determine the release of ¹⁴C from trichloroacetic acid-precipitable material. In the absence of PP_i such a release was assumed to be due to nucleases, rather than to reverse action

of the nucleotidyltransferase. The results in Fig. 3 show that there was no such nuclease activity in both types of tRNA nucleotidyltransferase, since the degradation was negligible over a period of 120 min.

Fig. 3. *Nuclease activity in tRNA nucleotidyltransferase from myopathic and control muscle*

Control deacylated tRNA molecules lacking the terminal -pC-C-A-OH end group were labelled with [¹⁴C]ATP and [¹⁴C]CTP, by using incorporating enzyme from myopathic (○) or control (●) animals, separated from the free labelled nucleotides by Sephadex filtration, and incubated at 37°C for the time-periods shown, before analysis. A detailed description of these procedures is given in the Materials and Methods section.

Fig. 4. Effects of different pH5 enzymes on the release of [³H]CTP and [¹⁴C]ATP from labelled tRNA

tRNA (150 µg) was incubated at pH 9.0, for 60 min at 37°C, with 60 µg of tRNA nucleotidyltransferase, 16 mM-KCl, 0.1 M-sucrose, 5 mM-MgCl₂, 20 µg of pyruvate kinase, 5 mM-phosphoenolpyruvate, 70 mM-glycine, 2.5 µCi of [¹⁴C]ATP (4.5 nmol) and 5.0 µCi of [³H]CTP (4.5 nmol), in a total reaction volume of 0.5 ml. Labelled tRNA was separated on a Sephadex column from the free labelled nucleotides (see the Materials and Methods section) and divided into four equal amounts containing 9252 c.p.m. (³H) and 3406 c.p.m. (¹⁴C). At zero time, each sample was supplemented with 200 mg of the indicated pH5 enzymes, and incubated at 37°C. The percentage of ³H (----) and ¹⁴C (—) remaining acid-precipitable was determined as outlined in the Materials and Methods section. pH5 enzymes used were (a) from control muscle, prepared without bentonite, (b) from myopathic muscle, prepared without bentonite, (c) from control muscle, treated with bentonite, and (d) from myopathic muscle, treated with bentonite.

The same test was then applied to the two different pH5 enzyme fractions. These were prepared from control and myopathic hamster skeletal muscles in the absence and in the presence of bentonite (5.0 mg/ml of homogenizing medium), relying on the ability of bentonite to absorb general nucleases (Littauer & Sela, 1962), and were incubated with labelled tRNA as in the preceding experiment to determine the rate of release of radioactivity. With pH5 enzyme from control muscle (prepared without bentonite) an unequal rate of removal of terminal labelled adenine and cytidine nucleotides was found (Fig. 4a). However, pH5 enzyme from myopathic muscle (prepared

without bentonite) removed [¹⁴C]adenylate at a proportionally faster rate than it did [³H]cytidylate (Fig. 4b). pH5 enzymes from control muscle, prepared in the presence of bentonite, carried out very little end-group removal (Fig. 4c), making comparison of the rate of removal of ¹⁴C- and ³H-labelled nucleotides difficult. Again, pH5 enzyme from myopathic muscle, prepared in the presence of bentonite (Fig. 4d), catalysed a higher rate of removal of the ¹⁴C- and ³H-labelled nucleotides, and more ¹⁴C label was liberated. This suggested that an active 3'-exonuclease was present in myopathic preparations and also to a lesser extent in controls, although the

results did not permit the specification of the susceptible phosphodiester bonds. Use of more bentonite did not change this pattern of results, suggesting that incomplete removal of endonucleases was not responsible for the differences.

Effect of bentonite treatment on endogenous aminoacylation of tRNA in pH5 enzymes

Aminoacylation of endogenous tRNA was assayed in pH5 enzyme fractions prepared in the presence and in the absence of bentonite, to determine aminoacyl-acceptor activities. Use of bentonite increased the amount of tRNA aminoacylated in both systems derived from myopathic and control muscle respectively (Fig. 5). This indicates that tRNA molecules in both pH5 enzymes prepared in the absence of bentonite lacked aminoacyl-acceptor sites, either because they were already aminoacylated or for other reasons. However, bentonite treatment did not eliminate the impairment in the performance of fractions in aminoacylation from myopathic muscle (–23%), and a proportion of the endogenous tRNA molecules of myopathic muscles were thus still defective, either with respect to the intactness of end-groups or to some other property.

Fig. 5. Effect of bentonite treatment of pH5 enzymes on the subsequent aminoacylation of endogenous tRNA

pH5 enzymes were prepared in the absence and in the presence of bentonite (5.0mg/ml of homogenizing medium). ^{14}C -labelled aminoacyl-tRNA formation, measured as described in the Materials and Methods section, was determined for pH5 enzyme from myopathic muscle prepared in the presence ($\blacktriangle$) and in the absence ($\circ$) of bentonite, and for pH5 enzyme from control muscle prepared in the presence ($\triangle$) and in the absence ($\bullet$) of bentonite.

It is believed that aminoacylation of the 3'-terminal trinucleotide $-\text{pC-C-A-OH}$ sequence of tRNA causes it to become resistant to exonucleolytic attack from the 3'-end (Rosset & Monier, 1963), and an experiment was carried out to study the aminoacylation of endogenous tRNA present in pH5 enzymes from myopathic and control muscles, with and without the addition of CTP, ATP and tRNA nucleotidyltransferase after 40min of incubation (Fig. 6). The regular hyperbolic pattern of aminoacyl-tRNA formation was altered by the addition of CTP, ATP and tRNA nucleotidyltransferase in both systems, more markedly in the system derived from myopathic muscle than in that from control muscle. After 40min of preincubation, the extent of aminoacylation in the control system was 35% greater than that in the myopathic muscle preparation, whereas 20min after the addition of CTP, ATP and the nucleotidyltransferase the difference was only 22%. It again appeared thus that some of the tRNA from myopathic muscle could not be aminoacylated, even in the presence of excess of exogenous CTP, ATP and incorporating enzyme. This is consistent with the failure of preparation in the presence of bentonite, a

Fig. 6. Effect of added ATP, CTP and tRNA nucleotidyltransferase on the aminoacylation of endogenous tRNA from myopathic and control muscle

Reaction conditions for the aminoacylation of endogenous tRNA are given in the Materials and Methods section. After 40min ($\downarrow$) of aminoacylation, tRNA nucleotidyltransferase (20 μg), 1 mM-ATP and 1 mM-CTP were added to each system, before further incubation for 20min. The curves represent: pH5 enzyme from myopathic muscle ($\blacktriangle$); pH5 enzyme from myopathic muscle plus added CTP, ATP and incorporating enzyme ($\triangle$); pH5 enzyme from control muscle without any supplementation ($\bullet$) and with the above additions ($\circ$).

potent ribonuclease inhibitor, to eliminate this difference between the two extracts. A further test of this situation was made by testing the effects of end-group restitution on the defect of pH5 enzyme from myopathic muscle in cell-free polypeptide synthesis, when preparations had been carried out in the presence of bentonite. The pH5 enzyme fractions from myopathic and control hamster skeletal muscles, prepared in the presence of bentonite, were preincubated for 60min at 37°C with and without exogenous CTP, ATP and tRNA nucleotidyltransferase, before being recombined with control polyribosomes to study polypeptide synthesis dictated by endogenous mRNA (Table 3).

When the pH5 enzyme fractions were not preincubated, the pH5 enzyme from myopathic muscle was 30% less active than the control in polypeptide synthesis. Preincubation with CTP, ATP and incorporating enzyme improved the relative performance of the defective pH5 enzyme, but residual impairment was still found.

The endogenous nucleotide-acceptor activities of tRNA from myopathic and control muscle, prepared in the presence of bentonite, were determined with [¹⁴C]ATP, [³H]CTP and partially purified tRNA nucleotidyltransferase (Table 4). tRNA in the pH5 enzyme from myopathic muscle had 20% more nucleotide-acceptor sites than control tRNA. The incorporation of ATP into endogenous tRNA of myopathic muscle was about 16% higher, whereas that of CTP was 21% higher than in the controls. The incorporation of CTP was greater than that of ATP in both systems, and, unexpectedly, the continuing incorporation of CTP was not accompanied by that of ATP. This does not conform to a process where two cytidylates and one adenylate are added sequentially in obligatory order. Contaminating poly(C)

polymerase activity may be responsible (Page *et al.*, 1967).

The possibility that some turnover of -pCpCpA-OH termini occurs *in vitro* has not been excluded, nor has the possible effect of various degrees of aminoacylation of tRNA been taken into account. This remains to be examined in detail.

It appears possible that removal of the trinucleotide end group of tRNA by the hydrolytic action of an

Table 3. *Effect of preincubation, with CTP, ATP and tRNA nucleotidyltransferase, on bentonite-treated pH5 enzymes from myopathic and control muscles in protein synthesis catalysed by control polyribosomes*

pH5 enzymes (0.5mg of RNA) were preincubated with tRNA nucleotidyltransferase (20 µg of protein), 1 mM-ATP, 1 mM-CTP, 5 mM-phosphoenolpyruvate, 50 µg of pyruvate kinase, 16 mM-KCl, 5 mM-MgCl₂ and 0.1 M-sucrose, for 60min or 0min at 37°C. The pH of the above mixture was adjusted to 9.0 with glycine. After the preincubation, control polyribosomes (0.4mg of RNA), 0.6 mM-GTP, 10 mM-mercaptoethanol and ¹⁴C-labelled protein hydrolysate (1.25 µCi) were added and the pH was readjusted to 7.8. The total reaction volume was 0.25 ml. Incubation was carried out for a further 30min at 37°C.

Source of pH5 enzyme	¹⁴ C-labelled amino acids incorporated (c.p.m.)
Control*	7037
Myopathic*	5692
Control	6397
Myopathic	4856

* pH5 enzymes were preincubated.

Table 4. *Endogenous incorporation of labelled ATP and CTP into RNA by pH5 enzymes*

The reaction mixture contained, in 0.25 ml: pH5 enzyme (0.5mg of RNA), tRNA nucleotidyltransferase (30 µg of protein), 5 mM-phosphoenolpyruvate, 50 µg of pyruvate kinase, 16 mM-KCl, 5 mM-MgCl₂, 0.1 M-sucrose, 1.25 µCi of [¹⁴C]ATP (2.25 nmol) and 2.5 µCi of [³H]CTP (2.25 nmol). The pH of the reaction mixture was adjusted to 9.0 with glycine. Incubation was carried out at 37°C. For further details, see the Materials and Methods section.

pH5 enzyme	Incorporation time (min)	Nucleotides incorporated (pmol)	
		[¹⁴ C]ATP	[³ H]CTP
Control	15	5.35	9.93
	30	5.88	16.8
	60	7.22	23.3
Myopathic	15	6.57	12.7
	30	7.25	20.7
	60	8.49	29.4

Table 5. *Incorporation of different nucleotides into pH5 enzymes prepared in the presence of bentonite from myopathic hamster skeletal muscles*

The reaction mixture contained in 0.25 ml: pH5 enzyme (0.5 mg of RNA), tRNA nucleotidyltransferase (30 μ g of protein), 5 mM-phosphoenolpyruvate, 50 μ g of pyruvate kinase, 16 mM-KCl, 5 mM-MgCl₂, 0.1 M-sucrose, with the following combinations of labelled nucleotides: (i) 2.5 μ Ci of [³H]CTP (4.5 nmol) and 0.5 μ Ci of [¹⁴C]GTP (10 nmol); (ii) 2.5 μ Ci of [³H]CTP (2.25 nmol) and 1.0 μ Ci of [¹⁴C]UTP (2.12 nmol); (iii) 2.5 μ Ci of [³H]CTP (2.25 nmol) and 5.0 μ Ci of [¹⁴C]ATP (9.0 nmol). The pH of the above mixture was adjusted to 9.0 with glycine. Incubations were carried out at 37°C for the time-periods shown. For further details, see the Materials and Methods section.

Time of incorporation (min)	Nucleotides incorporated (pmol)	
	[³ H]CTP	[¹⁴ C]GTP
15	80.8	64.4
30	127.0	90.7
60	184.0	113.0
	[³ H]CTP	[¹⁴ C]UTP
15	46.1	7.61
30	73.9	9.76
60	102.0	12.8
	[³ H]CTP	[¹⁴ C]ATP
15	38.1	26.1
30	59.9	27.2
60	85.9	27.3

exonuclease could be accompanied by the removal of another base or bases further along the RNA strand. This could involve the adenylate or guanylate residue always adjacent to the double-stranded region formed by complementary base-pairing between the 5'- and 3'-strands. An experiment was accordingly designed to find out whether only ATP and CTP were incorporated. pH5 enzymes, prepared in the presence of bentonite, were incubated with different combinations of ATP, CTP, UTP and GTP. Somewhat surprisingly, not only ATP and CTP, but also the other nucleotides, were incorporated (Table 5). The rates of incorporation, however, differed; in the presence of CTP, GTP was extensively incorporated, but when GTP was replaced by UTP, little incorporation of the latter was observed. In the presence of CTP, ATP was incorporated, but again not to the same extent as CTP, or for as long. These results suggested that the preparations used contained other enzymes catalysing the incorporation of nucleotides into acid-precipitable material. On the other hand, when [¹⁴C]ATP was incubated in the absence and in the presence of CTP and GTP, with pH5 enzyme from myopathic muscle as source of endogenous tRNA (Fig. 7), ATP was only incorporated at a high rate when CTP was present, as would be expected if there were a specific sequence of nucleotide addition. It follows that the major part of the incorporation of these two nucleotides was contributed by additions at the 3'-termini of tRNA molecules by the relevant transferase.

A relatively small proportion of the tRNA species from myopathic muscle appears to have lacked the 3'-terminal adenosine group, and a very high proportion lacked the adjacent cytidylates in addition. The small difference between ATP incorporation into tRNA when either CTP alone or both CTP and GTP were present did not support or refute the idea that guanylate residues may have been incorporated into defective 3'-termini of tRNA molecules, thus permitting the subsequent addition of cytidylate and adenylate units. Previous studies of tRNA nucleotidyltransferase had not dealt with this possibility, because of the choice of substrates (Deutscher, 1972a,b,c).

Discussion

tRNA from myopathic muscle appears to be qualitatively and quantitatively defective, and this fact is responsible for its impaired efficiency in supporting polypeptide synthesis on heterologous and homologous polyribosomes (Bester & Gevers, 1973).

The best explanation for our findings appears to be the presence of a 3'-exonuclease activity in myopathic muscle which attacks tRNA molecules in such a way that they remain substrates for tRNA nucleotidyltransferase within the limits of the latter's substrate specificity, and cease to be substrates when the hydrolysis proceeds beyond a certain stage. This cannot involve a ribonuclease I of the pancreatic type, because the exposed pyrimidine 3'-phosphate

Fig. 7. Incorporation of [^{14}C]ATP into endogenous tRNA in the presence of other nucleotides

The reaction mixtures contained in 0.25 ml: pH 5 enzyme (0.5 mg of RNA), tRNA nucleotidyltransferase (30 μg of protein), 5 mM-phosphoenolpyruvate, 30 μg of pyruvate kinase, 16 mM-KCl, 5 mM-MgCl₂, 0.1 M-sucrose, 5.0 μCi of [^{14}C]ATP (40 nmol), CTP (200 nmol) and GTP (200 nmol). The pH was adjusted to 9.0 with glycine. Incubations were carried out at 37°C. For further experimental details see the Materials and Methods section. Incorporation of [^{14}C]ATP into endogenous tRNA from myopathic muscle in the absence of CTP and GTP (○), in the presence of CTP (●), in the presence of GTP (△), and in the presence of CTP and GTP (▲), is indicated.

termini would not be substrates for tRNA nucleotidyltransferase. Again it would not be surprising that such an enzyme would fail to be absorbed by bentonite, because of its exonuclease nature. This enzyme would also be expected to leave in its wake a heterogeneous population of tRNA molecules lacking either one, two, three or more nucleotides at the 3'-terminus, rather than the trinucleotide sequence as a unit. It is possible, but not necessary, that the exonuclease attack would cease when double-stranded regions of RNA were reached; in tRNA this occurs after the fourth nucleotide, which is always an adenylate or guanylate residue (Philipps, 1969).

It is significant that Herrington & Hawtrey (1969) described the defectiveness of tRNA from non-lactating mammary glands to a nuclease activity present largely in the partially purified tRNA nucleotidyltransferase fraction, prepared by almost exactly the same method we have used. However, our pre-

parations from both control and myopathic muscle had little activity in the removal of [^{14}C]adenylate and [^{14}C]cytidylate residues from tRNA. On the other hand, an activity of this type was detectable in the unresolved pH 5 enzyme fractions from both sources of muscle, which was higher in the myopathic muscle, irrespective of whether bentonite was used during the preparations.

The problem now arises as to whether the tRNA from myopathic muscle becomes defective after isolation from the animals as the result of the activity of some process in the extracts, or whether the molecules are already defective *in vivo*. Preparation of pH 5 enzyme fractions from both types of animals in the presence of the ribonuclease-absorbent bentonite leads to an increased capacity of endogenous tRNA to accept amino acids in both control and myopathic animals, but does not affect the difference between the two. Similarly the defect of myopathic muscle in polypeptide synthesis is still evident when bentonite is used during the preparation. This suggests, but does not prove, that the biochemical lesion is already present before the cells are homogenized.

Our results therefore suggest that cell-free protein synthesis in preparations from myopathic hamster muscles is limited by the supply of intact tRNA molecules to a much greater extent than in those from controls. This is because a large number of the tRNA molecules lack either part or the whole of the necessary trinucleotide aminoacyl-acceptor terminus, or because they are damaged in some other way. The most likely reason for this is the activity of a 3'-exonuclease, which appears to be active in the soluble preparations from myopathic muscle. Whether the situation as found in our extracts applies to the intact myopathic muscle cells remains to be established, as does the relationship of these defects to the primary autosomally recessive genetic defect, and to the failure of affected muscle cells to attain normal functional status.

These studies were supported by grants from the Harry Crossley Foundation, the Association of South African Round Tables and the South African Atomic Energy Board.

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