Protein Synthesis in Muscles from Normal and Dystrophic Hamsters

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Homogenates of hindleg muscle were obtained from control and dystrophic male hamsters, 30 and 190 days of age, and were used to prepare the postmicrosomal pH 5-supernatant fraction. The activity of this fraction in the incorporation of [14 C]phenylalanyl-tRNA into peptides was increased in the dystrophic-muscle preparations. No such increase was found in brain or liver preparations from dystrophic hamsters. The increased capacity for aminoacyl-tRNA binding that was observed in preparations from dystrophic animals is discussed.

The Bio 14.6 strain of hamsters bearing hereditary myopathy and cardiomyopathy are frequently said to have muscular dystrophy. The dystrophy in hamsters, according to some workers, may resemble the Duchenne dystrophy of humans and the muscular dystrophy in the ReJ 129 dy/dy strain of mouse (Homburger *et al.*, 1966; Michelson *et al.*, 1955). In the dystrophic mouse there is a marked decrease in the weight of the hindleg muscle (Rowe & Goldspink, 1969*a,b*; Petryshyn & Nicholls, 1976), and a difference from the controls in the muscle enzyme activities (Pennington, 1963) and muscle tension (Harris & Wilson, 1971; Montgomery, 1975*a*). However, these changes were not found in dystrophic hamsters (Bradley & Jenkison, 1973; Montgomery *et al.*, 1975; Montgomery, 1975*b*).

In the wasted hindlimb of dystrophic mice, studies of protein synthesis *in vivo* have revealed little change except for an increased turnover of two soluble proteins, creatine kinase and adenylate kinase (Kitchin & Watts, 1973). However, cell-free preparations of muscle of dystrophic mice were reported to exhibit an increase in ribosomal activity together with a decrease in the labelling of myosin in polyribosomes translating the mRNA for this protein (Nwagwu, 1975; Srivastava, 1972). In our laboratory a proteinaceous inhibitor of peptide elongation was purified from the postmicrosomal-supernatant fraction of the muscle of dystrophic mice and accounted for a marked decrease in the capacity of cell-free preparations to carry out the elongation of peptides (Petryshyn & Nicholls, 1976; Petryshyn, 1977; Petryshyn & Nicholls, 1978).

In dystrophic hamsters, whole cell preparations of leg muscle, incubated *in vitro*, exhibited an increased incorporation of labelled amino acids into

soluble proteins and into actomyosin at both 60 days and 180–240 days of age. Similar changes in heart and diaphragm occurred in the older age group (Lochner *et al.*, 1971). Our results after the administration of labelled amino acids *in vivo* confirm these observations (Saleem *et al.*, 1978). Moreover, in the hypertrophied diaphragm of dystrophic hamsters there was a marked increase in synthesis and a smaller increase in degradation of total proteins (Goldspink & Goldspink, 1977). In newborn hamsters, Wrogemann *et al.* (1977) studied the incorporation *in vivo* of labelled leucine into the soluble proteins of liver and found an increased synthesis of haemoglobin in dystrophic hamsters younger than 3 days and a decreased synthesis in 6-day-old dystrophic hamsters. Bester & Gevers (1973*a,b*) obtained evidence for a defective tRNA, demonstrable in embryonic, 60-day-old and 180-day-old dystrophic hamsters, which appeared to result from a 3'-exonuclease activity and which limited cell-free protein synthesis in several muscle types, in brain and in liver of the dystrophic animals.

The results of Bester & Gevers (1973*b*), taken together with the results of the whole-cell studies cited above, suggested that *in vivo* some mechanism in the intact cell prevents the action of the exonuclease and the consequent production of the defective tRNA species. Because of the reported differences between dystrophic mice and dystrophic hamsters, it seems unlikely that cell-free preparations of leg muscle from dystrophic hamsters contain an inhibitor of elongation such as we have isolated from dystrophic mice (Petryshyn & Nicholls, 1976, 1978). In order to examine the activity of that cytosol fraction which contains most of the elongation-factor activity for peptide synthesis (Girgis & Nicholls, 1972), and to avoid the results of any liberated nuclease activity, we have examined the capacity of the pH 5-supernatant fraction for elongation of peptides in 30- and 190-day-old dystrophic hamsters.

Abbreviations used: EF 1, elongation factor 1; EF 2, elongation factor 2.

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Materials and Methods

Chemicals

L-[U- 14 C]Phenylalanyl-tRNA (specific radioactivity 385 Ci/mol, 0.156 μ Ci/mg of tRNA) containing 19 other unlabelled aminoacyl-tRNA species from *Escherichia coli* strain B was obtained from New England Nuclear, Boston, MA, U.S.A. Other chemicals were obtained as previously described (Girgis & Nicholls, 1972; Young & Nicholls, 1978).

Animals

Normal (strain RB) and dystrophic (strain Bio 14.6) male Syrian hamsters, 30 and 190 days of age, were obtained from the Trenton Experimental Laboratory Animal Co., Bar Harbor, ME, U.S.A., and were allowed free access to water and fox cubes (Teklad Inc., Monmouth, IL, U.S.A.) before the experiment. Male rats of the Wistar strain, weighing 180–220 g, had free access to water and the same food.

At the time of the experiment the hamsters were decapitated and the tissues (liver, brain and hindleg muscle) rapidly removed, dissected free of connective tissue, weighed and then placed in ice-cold buffer. Liver was the only tissue obtained from the rats.

Preparation of microsomal and postmicrosomal supernatant fractions

All work was performed on ice at 2–4°C. The tissues were homogenized in a glass homogenizer with 12–15 passes of a Teflon pestle in 4 vol. of buffer I containing 0.25 M-sucrose, 50 mM-Tris/HCl (pH 7.8 at 22°C), 80 mM-KCl, 6 mM-MgCl₂ and 10 mM-2-mercapto-ethanol. For muscle, the tissue was treated for 30 s in a mixer (model SDT, Tekmar, Cincinnati, OH, U.S.A.) before the homogenization step. Muscle homogenates were filtered through several layers of gauze to remove debris. Tissue homogenates were centrifuged at 12000g for 10 min at 2°C in a Spinco type 40 rotor (r_{av} , 5.9 cm). The resulting mitochondrial pellet was discarded and the postmitochondrial supernatant (S 15) was centrifuged at 105000g for 90 min to sediment the microsomal fraction. For rat liver, the microsomal fraction was used to prepare preincubated salt-washed ribosomes as described by Girgis & Nicholls (1971). The postmicrosomal-supernatant fraction (S 105) obtained from the 90 min centrifugation step above was stored at –20°C or was used for the preparation of the pH 5-supernatant fraction as described previously (Petryshyn & Nicholls, 1976).

Preparation of EF 1 from rat liver

The pH 5-supernatant fraction of liver obtained from normal rats was treated as described previously with heat and *N*-ethylmaleimide to remove the activity of EF 2 (McKeehan & Hardesty, 1969; Petryshyn & Nicholls, 1976).

Determination of protein and radioactivity

Protein concentration was measured in duplicate tubes by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard, or as previously described in preparations freed of RNA (Girgis & Nicholls, 1972). RNA was determined from the A_{260} ($A_{260}^{1\%} = 230$). The incorporation of labelled amino acids into peptide was measured in duplicate or triplicate tubes after incubation as described in the legend to Fig. 1 and after precipitation of the protein by 10% (w/v) trichloroacetic acid, heating in 5% trichloroacetic acid at 90°C for 20 min, and washing as described by Girgis & Nicholls (1971). The protein was solubilized in 1.0 ml of Protosol (obtained from New England Nuclear) and transferred to scintillation vials with scintillation fluid for radioactivity counting in a Nuclear-Chicago model 6860 liquid-scintillation spectrometer at 85% counting efficiency. Activity was expressed as d.p.m./mg of RNA. Samples were corrected by subtraction of values for non-incubated tubes, which gave approx. 100 d.p.m. per tube and which were similar to those for tubes lacking either ribosomes or supernatant fraction. The results shown are representative of those obtained in three or more separate experiments for each different age group by using dystrophic hamsters and age-matched control hamsters.

Results

In the dystrophic hamsters aged 190 days, no statistically significant differences were noted in the body weight and muscle weight, nor in the protein concentrations of the postmicrosomal supernatant and pH 5-supernatant fractions (Table 1). Similarly, the 30-day-old dystrophic animals showed no change in weight or in protein concentration of the soluble cytosol fractions.

Table 1. *Protein concentration of the postmicrosomal-supernatant fractions of the hindleg muscle of dystrophic hamsters*

The hindleg-muscle fractions of 190-day-old hamsters were prepared as described in the Materials and Methods section. The protein concentration of duplicate samples was determined by the method of Lowry <i>et al.</i> (1951). Values are means $\pm$ S.E.M. for four pairs of control and dystrophic hamsters. <i>P</i> values by Student's <i>t</i> test were not significant at <0.05 level.		
	Control	Dystrophic
Body wt. (g)	108 $\pm$ 6.6	98.5 $\pm$ 7.4
Muscle wt. (g)	7.45 $\pm$ 0.37	6.72 $\pm$ 0.74
Protein concn. of S 105 fraction (mg/100 mg)	10.2 $\pm$ 0.61	10.1 $\pm$ 0.63
Protein concn. of pH 5-supernatant fraction (mg/100 mg)	6.35 $\pm$ 1.04	5.80 $\pm$ 0.91

Fig. 1. Incorporation of [14 C]phenylalanyl-tRNA into peptide by the postmicrosomal-supernatant fraction (S 105) obtained from (a) 30- and (b) 190-day-old dystrophic hamsters

The postmicrosomal-supernatant fractions were prepared as described in the Materials and Methods section. Incubation was for 10 min at 37°C in duplicate tubes with various concentrations of S105 fraction in 0.40 ml of medium containing 50 mM-Tris/HCl (pH 7.8 at 25°C), 80 mM-KCl, 6 mM-MgCl₂, 10 mM-2-mercaptoethanol, 0.4 mM-GTP, [14 C]phenylalanyl-tRNA (13700 d.p.m., 33 μg of tRNA), excess salt-washed rat liver ribosomes (50 μg of RNA) and 100 μg of poly(U). The incubation was terminated, the protein precipitated, washed and counted for radioactivity as described in the Materials and Methods section. Incorporation is expressed as $10^{-3} \times$ d.p.m./mg of RNA and values were corrected for unincubated blanks as described in the Materials and Methods section. ●, Control; ○, dystrophic hamsters.

When the incorporation of [14 C]phenylalanyl-tRNA into peptide was measured in the postmicrosomal (Fig. 1) and pH 5-supernatant fractions (Fig. 2) of the dystrophic hamsters, an increase was always observed relative to the control hamsters when low concentrations of the cytosol fractions were used. This was found both for the 30-day-old dystrophic hamsters (Figs. 1a and 2a) and for the 190-day-old dystrophic animals (Figs. 1b and 2b).

This increase in dystrophic animals occurred in the presence of an excess of normal rat liver ribosomes and of other reactants. It was evident at limiting concentrations of the soluble cytosol fractions from dystrophic animals, but not when excess amounts were present in the incubation mixture, suggesting that control preparations are deficient in some reactant for peptide elongation. Preparations of pH 5-supernatant fractions obtained from the liver or brain of the dystrophic animals, however, showed the same incorporation as the controls at limiting concentrations (Figs. 3a and 3b). Fig. 4 shows the kinetics of the reaction when limiting amounts of muscle pH 5-supernatant fraction were used. The rate and extent of the reaction remained greater with the pH 5-supernatant fractions from dystrophic animals. In all of these experiments, the young dystrophic animals resembled the 190-day-old hamsters. Moreover, when hamsters of an intermediate age (60, 90, 130 and 170 days of age) were studied, the muscle supernatant fractions of the dystrophic animals exhibited an increased capacity for peptide synthesis relative to the control hamsters. Muscle preparations from dystrophic female hamsters (190 days of age) similarly exhibited an increased peptide synthesis compared with female controls.

Because of the possibility that different amounts of Ca²⁺ present in the dystrophic-muscle preparation might influence incorporation, the Ca²⁺ was removed by the addition of the calcium chelator, EGTA. The addition of EGTA to the reaction mixture up to a concentration of 1.0 mM did not change the increase in [14 C]phenylalanyl-tRNA incorporation observed with the dystrophic-muscle preparations. Furthermore, it should be stressed that the volume of the supernatant fractions was usually less than 5% of the incubation volume, so that ionic changes in the muscle preparations are unlikely to affect the incorporation.

Increasing the GTP concentration from 0.2 to 1.0 mM in the presence or absence of 10 mM-2-mercaptoethanol, had no effect on the difference between the dystrophic-muscle and control preparations. Thus the increased incorporating activity of the dystrophic-leg-muscle preparations cannot be due to a decreased activity of a GTP- and thiol-sensitive inhibitor such as reported for rat liver (Scornik *et al.*, 1967; Rupniak & Quincey, 1973). Since excess amounts of [14 C]-phenylalanyl-tRNA, ribosomes and poly(U) were used in the reaction mixture, it is most unlikely that differences in ribonuclease activity, should they occur, could explain the difference between control and dystrophic-muscle preparations.

Because of the possibility that the dystrophic-muscle preparation might contain decreased amounts of inhibitory substances or increased amounts of activating substances of low molecular weight, the pH 5-supernatant fractions of dystrophic and control

Fig. 2. Incorporation of $[^{14}\text{C}]$ phenylalanyl-tRNA into peptide by the pH 5-supernatant fraction from the hindleg of control and dystrophic hamsters. Incubation and other details were as for Fig. 1. (a) 30-day-old hamsters; (b) 190-day-old hamsters. ●, Control; ○, dystrophic hamsters.

Fig. 3. Incorporation of $[^{14}\text{C}]$ phenylalanyl-tRNA into peptide by the pH 5-supernatant fraction of (a) brain and (b) liver. Incubation and other details were as for Fig. 1. Hamsters were 190 days old. ●, Control; ○, dystrophic hamsters.

muscle were subjected to gel filtration on Sephadex G-25. The $[^{14}\text{C}]$ phenylalanyl-tRNA incorporation remained higher in the dystrophic-muscle preparations than it was in the control preparations, making

it most unlikely that the difference was due to ions, small protein molecules or other substances of low molecular weight.

Previously Bester & Gevers (1973a,b) observed a

Fig. 4. Kinetics of [^{14}C]phenylalanyl-tRNA incorporation into polyphenyl-alanine

Incubation and other details were as for Fig. 1, except that 300 μg of pH 5-supernatant fraction was used. Hamsters were 190 days old. ●, Control; ○, dystrophic hamsters.

defective tRNA in cell-free preparations of dystrophic hamster muscle, believed to result from the release of exonuclease activity during preparation of the pH 5-enzyme fraction. This fraction contains tRNA and synthetases, and is also used as a source of EF 1 and EF 2, although most of the elongation-factor activity remains in the pH 5-supernatant fraction. In the experiments described above, an excess of exogenous charged tRNA was used in the incubation mixture, so that only elongation-factor activity (rather than elongation activity plus charging activity) was measured and found to be increased in the dystrophic-muscle preparations. When the postmicrosomal-supernatant (S 105) fraction, which contains tRNA, synthetases and EF 1 and EF 2, was incubated with [^{14}C]phenylalanine, rather than with [^{14}C]phenylalanyl-tRNA, the increase was no longer present in the dystrophic-muscle preparations. This result would be expected if an exonuclease acting on tRNA species is liberated during the preparation of the homogenate fractions from dystrophic muscle and results in a defective charging of tRNA.

Previous results in this laboratory showed that EF 1, which catalyses the binding of aminoacyl-tRNA to ribosomes, is limiting relative to EF 2 in cytosol preparations of liver, kidney, brain, cardiac muscle and skeletal muscle (Girgis & Nicholls, 1972;

Fig. 5. Binding of [^{14}C]phenylalanyl-tRNA to ribosomes in pH 5-supernatant fraction obtained from the hindleg muscle of control and dystrophic hamsters

An excess of salt-washed preincubated ribosomes (100 μg of RNA) from rat liver were incubated for 7 min at 37°C with various amounts of pH 5-supernatant protein obtained from muscle of 30-day old hamsters. The incubation mixture had a final volume of 0.4 ml and contained 0.25 M-sucrose, 50 mM-Tris/HCl buffer (pH 7.8), 80 mM-KCl, 0.4 mM-GTP, 6 mM-MgCl₂, [^{14}C]phenylalanyl-tRNA (13700 d.p.m.) and 4 mM-sodium fusidate as described by Malkin & Lipmann (1969). The reaction was terminated by adding ice-cold buffer I (see the Materials and Methods section), and the samples were washed with three portions of buffer on Millipore filters and counted for radioactivity as described (Young & Nicholls, 1978). Blanks containing all the reactants except supernatant were subtracted. (a) Effect of pH 5-supernatant concentration; (b) the pH 5-supernatant (80 μg of protein) was supplemented with elongation factor prepared from control rat liver. ●, Control; ○, dystrophic hamsters.

Petryshyn & Nicholls, 1976; Petryshyn *et al.*, 1977). Similar experiments showed this to be the case in control and dystrophic hamster muscle (results not shown). The binding activity of EF 1 was measured directly in the presence of fusidic acid as described by Malkin & Lipmann (1969), in the control and dystrophic hamster leg-muscle preparations (Fig. 5a), and was markedly greater in the dystrophic-muscle preparation. A preparation of EF 1 that had been freed of EF 2 activity was added to the binding reaction. When EF 1 was added to a limiting concentration of control pH 5-supernatant fraction, the binding reaction was increased to extents above that of the unsupplemented dystrophic-muscle preparation (Fig. 5b). When EF 1 was added to the same limiting concentration of pH 5-supernatant fraction from dystrophic muscle, the binding, which was greater initially, was further increased. Additional high concentrations of proteins in the binding assay (80 µg of pH 5-supernatant fraction and over 200 µg of EF 1 preparation, together with 100 µg of ribosomal protein) overload the filters. Thus it was not possible to increase the binding of the control preparation to the value for the supplemented dystrophic-muscle preparation. Binding was always greater in the dystrophic hamsters regardless of age (30–190 days).

Since the increase in EF 1 activity in the pH 5-supernatant fraction from dystrophic animals might be due to the absence of an inhibitor (normally present in control muscle), or to the presence of an activator, experiments were carried out combining various amounts of pH 5-supernatant fractions from control and dystrophic animals prepared by passage through a Sephadex G-25 gel-filtration step. The incorporation of [¹⁴C]phenylalanyl-tRNA into peptide by using various amounts of mixed pH 5-supernatant fractions (control plus dystrophic) was similar to the theoretical value. Similar results were also found before passage of the preparations through Sephadex G-25. These observations do not provide evidence that inhibitors or activators are involved in the increased incorporating capacity of the dystrophic-muscle preparation.

Discussion

The results obtained here with the hindleg muscle of dystrophic hamsters contrast markedly with those reported previously for dystrophic mice, where the cytosol fractions showed a decreased capacity for protein synthesis which resulted from a high-molecular-weight cytosolic protein that was inhibitory to peptide elongation (Petryshyn & Nicholls, 1976, 1978; Petryshyn, 1977). In dystrophic hamsters, the results obtained with the cell-free preparations resemble those obtained in studies with whole cells

and whole animals (Lochner *et al.*, 1971; Goldspink & Goldspink, 1977; Saleem *et al.*, 1978), where an increased synthesis of proteins was reported.

By using exogenous phenylalanyl-tRNA in the protein-synthesis assays, the possibility of nuclease modification of endogenous tRNA, such as proposed by Bester & Gevers (1973b), has been avoided. Thus, although a defective tRNA may be present in homogenates, owing perhaps to the release of an exonuclease during homogenization of the leg muscle (Bester & Gevers, 1973b), the provision of excess [¹⁴C]phenylalanyl-tRNA in the reaction mixture and the removal of the tRNA and synthetases by the pH 5 precipitation step makes it possible to assay the elongation-factor activity of the cytosol.

The increase in peptide elongation in dystrophic hamster muscle was found in all of the preparations studied, regardless of the age or sex of the animals, and irrespective of prior Sephadex G-25 gel filtration. The latter step was to remove substances of low molecular weight that might affect peptide synthesis, e.g. Ca²⁺, GTP and thiol agents.

In some regards, the results for dystrophic hamsters resemble those for breast muscle of dystrophic chickens, where an increased elongation was observed. However, the increase in chicks was due primarily to increased EF 2 activity and only secondarily to EF 1 activity (Weinstock & Markiewicz, 1974). On the other hand, in the heart of 75-day-old dystrophic mice, we have found that the increase in peptide synthesis that occurs in the postmicrosomal-supernatant fractions is the result of an increase in EF 1 activity (Petryshyn *et al.*, 1977). The results obtained here with dystrophic hamster skeletal muscle closely resemble the results with dystrophic mouse heart.

By using a heterologous system containing an excess of all of the other factors required for peptide synthesis, the capacity of the pH 5-supernatant fraction can be tested. The increased incorporation and binding by phenylalanyl-tRNA with the dystrophic-muscle preparations could result either from changes in the amounts or activity of EF 1 or EF 2 or from changes in factors affecting the activity of EF 1 or EF 2, since initiation factors are not required for this test system (Capon & Nicholls, 1974). Since EF 1 is limiting, relative to EF 2, and since supplementation of the control preparation with EF 1 was able to elevate the incorporation to that of the dystrophic-muscle preparations, the effect was localized to the EF 1 step. Such supplementation studies have been used by a number of workers as well as ourselves (Girgis & Nicholls, 1971; Hassell & Engelhardt, 1976; Smith *et al.*, 1976). Indeed the EF 1-dependent binding capacity of the dystrophic-muscle preparations was also greater when it was measured directly, and supplementing the control preparation brought the binding to the value in the dystrophic-muscle preparation.

Similar observations of increased peptide synthesis and aminoacyl-tRNA binding have been made for liver (Cappon & Nicholls, 1974; Nicholls *et al.*, 1977) and for kidney (Girgis & Nicholls, 1971; Nicholls *et al.*, 1975) undergoing hypertrophy and hyperplasia. For liver, the EF 1 was purified extensively and an increased reactivity of the low-molecular-weight form (EF 1_L), but not the high-molecular-weight form (EF 1_H), detected (Young & Nicholls, 1978). Changes in the relative proportion of the low-molecular-weight form, the species involved in aminoacyl-tRNA binding, have been observed during development (Slobin & Möller, 1976; Bolla & Brot, 1975), and changes in the specific activity of fractions containing EF 1 have been reported in the liver of various species after different stimuli (Nicholls *et al.*, 1974; Liu *et al.*, 1974; Bolla *et al.*, 1975; Smith *et al.*, 1976; Nielsen & Haschemeyer, 1977).

The purification of EF 1 from muscle of control and dystrophic hamsters will have to be carried out in order to see whether there are changes in the ratio of the heavy to light forms and/or whether there are changes in the specific activity or quantity of EF 1. Tightly bound activators (or inhibitors) may occur in muscle EF 1 such as those reported in liver EF 1 (Iwasaki *et al.*, 1973; Kotsiopoulos & Mohr, 1975). Another possibility might be that in dystrophy there is an increased turnover (i.e. synthesis and degradation) of EF 1 that results in a more reactive form of EF 1. An increased turnover of muscle proteins has been found in the diaphragm of dystrophic hamsters (Goldspink & Goldspink, 1977) at 100 and 230 days of age, but not at 30 days of age. In the present experiments an increased elongation of peptides has been detected in cell-free preparations obtained from the leg muscle of the dystrophic animals studied (30–190 days old), suggesting that, as with diaphragm and other tissues, fibre regeneration is accompanied by an increased capacity for peptide synthesis.

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