Effects of starvation, diabetes and acute insulin treatment on the regulation of polypeptide-chain initiation in rat skeletal muscle

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The rate of protein synthesis in skeletal muscle is greatly decreased in response to diabetes and starvation. Analysis of polyribosome profiles indicates that polypeptide-chain initiation is impaired under these conditions. To identify the step in initiation that is affected, we assayed the incorporation of [³⁵S]methionyl-tRNA^{fMet} into [³⁵S]methionyl-tRNA^{fMet}-40S-ribosomal-subunit initiation complexes in cell-free extracts based on postmitochondrial supernatants prepared from gastrocnemius muscle. Extracts from either starved or diabetic rats were 30–40% less active in forming these complexes compared with those derived from fed or insulin-maintained controls respectively. This change could be reversed by treatment of either starved or diabetic rats with insulin *in vivo* 30 min before death. Formation of 40S initiation complexes by extracts from either fed or starved rats could be stimulated by the addition of exogenous purified initiation factor eIF-2, but extracts from starved or diabetic rats were more sensitive than controls to stimulation by low concentrations of the factor. These results provide evidence for the acute regulation by insulin of protein synthesis in skeletal muscle at the level of polypeptide-chain initiation, and suggest that in this tissue, as in certain other eukaryotic systems, control of initiation appears to be mediated by changes in the activity of initiation factor eIF-2.

There is now much evidence from studies *in vivo* and *in vitro* that a decrease in the rate of skeletal-muscle protein synthesis is an important component of the changes in protein metabolism after starvation (Rannels *et al.*, 1978) and induction of diabetes (Pain & Garlick, 1974; Flaim *et al.*, 1980). In both cases this inhibition has two components: a lower overall capacity to synthesize proteins, owing to a decrease in tissue ribosome content, and a fall in the rate of protein synthesis per ribosome (Henshaw *et al.*, 1971; Pain & Garlick, 1974; Flaim *et al.*, 1980). The latter effect is the more rapid in onset, can be reversed by acute treatment with insulin of muscle from either starved (Li *et al.*, 1979) or diabetic (Flaim *et al.*, 1980) rats, and has

been interpreted in terms of an inhibition of polypeptide-chain initiation on the basis of changes in the proportion of ribosomes in polysomes. In the perfused hemicorpus preparation, skeletal-muscle protein synthesis declined after the first 1 h of perfusion, in parallel with an accumulation of inactive ribosomes, presumably at the expense of polysomes (Jefferson *et al.*, 1977). These changes were prevented if insulin was included in the perfusion medium.

In other eukaryotic systems studied, peptide-chain initiation is regulated at the level of eIF-2-dependent formation of [40S·Met-tRNA^{fMet}] complexes. The shut-off of protein synthesis in reticulocyte lysates deprived of haem is preceded by a decrease in the extent of binding of Met-tRNA^{fMet} to 40S subunits (Legon *et al.*, 1973). Similarly, in cultured Ehrlich ascites-tumour cells, deprivation of an essential amino acid results in a rapid fall in the number of native 40S subunits bearing Met-tRNA^{fMet} (Pain & Henshaw, 1975). These defects can be reversed by the addition of exogenous eIF-2

Abbreviations used: eIF-2, eukaryotic initiation factor 2; [40S·Met-tRNA^{fMet}], 40S ribosomal subunit·Met-tRNA^{fMet} initiation complex.

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to cell-free extracts of either reticulocytes (Clemens *et al.*, 1974) or Ehrlich cells (Pain *et al.*, 1980). In reticulocytes, inhibition of initiation correlates closely with activation of a protein kinase, which phosphorylates the α -subunit of eIF-2 (Ranu & London, 1976; Farrell *et al.*, 1977). This covalent modification results in the loss of the ability of eIF-2 to exchange GDP for GTP and hence to recycle between successive rounds of protein synthesis (Clemens *et al.*, 1982; Siekierka *et al.*, 1982).

The mechanism of control of initiation of protein synthesis in skeletal muscle is, in contrast, poorly understood. eIF-2 activity of postribosomal supernatants from psoas muscle, assayed by ternary-complex formation, has been reported to be unaffected by starvation when the results are expressed relative to the total muscle RNA concentration (Rannels *et al.*, 1978). In the present paper we show that the formation of [40S-Met-tRNA^{fMet}] complexes is impaired in postmitochondrial supernatants from starved or diabetic animals, and that this effect is reversed by acute insulin administration *in vivo* or by addition of eIF-2 *in vitro*.

Materials and methods

Materials

[³⁵S]Methionine (500–1000 Ci/mmol) was obtained from Amersham International (Amersham, Bucks., U.K.). Calf liver tRNA was from Boehringer (Lewes, E. Sussex, U.K.). GTP, ATP, phosphocreatine, streptozotocin and bovine pancreatic insulin were from Sigma Chemical Co. (Poole, Dorset, U.K.) and protamine–zinc insulin was obtained from Wellcome (Beckenham, Kent, U.K.). Cetyltrimethylammonium bromide was purchased from BDH Chemicals (Poole, Dorset, U.K.). Glass-fibre filters (GF/C) were a product of Whatman (Maidstone, Kent, U.K.).

[³⁵S]Methionyl-tRNA^{fMet} was prepared by using *Escherichia coli* aminoacyl-tRNA synthetases (a gift from Dr. M. J. Clemens, Department of Biochemistry, St. George's Hospital Medical School, London) as described by Clemens *et al.* (1974). Rat liver eIF-2 was prepared as described by Proud *et al.* (1982) and was approx. 90% pure.

Experimental animals

Male Sprague–Dawley rats (110–130 g) were provided with food (Spratts, Croydon, U.K.) and water *ad libitum*. Starved rats were deprived of food at 09:00–11:00 h and kept in wire-bottomed cages for 48 h before death. For experiments on the effects of diabetes, rats were made diabetic on day 0 by intraperitoneal injection of streptozotocin (12–13 mg/100 g body wt.) dissolved in 10 mM-sodium citrate, pH 5.0, and were injected subcu-

taneously with 2 units of protamine–zinc insulin on day 1. They were then divided into two groups. Control (insulin-maintained) rats were given a further 3 units of insulin/day on days 2–6, whereas animals for the diabetic group were given 3 units of insulin/day on days 2–4 and were then withdrawn from insulin on days 5 and 6. All injections were between 09:00 and 11:00 h, and animals were killed in the morning of day 7.

Normal and insulin-maintained diabetic rats gained weight at approx. 7%/day, whereas 2-day-starved rats lost weight at approx. 11%/day and 2-day-diabetic rats at approx. 2%/day. Plasma glucose concentrations for diabetic rats were 17.6 ± 0.7 mM and for insulin-maintained diabetic rats were 6.0 ± 0.2 mM (means $\pm$ S.E.M., $n = 6$ –8). In some experiments, starved and diabetic rats were each injected intravenously with 5 units of soluble insulin (0.2 ml) 30 min before death.

Tissue preparation

Rats were killed by decapitation, heparinized blood samples were collected and the lower limbs were removed and placed in ice water within 15 s. Both whole gastrocnemius muscles were then removed and frozen in liquid N₂ within a further 30 s. Muscles were stored at -80°C until analysis. Plasma glucose was determined by using glucose oxidase (Bergmeyer & Bernt, 1974).

Preparation of postmitochondrial supernatants

Frozen muscles were ground to a powder with a pestle and mortar under liquid N₂ and homogenized in 6 vol. of ice-cold 50 mM-triethanolamine hydrochloride, pH 7.8, containing 120 mM-KCl, 5 mM-magnesium acetate, 0.2 mM-EGTA, 4 mM-sodium pyrophosphate and 2.3% (w/v) glycerol, with a Polytron homogenizer (Kinematica, Lucerne, Switzerland) set at position 4, with three bursts of 5 s and cooling rapidly between operations. In some experiments 0.2 ml samples of homogenate were removed for RNA assay as described below. Homogenates were then centrifuged at 10000 *g* for 10 min at 4°C . Samples of postmitochondrial supernatants were immediately transferred to micro-centrifuge tubes on ice and stored in liquid N₂. RNA was determined on 0.2 ml samples of homogenate or postmitochondrial supernatant by the method of Munro & Fleck (1969), assuming that, after alkaline hydrolysis 1 mg of RNA is equivalent to 32.5 A_{260} units. A correction for absorption at 260 nm by non-RNA material in 0.35 M-HClO₄ supernatants of alkali digests was applied (Munro & Fleck, 1969), based on peptide content measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Formation of [40S·Met-tRNA^{fMet}] initiation complexes by muscle extracts

Conditions used for the formation of [40S·Met-tRNA^{fMet}] complexes were similar to those employed by Pain *et al.* (1980) for cell-free protein synthesis in extracts from Ehrlich ascites-tumour cells. Reagent 'cocktail' (24 μ l) was added to 200 μ l of muscle postmitochondrial supernatant such that final concentrations were as follows: 40 mM-triethanolamine hydrochloride, pH 7.8, 1.5 mM-magnesium acetate, 120 mM-KCl, 3.3 mM-sodium pyrophosphate, 1.9% glycerol, 6 mM-2-mercaptoethanol, 0.4 mM-spermidine, 1 mM-ATP, 0.25 mM-GTP and 10 mM-phosphocreatine. The reaction was started by addition of 16 μ l of [³⁵S]Met-tRNA^{fMet} (0.1–0.3 μ Ci) and the mixture incubated at 30°C. At the end of the incubation the tubes were placed in ice water and the incubated samples were layered on to pre-cooled 11 ml linear 20–50% (w/v) sucrose gradients, prepared in 0.1 mM-sodium cacodylate, pH 6.5, containing 120 mM-KCl and 2 mM-magnesium acetate (Crystal *et al.*, 1974). The gradients were centrifuged for 16 h at 110 000 g_{av} in a Beckman SW40 rotor at 4°C. The procedure used for fractionation and counting of 40S-bound radioactivity has been described (Pain *et al.*, 1980). In early experiments the complete gradient (18 fractions) was assayed. However, since radioactivity separated from the top of the gradient was found exclusively in a single peak in the 40S region, in subsequent experiments only eight fractions were measured, which included this region. Total radioactivity in the 40S-associated peak was calculated by summation, after subtraction of background, and was expressed in terms of extract RNA content.

Met-tRNA^{fMet} deacylase activity in muscle extracts

Muscle extracts were incubated under the conditions described above for assay of [40S·Met-tRNA^{fMet}] complex-formation. Samples (10 μ l) were spotted on to Whatman no. 1 filter-paper discs at timed intervals during the incubation at 30°C, placed in ice-cold 10% trichloroacetic acid, washed successively with 10% trichloroacetic acid, ethanol and acetone, dried and counted for radioactivity in scintillant (0.4% 2,5-diphenyloxazole in toluene).

Distribution of ribosomes between active and inactive pools

Postmitochondrial supernatants (50–80 μ l, 0.8–1.4 A_{260} units) were layered on linear 5 ml 20–50% sucrose gradients in 50 mM-triethanolamine hydrochloride, pH 7.8, containing 120 mM-KCl and 2 mM-magnesium acetate. Gradients were centrifuged at 170 000 g_{av} for 90 min at 4°C in a Beckman SW50.1 rotor, pumped through an ISCO type 6

flow-cell and monitored for absorbance at 254 nm with an ISCO UA-5 recording spectrophotometer. The relative amounts of active (polysomal) ribosomes and inactive (80S+dimer) ribosomes were determined by cutting out and weighing the appropriate areas of the peaks on the recorder paper. Since the dimeric ribosomes could be completely dissociated into 40S and 60S subunits by centrifugation in buffer containing 500 mM-KCl (results not shown), these were taken to be inactive in protein synthesis and included with the 80S ribosomes.

Results and discussion

Gastrocnemius muscle was chosen for this study since protein-synthesis rates in this tissue *in vivo* have been shown to be decreased markedly in diabetes induced by streptozotocin (Pain & Garlick, 1974) or alloxan (Hay & Waterlow, 1967). The content of inactive ribosomes (relative to total tissue RNA) in postmitochondrial extracts from medial gastrocnemius muscle is increased in diabetic rats (Flaim *et al.*, 1980), implying a block in peptide-chain initiation. In our study, diabetes was induced by withdrawal of insulin from rats maintained on insulin for 4 days after streptozotocin treatment, in an attempt to decrease toxic effects of the drug at the time of the experiment.

RNA content and proportion of ribosomes in polysomes

Table 1 shows that the RNA concentration of rat gastrocnemius muscle was significantly decreased after either 2 days of starvation or 2 days of diabetes, by 19% and 13% respectively. Since approx. 80% of tissue RNA is rRNA, these results indicate a decreased capacity for muscle protein synthesis under these conditions. However, these changes alone are insufficient to account for the degree of inhibition of protein synthesis *in vivo* in response to starvation (Millward *et al.*, 1973) and induction of diabetes (Pain & Garlick, 1974). Table 1 also shows that the proportion of RNA that was recovered in the postmitochondrial supernatant fraction was unaffected by either starvation or diabetes.

Typical polysome profiles obtained with muscle postmitochondrial extracts from fed, starved and insulin-treated starved rats are given in Fig. 1, which confirms previous reports that there is a shift of rRNA from polysomes to inactive particles in starvation, and that acute insulin treatment reverses this change (Li *et al.*, 1979). The shift in polysome size distribution resulting from diabetes was similar to that produced by starvation, and was also reversed by acute insulin treatment (results not shown). The ratio of inactive to active

Table 1. *Effects of 2 days of starvation and 2 days of diabetes on rat gastrocnemius-muscle RNA concentration and RNA recovery in postmitochondrial extracts*

Postmitochondrial extracts were prepared as described in the Materials and methods section. Results are means $\pm$ s.e.m. for six observations. Significance of difference from appropriate control: * $P < 0.05$, ** $P < 0.01$.

Condition	RNA concn. (mg/g wet wt.)	RNA recovery in extract (%)
Expt. 1		
Control (fed)	1.58 $\pm$ 0.16	33.7 $\pm$ 2.1
Starved	1.28 $\pm$ 0.14**	32.8 $\pm$ 3.2
Expt. 2		
Control (insulin-maintained)	1.10 $\pm$ 0.06	41.8 $\pm$ 3.4
Diabetic	0.96 $\pm$ 0.10*	46.1 $\pm$ 3.7

Fig. 1. *Polysome size distribution: effects of starvation and insulin treatment*

Preparation of postmitochondrial extracts and analysis on 20–50% sucrose gradients were as described in the Materials and methods section. Sedimentation was from left to right. Muscle extracts were obtained from (a) fed rats, (b) 2-day-starved rats and (c) 2-day-starved rats 30 min after intravenous administration of 5 units of insulin. The position of 80S monomeric ribosomes is indicated by the arrows.

ribosomes (Table 2) shows a significant increase after starvation or diabetes and a decrease when either starved or diabetic rats are treated acutely with insulin. These findings suggest that in starvation or diabetes the rate of initiation of protein synthesis is decreased relative to that of chain elongation, although decreases in polysomes cannot be interpreted unambiguously as a decrease in initiation rate alone. To determine more directly the effects of diabetes and starvation on the rate of initiation, we have used an assay based on the formation of $[40S \cdot \text{Met-tRNA}^{\text{Met}}]$ initiation complexes by muscle postmitochondrial extracts.

Formation of $[40S \cdot \text{Met-tRNA}^{\text{Met}}]$ initiation complexes

Fig. 2 shows that $[^{35}\text{S}]\text{Met-tRNA}^{\text{Met}}$ is incorporated exclusively into the 40S region of 20–50% sucrose gradients after incubation of muscle postmitochondrial extracts under the conditions described in the Materials and methods section. There was no evidence for association of added

$[^{35}\text{S}]\text{Met-tRNA}^{\text{Met}}$ with 80S ribosomal monomers or polysomes, which implies a block in the initiation sequence in these extracts at the level of attachment of mRNA or 60S subunits. The time course of $[40S \cdot \text{Met-tRNA}^{\text{Met}}]$ complex-formation is given in Fig. 3; 40S-associated radioactivity increased linearly for 4–5 min and then declined. In the subsequent experiments the standard incubation time used for assay of the rate of initiation-complex formation was 2 min.

Effect of starvation and diabetes on $[40S \cdot \text{Met-tRNA}^{\text{Met}}]$ initiation-complex formation

The ability of muscle postmitochondrial extracts from starved and diabetic rats to form $[40S \cdot \text{Met-tRNA}^{\text{Met}}]$ complexes *in vitro*, expressed on the basis of the RNA concentration of the extracts, was decreased by 30–40% compared with fed and insulin-maintained controls (Table 3, Fig. 2b). This is direct evidence, therefore, for inhibition of initiation at the level either of $[\text{eIF-2} \cdot \text{GTP} \cdot \text{Met-tRNA}^{\text{Met}}]$ ternary-complex formation or of

binding of these complexes to 40S subunits in skeletal muscle of starved and diabetic animals. Furthermore, the extent of the decrease in 40S initiation-complex formation *in vitro* shown here is comparable with that previously found for skeletal-muscle protein-synthesis rate on an RNA basis *in vivo* after 2 days starvation (Millward *et al.*, 1973) or 2 days diabetes (Flaim *et al.*, 1980). This agreement supports the view that muscle protein-synthetic activity is controlled at an early step in the initiation sequence. Our conclusions are substantially strengthened by the results of experi-

ments carried out simultaneously in another laboratory (F. J. Kelly & L. S. Jefferson, personal communication), showing that labelling of native [40S-Met-tRNA^{fMet}] complexes with [³⁵S]-methionine in the perfused hind-limb system is impaired in preparations from diabetic rats.

Since tracer doses of Met-tRNA^{fMet} were used in our experiments, it is important to exclude the possibility that the changes in labelling of initiation complexes found were due to altered amounts of endogenous Met-tRNA^{fMet} resulting in different specific radioactivities of the substrate in extracts from starved and diabetic rats compared with controls. Endogenous Met-tRNA^{fMet} contents in muscle extracts from normal, starved and diabetic rats were therefore compared by the method of isotope dilution, with extracts of equal total RNA concentration (Fig. 4); there were no differences in the concentration of endogenous Met-tRNA^{fMet} in the three types of extract.

A possible explanation for the loss of bound Met-tRNA^{fMet} after longer incubation times (Fig. 3) is the presence of Met-tRNA^{fMet} deacylase activity in the muscle extracts. This would be a problem if either (a) deacylase acts directly on 40S-bound Met-tRNA^{fMet} or (b) there is rapid exchange between 40S-bound and free Met-tRNA^{fMet} (Darnbrough *et al.*, 1973; Pain & Clemens, 1983; Erni & Staehelin, 1983), and endogenous Met-tRNA^{fMet} charging continues during the incubation. Since muscle extracts under the conditions described do not form active 80S initiation complexes and apparently cannot carry out polypeptide-chain elongation (results not shown), loss

Table 2. *Distribution of active and inactive ribosomes in postmitochondrial extracts of gastrocnemius muscle*

Treatment of rats, preparation of postmitochondrial extracts and analysis on 20–50% sucrose gradients were as described in the Materials and methods section. Results are means $\pm$ S.E.M. for groups of six rats (**P* < 0.01 versus appropriate control, ***P* < 0.001 versus untreated diabetic).

	Ribosome distribution (% of inactive ribosomes)
(a) Effects of starvation and acute insulin treatment	
Expt. 1	
Control (fed)	41.9 $\pm$ 1.7
Starved	49.0 $\pm$ 1.4*
Expt. 2	
Control (starved)	55.5 $\pm$ 2.7
Starved, insulin-treated	42.8 $\pm$ 2.7*
(b) Effects of diabetes and acute insulin treatment	
Control	49.4 $\pm$ 5.2
Diabetic	59.4 $\pm$ 7.0*
Diabetic, insulin-treated	42.7 $\pm$ 2.2**

Fig. 2. Formation of [40S-Met-RNA^{fMet}] initiation complexes by postmitochondrial supernatants from muscle of (a) fed and (b) starved rats

Postmitochondrial supernatants were prepared from gastrocnemius muscle and incubated with [³⁵S]Met-tRNA^{fMet} for 2 min under the conditions described in the Materials and methods section. They were then analysed on sucrose gradients as described, the conditions being such that polysomes were pelleted. Gradient fractions were treated with cetyltrimethylammonium bromide to precipitate RNA (Pain *et al.*, 1980). ●, Precipitable radioactivity; ····, A₂₅₄.

Fig. 3. Time course of $[40S \cdot Met-tRNA^{fMet}]$ initiation-complex formation in muscle postmitochondrial supernatants

Incubations were carried out as described in the Materials and methods section for various times. Gradient analysis was as described in Fig. 2. The result of a typical experiment is shown.

of total acid-precipitable radioactivity from the incubation can be taken as an indicator of $Met-tRNA^{fMet}$ deacylation. Fig. 5 shows that muscle postmitochondrial extracts do contain deacylase activity, and this may indeed explain the time course of $[^3S]Met-tRNA^{fMet}$ binding to 40S subunits, since approx. 50% of added $Met-tRNA^{fMet}$ was deacylated after 10 min. However, after the standard incubation time used in $[40S \cdot Met-tRNA^{fMet}]$ complex-formation assays (2 min), the extent of $Met-tRNA^{fMet}$ deacylation was small and very similar (about 8%) in all experimental groups studied. Thus deacylation is unlikely to have a significant effect on either the extent of binding of added $[^3S]Met-tRNA^{fMet}$ to 40S subunits in our assays or the differences in binding activity between the groups studied.

Effect of acute insulin administration on $[40S \cdot Met-tRNA^{fMet}]$ initiation-complex formation

It seems likely that at least a part of the inhibition of skeletal-muscle protein synthesis in starvation as well as in diabetes is a consequence of a decrease in circulating insulin. Insulin treatment of diabetic rats stimulates muscle protein synthesis *in vivo* (Pain & Garlick, 1974). Acute administration of insulin to postabsorptive rats stimulates the

Fig. 4. Effect of addition of unlabelled $Met-tRNA^{fMet}$ on the formation of $[40S \cdot Met-tRNA^{fMet}]$ initiation complex. Equal volumes of three postmitochondrial extracts were pooled, and $45 \mu l$ portions of each mixture ($5 \mu g$ of RNA) were incubated in a volume of $90 \mu l$ under the conditions described in the Materials and methods section, in the presence of $0.07 \mu Ci$ of $[^3S]Met-tRNA^{fMet}$ and various amounts of unlabelled $Met-tRNA^{fMet}$. 3S -labelled $[40S \cdot Met-tRNA^{fMet}]$ complex formation is expressed as a percentage of the control activity observed in the absence of added unlabelled $Met-tRNA^{fMet}$. Muscle extracts were obtained from (a) fed rats, (b) 2-day-diabetic rats and (c) 2-day-starved rats. From these data the endogenous concentrations of $Met-tRNA^{fMet}$ in the extracts are estimated to be $1.1 \mu M$, $1.0 \mu M$ and $1.1 \mu M$ in samples from fed, diabetic and starved rats respectively.

Table 3. *Effects of starvation and diabetes on [40S·Met-tRNA^{fMet}] initiation-complex formation in postmitochondrial extracts of gastrocnemius muscle*

Preparation of postmitochondrial extracts from 2-day-starved and 2-day-diabetic rats and assay of initiation complex formation were as described in the Materials and methods section. The variation between Expts. 1 and 2 in radioactivity bound is due to differences in specific radioactivity of added [³⁵S]Met-tRNA^{fMet}. Results are means ± S.E.M. of groups of six.

	[40S·Met-tRNA ^{fMet}] complex-formation (c.p.m./μg of RNA)	Change (%)
Expt. 1		
Fed	101.4 ± 6.2	
Starved	63.8 ± 6.9	− 37.1 (<i>P</i> < 0.001)
Expt. 2		
Insulin-maintained	119.5 ± 9.5	
Diabetic	81.7 ± 10.1	− 32.4 (<i>P</i> < 0.01)

Fig. 5. *Time course of deacylation of added [³⁵S]-Met-tRNA^{fMet} by muscle postmitochondrial extracts*
Muscle postmitochondrial extracts were incubated with [³⁵S]Met-tRNA^{fMet}, and at the times indicated 10 μl samples were precipitated with 10% trichloroacetic acid and radioactivity was measured as described in the Materials and methods section. Acid-precipitable radioactivity is expressed as the percentage of the value at zero time. (a) Samples from fed rats (○), 2-day-starved rats (●) and starved rats treated with insulin 30 min before death (△). (b) Extracts from control (insulin-maintained diabetic) rats (○), 2-day-diabetic rats (●) and diabetic rats treated with insulin 30 min before death (△). In both experiments incubations were also performed in the absence of tissue extract (□). Each point represents the man of five or six determinations, and the S.E.M. is less than 5% of the mean in each case.

rate of protein synthesis in gastrocnemius muscle within 30 min (Garlick *et al.*, 1983), and the decrease in protein-synthesis rates seen in perfused hemicorpus preparations from starved rats is reversed by addition of insulin to the medium (Li *et al.*, 1979). Here we have investigated the effects of acute administration of soluble insulin to 2-day-diabetic and 2-day-starved rats. Table 4 demonstrates that postmitochondrial supernatants prepared within 30 min of insulin treatment of either starved or diabetic rats shows a significant (40–50%) stimulation of [40S·Met-tRNA^{fMet}] complex-

formation. This suggests that insulin can act acutely to reverse the effects of starvation and diabetes on initiation of protein synthesis in muscle.

Effect of addition of exogenous eIF-2 to muscle extracts

Addition of exogenous purified eIF-2 to cell lysates reverses the impairment of initiation that occurs when the reticulocyte system is deprived of haemin (Clemens *et al.*, 1974) or when Ehrlich ascites-tumour cells are deprived of essential

Table 4. *Effects of acute insulin administration on [40S·Met-tRNA^{fMet}] initiation-complex formation in postmitochondrial extracts of starved and diabetic rats*

Rats (2-day-starved and 2-day-diabetic) were treated 30 min before death with insulin (5 units, intravenously). Preparation of postmitochondrial extracts and assay of [40S·Met-tRNA^{fMet}] complex-formation were as described in the Materials and methods section. The variation between Expts. 1 and 2 in radioactivity bound is due to differences in specific radioactivity of added [³⁵S]Met-tRNA^{fMet}. Results are means ± S.E.M. for groups of six.

	[40S·Met-tRNA ^{fMet}] complex-formation (c.p.m./μg of RNA)	Change (%)
Expt. 1		
Starved	59.6 ± 4.2	
Starved, insulin-treated	88.1 ± 8.0	+47.8 (<i>P</i> < 0.01)
Expt. 2		
Diabetic	148 ± 22	
Diabetic, insulin-treated	216 ± 23	+46.3 (<i>P</i> < 0.05)

Fig. 6. *Stimulation of [40S·Met-tRNA^{fMet}] complex-formation in extracts from muscle of fed and starved rats in response to addition of exogenous eIF-2*

Pooled muscle samples from fed and starved rats were obtained by mixing equal volumes of three extracts in each case. Incubations for measurement of [40S·Met-tRNA^{fMet}] complex formation were as described in the Materials and methods section, and were carried out in the presence of increasing concentrations of exogenous purified eIF-2. Results are expressed as the percentage of the complex-formation obtained in the absence of added eIF-2. Extracts were from muscle of fed (●) and 2-day-starved (○) rats.

amino acids (Pain *et al.*, 1980). This suggests that availability of active eIF-2 limits the ability of these systems to form 40S initiation complexes. Fig. 6 shows the effects of addition of increasing concentrations of exogenous purified eIF-2 to postmitochondrial extracts of muscle from fed and 2-day-starved rats. Addition of small amounts of

Table 5. *Effects of exogenous eIF-2 on [40S·Met-tRNA^{fMet}] initiation-complex formation in postmitochondrial extracts of gastrocnemius muscle*

Preparation of postmitochondrial extracts and conditions for initiation-complex formation were as described in the Materials and methods section. Each extract was incubated in the presence and absence of exogenous eIF-2 (4.7 μg/ml). The eIF-2 stimulation was calculated as the radioactivity bound to the 40S ribosomal subunit in each extract in the presence of added eIF-2, expressed as a percentage of that obtained with the same extract in the absence of added initiation factor. Results are expressed as means ± S.E.M. for groups of six.

Condition	Stimulation by eIF-2 (%)
Expt. 1	
Control (fed)	108 ± 11
Starved	148 ± 8
Expt. 2	
Control (insulin maintained)	113 ± 3
Diabetic	134 ± 4

eIF-2 resulted in a greater proportional stimulation of formation of 40S initiation complexes in extracts from starved rats than in those from fed rats. The difference between extracts in their response was less marked at higher concentrations of eIF-2. A more extensive comparison, with an eIF-2 concentration of 4.7 μg/ml, was made of the responses of extracts from groups of fed, starved, diabetic and insulin-maintained control rats. Table 5 shows that extracts from starved and diabetic rats consistently showed a greater stimulation in response to this concentration of eIF-2 than did the extracts from the corresponding fed and insulin-maintained controls. In these experiments we analysed each extract in the presence and absence of exogenous eIF-2 within the same batch of samples. Since rotor capacity limited the number of samples that we could analyse simultaneously, we have not

made a direct comparison of the absolute amount of complex formed in extracts from fed and starved (or normal and diabetic) rats in the presence of eIF-2. Hence our results do not show whether the stimulatory effect of exogenous eIF-2 on extracts from starved rats is sufficient to restore their capacity for 40S initiation-complex formation completely to that of extracts from fed controls. We therefore do not exclude the possibility that other components, in addition to eIF-2, are sensitive to impairment by starvation. However, our data do show that the percentage stimulation by exogenous eIF-2 *in vitro*, with extracts from either starved or diabetic rats (Table 5), is comparable with that resulting from insulin treatment *in vivo* (Table 4).

Previous studies (Rannels *et al.*, 1978) on the activity of muscle eIF-2, as assessed by the ability of postribosomal supernatants to form [Met-tRNA^{fMet}·eIF-2·GTP] ternary complexes, did not reveal an effect of starvation when the results were expressed as a function of tissue RNA content. It is likely that the data of Rannels *et al.* (1978) represent a measure of total cellular eIF-2, since the extracts were prepared by homogenizing the tissue in medium containing 0.25 M-KCl, which would release eIF-2 from 40S ribosomal subunits. However, such assays may not reveal differences in the state of activity of the initiation factor. Indeed, even in the reticulocyte system, where phosphorylation of eIF-2 is closely associated with loss of initiation activity, Benne *et al.* (1980) were unable to demonstrate impairment of ternary-complex formation by eIF-2 recovered from extracts subjected to haem deprivation. More recent work has shown that eIF-2 phosphorylation inhibits recycling of eIF-2 by preventing exchange of bound GDP for GTP, and that ternary-complex assays reveal an effect of eIF-2 phosphorylation only under certain conditions, i.e. when the cellular guanine-nucleotide exchange factor is present and when Mg²⁺ is present at a physiological concentration such that recycling of eIF-2·GDP requires interaction with a guanine-nucleotide exchange factor (Siekierka *et al.*, 1982). We do not yet know whether the activity of eIF-2 in skeletal muscle is regulated by phosphorylation, but this possibility would be consistent with the presence in this tissue of eIF-2 kinase activity (Proud & Pain, 1982) and of a phosphatase with activity towards eIF-2 (Ingebritsen & Cohen, 1983).

It has been suggested that peptide-chain initiation may be controlled through alterations in the 40S ribosomal subunits themselves, perhaps by phosphorylation of ribosomal protein S6 (Duncan & McConkey, 1982). However, evidence on this point is conflicting (Leader *et al.*, 1981) and, in any case, changes in S6-protein phosphorylation in

muscle have not been found to occur in response to diabetes (Nielsen *et al.*, 1982). Moreover, our finding that the impairment in 40S initiation-complex formation caused by either starvation or diabetes can be at least partially restored by the addition of exogenous eIF-2 argues against a major role for alterations in the 40S subunits in producing the effects we observe.

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