Purification and phosphorylation of initiation factor eIF-2 from rabbit skeletal muscle

Christopher G. Proud* and Virginia M. Pain

School of Biological Sciences, University of Sussex, Falmer, Brighton, BN1 9QG, England

Received 6 May 1982

Skeletal muscle *Initiation factor-2* *Protein phosphorylation*

1. INTRODUCTION

In mammalian skeletal muscle, the rate of protein synthesis is subject to regulation by a number of hormones, including insulin and glucocorticoids [1–5]. These hormones appear to exert their translational effects at the level of peptide-chain initiation [3,4,7]. One of the best characterised examples of the regulation of protein synthesis at initiation is the inhibition of this process which occurs when reticulocyte lysates are deprived of haemin (reviewed in [6]). Here, inhibition of protein synthesis is associated with increased phosphorylation of an initiation factor (eucaryotic initiation factor-2, eIF-2) by a haem-regulated protein kinase (haem-controlled repressor, HCR) [7]. The normal function of eIF-2 is to promote the binding of the initiator tRNA (Met-tRNA$_f$) to the 40 S ribosomal subunit. This process involves formation of a 'ternary complex' between eIF-2, Met-tRNA$_f$ and GTP [8]. It appears that phosphorylation of eIF-2 impairs its ability to function in these initiation reactions. Phosphorylation of eIF-2 may also be involved in the regulation of protein synthesis under other conditions and in cells other than reticulocytes (reviewed in [9]).

Abbreviations: Tris, Tris(hydroxymethyl)aminomethane; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol bis (β-aminoethyl ether)N,N,N',N'-tetraacetic acid; HCR, haem-controlled repressor; eIF-2, eucaryotic initiation factor-2; eIF-2α, eIF-2β, α,β subunits of eIF-2; SDS, sodium dodecylsulphate

* Present address, for correspondence: The Biological Laboratory, University of Kent, Canterbury, Kent CT2 7NJ, England

As a first step in investigating the possibility that similar mechanisms involving phosphorylation of eIF-2 mediate the hormonal regulation of initiation in skeletal muscle, we have purified eIF-2 from this tissue. The factor from skeletal muscle shows strong similarities to eIF-2 from other sources, and in particular it is a substrate for phosphorylation by the haem-controlled repressor from reticulocytes. Preliminary evidence suggests that skeletal muscle also contains a protein kinase capable of phosphorylating the same subunit (α) of eIF-2 which is phosphorylated by HCR.

2. MATERIALS AND METHODS

The radiochemicals L-[^{35}S]methionine and [γ-^{32}P]ATP were obtained from the Radiochemical Centre (Amersham). Phosphocellulose, GTP, ATP, pyruvate kinase (type II from rabbit muscle), phosphoenolpyruvate and reagents for polyacrylamide gel electrophoresis were from Sigma Chemical Co. (Poole). Cellulose nitrate filters (pore size 0.45 μm) were purchased from Sartorius (Belmont, Surrey). Calf liver tRNA was from Boehringer (Lewes). Ammonium sulphate (grade for enzyme work) was supplied by BDH (Poole).

[^{35}S]Methionyl-tRNA$_f$ was prepared as in [10] using *E. coli* aminoacyl-tRNA synthetases. The purified haem-controlled repressor was a generous gift from Dr Tim Hunt (University of Cambridge). Initiation factor eIF-2 (from rat liver) was kindly provided by Dr Michael Clemens (St George's Hospital Medical School, London).

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

2.1. *Assay of initiation factor eIF-2*

Initiation factor eIF-2 was assayed by its ability to form ternary complexes with GTP and Met-tRNA$_f$. The formation of these complexes, containing [^{35}S]Met-tRNA$_f$, was quantified by their retention on cellulose nitrate filters, essentially as in [11]. A typical incubation (50 μl) contained: 20 mM Tris–HCl (pH 7.6); 0.5 mM magnesium acetate; 1 mM dithiothreitol; 100 mM KCl; 0.5 mg/ml bovine serum albumin; 0.5 mM GTP; 3.0 mM phosphoenolpyruvate; 0.2 U pyruvate kinase; and 0.1 μCi [^{35}S]Met-tRNA$_f$. Incubations were at 30°C and after 15 min a 45 μl sample was removed and immediately diluted with 1.5 ml buffer containing 25 mM Tris–HCl (pH 7.6); 90 mM KCl and 5 mM magnesium acetate. Diluted samples were filtered through cellulose nitrate filters, and each was washed with a further 10 ml of buffer and then dried before measurement of radioactivity. Assays were carried out in duplicate and corrected for non-specific binding of [^{35}S]Met-tRNA$_f$ by subtraction of the radioactivity bound in assays carried out in the absence of GTP, pyruvate kinase and phosphoenolpyruvate. One unit of eIF-2 activity mediates the binding of one nanomole of Met-tRNA$_f$ in the standard assay.

2.2. *Polyacrylamide gel electrophoresis*

Polyacrylamide gel electrophoresis was performed using slab gels containing 10% acrylamide/0.1% methylene bis-acrylamide/0.1% SDS as described [12] and modified [13].

2.3. *Phosphorylation of eIF-2*

Phosphorylation of eIF-2 was carried out in incubations containing (in 25 μl total vol.): 20 mM Hepes (4-(2-hydroxyethyl)-1-piperazine ethane) (pH 7.1); 1 mM dithiothreitol; 0.1 mM EDTA; 0.03 mM EGTA; 2 mM magnesium acetate; 100 mM KCl and HCR, eIF-2 or other components as indicated. Reactions, at 30°C, were started by the addition of [γ-^{32}P]ATP (0.1 mM, 1.2 Ci/mmol). After 5 min an equal volume of gel electrophoresis buffer, containing 2% SDS and 40 mM dithiothreitol, was added, and the samples were heated at 95°C for 5 min. They were then layered onto polyacrylamide slab gels and subjected to electrophoresis, and subsequently stained with Coomassie blue/Crocein scarlet. Destained, dried gels were subjected to autoradiography using Kodak X-ray film.

2.4. *Preparation of muscle supernatant fractions*

Samples (~1 g) of rat gastrocnemius muscle were homogenised using a Polytron homogeniser (3 × 10 s) in ice-cold homogenisation medium comprising: 50 mM Tris–HCl (pH 7.8); 5 mM magnesium acetate; 0.2 mM EDTA; 4 mM sodium pyrophosphate; 0.25 M sucrose; 1 mM dithiothreitol and 0.25 M KCl. The homogenates were centrifuged at 4°C for 10 min at 20 000 × *g*. The resulting supernatants were then recentrifuged at 135 000 × *g* for 2.5 h at 4°C, and the postribosomal supernatants so obtained were stored in liquid nitrogen until required.

2.5. *Purification of eIF-2 from rabbit skeletal muscle*

The procedure used was derived from that in [14] for pig liver eIF-2. Male New Zealand White rabbits were given á lethal dose of Nembutal, and the muscle from the back and the hind legs was rapidly removed, frozen in liquid nitrogen and stored until use, at − 70°C. All subsequent steps were carried out at 4°C. Muscle (250 g) was homogenised in 3 vol. 20 mM triethanolamine–HCl (pH 7.5); 0.5 M KCl; 5 mM magnesium acetate; 2 mM sodium pyrophosphate; 10 mM β-mercaptoethanol; 0.5 mM EGTA in an Atomixer blender at top speed for 3 × 20 s. The homogenate was centrifuged at 20 000 × *g* for 25 min. The supernatant was filtered through glass wool, and then recentrifuged for 16 h at 37 000 × *g*.The resulting 'postribosomal' supernatant was then subjected to ammonium sulphate fractionation. Powdered $(NH_4)_2SO_4$ was added over 3 h to achieve 40% saturation. Precipitated proteins were removed by centrifugation at 20 000 × *g* for 30 min. The supernatant was made 52% saturated in ammonium sulphate (by the slow addition of powdered $(NH_4)_2SO_4$) and the precipitated proteins were collected by centrifugation for 30 min at 20 000 × *g*. The precipitates were dissolved in ~ 25 ml buffer A (20 mM Tris–HCl (pH 7.8); 10% (v/v) glycerol; 0.2 mM EDTA; 10 mM β-mercaptoethanol) containing 0.25 M KCl, dialysed extensively against this buffer. After dialysis the material was stored at − 70°C.

The stored ammonium sulphate fractions from 4 of the above purification procedures were thawed, combined and applied to a column of phospho-

Table 1

Purification of initiation factor eIF-2 from rabbit skeletal muscle

Step	Volume (ml)	Protein (mg)	Activity (U)	Spec. act. (mU/mg)	Purification (-fold)	Yield (%)
1. 20000 × *g* supernatant	1756	18636	7.45	0.40	1	100
2. 37000 × *g* supernatant	1427	12450	5.08	0.41	1.03	68.0
3. 40–52% $(NH_4)_2SO_4$ fraction	98	1254	2.35	1.87	4.68	31.4
4. Phosphocellulose (pH 7.8)	14.3	9.8	1.35	138	345	18.0
5. DEAE-cellulose (pH 7.8)	3.7	0.47	1.42	3020	7550	19.0

At all steps the protein concentration was determined by the Lowry method [15]; 880 g rabbit muscle was used in this preparation.

cellulose (6.5 × 2.6 cm) equilibrated in buffer A containing 0.25 M KCl. The column was thoroughly washed with this buffer, and the eIF-2 was then eluted with buffer A containing 0.7 M KCl. Fractions (~ 5 ml) were collected and those found to contain the highest eIF-2 activities were combined and dialysed against 2 × 400 ml buffer A containing 0.09 M KCl. The dialysed material was applied to a small (1.5 × 0.6 ml) DEAE-cellulose column. The column was then extensively washed with buffer A containing 0.09 M KCl and then with buffer A containing 0.12 M KCl. The eIF-2 was then eluted from the column with buffer A containing 0.18 M KCl. The fractions showing the highest eIF-2 activity were divided into 0.05 ml aliquots and stored at −80°C.

3. RESULTS AND DISCUSSION

3.1. *Purification procedure*

A summary of the purification of eIF-2 from rabbit skeletal muscle is shown in table 1. Several comments on the determination of eIF-2 activity should be made. At steps 1 and 2, dialysis of samples prior to assay resulted in an increase in the apparent eIF-2 activity, due perhaps to the removal of GDP which strongly inhibits ternary complex formation [16]. Also at step 1, eIF-2 activity appeared to increase as the dilution of the sample in the ternary complex assay increased. This is presumably due to the presence at this stage of non-dialysable factors, e.g., unlabelled Met-$tRNA_f$ or mRNA [17], which would inhibit the binding of the [^{35}S]Met-$tRNA_f$ in the eIF-2 assay. Accordingly, samples were diluted sufficiently to overcome this inhibition. At later stages (4,5) of the procedure, where protein concentrations were low, the measured eIF-2 activity was increased by the presence in the assays of bovine serum albumin (0.5 mg/ml). Addition of this protein has no effect on assays at earlier stages of the purification, and presumably prevents losses of eIF-2 on the walls of the reaction tube, as in [18]. When eIF-2 was assayed as described the overall yield was 15–20%. The purified preparation of eIF-2 was stable to storage at −80°C, showing no loss of activity over 6 months. The purification procedure described here has also been successfully applied to the isolation of eIF-2 from rat skeletal muscle and beef heart muscle (C.G.P., unpublished).

3.2. *Characterisation of rabbit muscle eIF-2*

The functional properties of eIF-2 from rabbit skeletal muscle were similar to those reported for this initiation factor from other sources [14,19–21], in terms of its ability to support formation of ternary complexes (eIF-2 · GTP · Met-$tRNA_f$) and to mediate binding of Met-$tRNA_f$ to 40 S ribosomal subunits (not shown). Formation of ternary complexes by muscle eIF-2 was dependent on the presence of GTP, was stimulated by a GTP-regenerating system (phosphoenolpyruvate plus pyruvate kinase) and inhibited by excess Mg^{2+} (0.5–5.0 mM; not shown).

When purified muscle eIF-2 was analysed on polyacrylamide gels under denaturing conditions, 3 major protein-staining bands were observed. In addition a number of minor components were evident

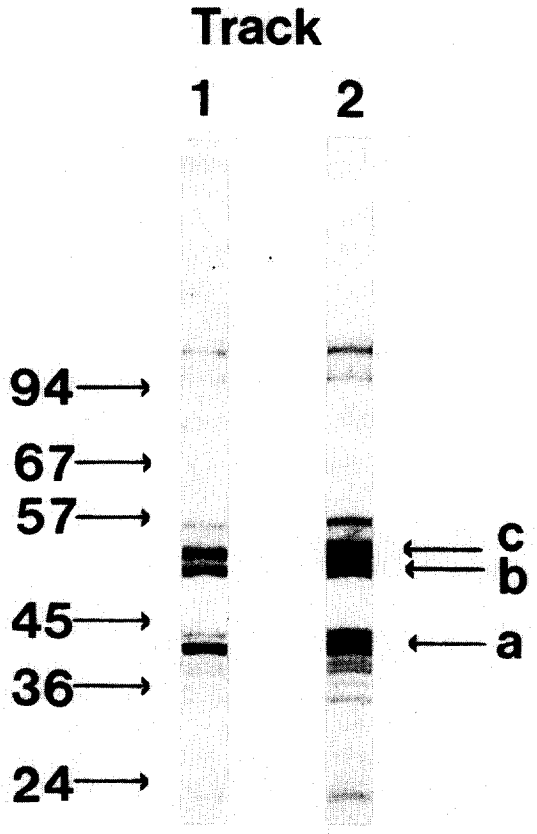

Fig.1. SDS–polyacrylamide gel electrophoresis of purified rabbit muscle eIF-2. Samples of eIF-2 from step 5 were subjected to electrophoresis as in section 2: track 1, ~ 11 μg eIF-2; track 2, ~ 21 μg eIF-2. GEls were stained with Coomassie blue/Croccin scarlet. Marker proteins (migration positions and M_r-values ($\times 10^{-3}$) indicated by arrows) were phosphorylase, bovine serum albumin, pyruvate kinase, ovalbumin, glyceraldehyde 3-phosphate dehydrogenase and trypsinogen. The arrows labelled a, b and c indicate the α, β and γ subunits of eIF-2, respectively.

(fig.1). The position of the 3 major components corresponded to M_r 39 000, 51 000 and 54 000: these components are generally termed the α-, β- and γ-subunits of eIF-2, respectively. Allowing for the well-characterised anomalies in the behaviour of the subunits on gel-electrophoresis [22], these M_r-values are similar to those reported for the 3 subunits of eIF-2 from other sources [14,19–21]. In our experience the resolution of the two larger subunits was variable and on some gels they comigrated, so that only two major protein-staining bands were observed. Isolation of a fully active preparation of eIF-2 containing only two subunits has been reported [23].

3.3. *Phosphorylation of rabbit skeletal muscle eIF-2*

When purified rabbit muscle eIF-2 was incubated with [γ-^{32}P]ATP and HCR, the M_r 39 000 component of the eIF-2, i.e., the α-subunit, became radiolabelled (fig.2, track 3). This was as expected, since HCR also phosphorylates the α-subunit of rabbit reticulocyte eIF-2 [24,25]. In the absence of HCR, only a trace of radioactivity was detected in the positions corresponding to the α-, β- and γ-subunits of eIF-2 (fig. 2, track 2). However, a minor component (M_r 100 000) present in the eIF-2 preparation became equally strongly phosphorylated in the presence or absence of HCR. The nature of this contaminant is unclear; its M_r does not correspond to that of any of the phosphorylatable proteins of muscle glycogen metabolism. When HCR was incubated alone, there was no detectable phosphorylation.

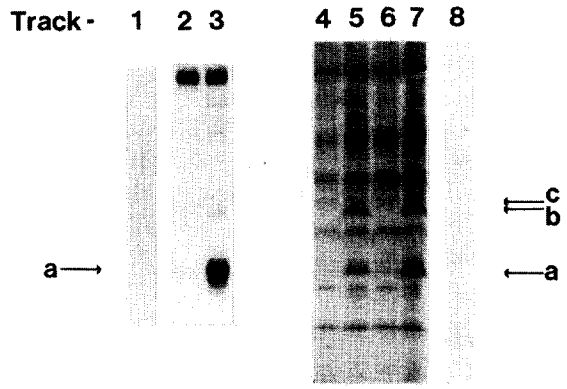

Fig.2. Phosphorylation of eIF-2. Incubations including [γ-^{32}P]ATP were done as in section 2. After denaturation samples were subjected to SDS–polyacrylamide gel electrophoresis and autoradiograms of the stained gels were prepared as in section 2. Autoradiogram: track 1, HCR (8 units/ml [25]) alone; track 2, step 5 rabbit skeletal muscle eIF-2 (3 μg) alone; track 3, step 5 eIF-2 (3 μg) plus HCR (8 units/ml); tracks 4–7: experiments with skeletal muscle supernatants (6 μl/incubation). Incubations also contained rat liver eIF-2 (~ 3 μg; tracks 5,7) or HCR (8 units/ml, tracks 6,7); track 8, rat liver eIF-2 (~ 3 μg) alone. The arrows labelled a, b and c indicate the migration positions of the α, β and γ subunits of eIF-2, respectively.

To investigate whether skeletal muscle itself contains a protein kinase capable of phosphorylating eIF-2α, purified eIF-2 (from rat liver) was incubated with samples of rat muscle supernatants in the presence of [γ-^{32}P]ATP (fig. 2, tracks 4–7). When supernatants or eIF-2 were incubated separately little or no incorporation of ^{32}P into eIF-2α was apparent (tracks 4,8). When eIF-2 and supernatants were incubated together, it was apparent that phosphorylation of eIF-2α had occured (track 5), indicating that rat muscle does contain eIF-2α kinase activity. Some phosphorylation in the position of eIF-2β was also evident, in line with [27] that skeletal muscle contains an eIF-2β kinase. Inclusion of HCR in the incubations resulted in an increase specifically in the level of phosphorylation of eIF-2α (track 7).

The observation that muscle eIF-2 is a substrate for HCR and that muscle appears to contain an eIF-2α kinase, taken with the presence in muscle of a potent eIF-2α phosphatase [28], suggest that the modulation of eIF-2 activity through changes in its phosphorylation state may represent a potential control point in the regulation of skeletal muscle protein synthesis.

ACKNOWLEDGEMENTS

We are grateful to Dr Michael Clemens (St George's Hospital Medical School, London) both for providing rat liver eIF-2 and for performing the eIF-2 phosphorylation experiment illustrated in fig. 2 (tracks 4–7). We thank Dr Tim Hunt (University of Cambridge) for HCR. This work was supported by a grant to V.M.P. from the Medical Research Council.

REFERENCES

[1] Pain, V.M. (1980) in: Biochemistry of Cell Regulation: Gene Expression (Clemens, M.J. ed) vol. 1, pp. 85–117, CRC Press Inc., Boca Raton FL.
[2] Pain, V.M. and Garlick, P.J. (1974) J. Biol. Chem. 249, 4510–4514.
[3] Flaim, K.E., Copenhaver, M.E. and Jefferson, L.S. (1980) Am. J. Physiol. 239, E88–E95.
[4] Jefferson, L.S. (1980) Diabetes 29, 487–496.
[5] Rannels, S.R. and Jefferson, L.S. (1980) Am. J. Physiol. 238, E564–E572.
[6] Gross, M. (1980) Mol. Cell. Biochem. 31, 25–36.
[7] Farrell, P.J., Balkow, K., Hunt, T., Jackson, R.J. and Trachsel, H. (1977) Cell 11, 187–200.
[8] Schreier, M.H. and Staehelin, T. (1973) Nature New Biol. 242 35–38.
[9] Austin, S.A. and Clemens, M.J. (1980) FEBS Lett. 110, 1–7.
[10] Clemens, M.J., Henshaw, E.C., Rahaminoff, H. and London, I.M. (1974) Proc. Natl. Acad. Sci. USA 71, 2946–2950.
[11] Gupta, N.K., Woodley, C.L., Chen, Y.C. and Bose, K.K. (1973) J. Biol. Chem. 248, 4500–4511.
[12] Laemmli, U.K. (1970) Nature 227, 680–685.
[13] Anderson, C.W., Baum, P.R. and Gesteland, R.F. (1973) J. Virol. 12, 241–252.
[14] Harbitz, I. and Hauge, J.G. (1976) Arch. Biochem. Biophys. 176, 766–778.
[15] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[16] Walton, G.M. and Gill, G.N. (1976) Biochim. Biophys. Acta 418, 195–203.
[17] Kaempfer, R., Rosen, H. and Israeli, R. (1978) Proc. Natl. Sci. USA 75, 650–654.
[18] Benne, R., Amesz, H., Hershey, J.W.B. and Voorma, H.O. (1979) J. Biol. Chem. 254, 3201–3207.
[19] Benne, R., Wong, C., Luedi, M. and Hershey, J.W.B. (1976) J. Biol. Chem. 251, 7675–7681.
[20] Trachsel, H., Erni, B., Schreier, M.H., Braun, L. and Staehelin, T. (1979) Biochim. Biophys. Acta 608, 484–490.
[21] Nygard, O., Westerman, P. and Hultin, T. (1980) Biochim. Biophys. Acta 608, 196–200.
[22] Lioyd, M.A., Osborne, J.C., Safer, B., Powell, G.M. and Merrick, W.C. (1980) J. Biol. Chem. 255, 1189–1193.
[23] Stringer, E.A., Chaudhuri, A. and Maitra, U. (1979) J. Biol. Chem. 254, 6845–6848.
[24] Kramer, G., Cimadevilla, J.M. and Hardesty, B. (1976) Proc. Natl. Acad. Sci. USA 73, 3078–3082.
[25] Levin, D.H., Ranu, R.S., Ernst, V. and London, I.M. (1976) Proc. Natl. Acad. Sci. USA 73, 3112–3116.
[26] Clemens, M.J., Pain, V.M., Wong, S.T. and Henshaw, E.C. (1982) Nature 276, 93–95.
[27] DePaoli-Roach, A.A., Roach, P.J., Pham, K., Kramer, G. and Hardesty, B. (1981) J. Biol. Chem. 256, 8871–8874.
[28] Stewart, A.A., Hemmings, B.A., Cohen, P., Goris, J. and Merlevede, W. (1981) Eur. J. Biochem. 115, 197–205.