The Journal of Experimental Biology 206, 2363-2371

doi:10.1242/jeb.00422

The role of eukaryotic initiation factor 2α during the metabolic depression associated with estivation

Julian L. Pakay[1,*], Andrew A. Hobbs[1], Scot R. Kimball[2] and Michael Guppy[1]

[1]*Biochemistry and Molecular Biology, School of Biomedical and Chemical Sciences, University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia* and [2]*Department of Cellular and Molecular Physiology, Penn State College of Medicine, 500 University Drive, Hershey, PA 17033, USA*

*Author for correspondence at present address: Cellular Regulation, MRC Dunn Human Nutrition Unit, Wellcome Trust/MRC Building, Hills Road, Cambridge, CB2 2XY, UK (e-mail: jlp@mrc-dunn.cam.ac.uk)

Accepted 31 March 2003

Summary

We have investigated the role of eukaryotic initiation factor 2α (eIF2α) in two estivating organisms previously shown to downregulate protein synthesis during metabolic depression, the land snail *Helix aspersa* Müller and the desert frog *Neobatrachus sutor* Main 1957. We have developed a method using a single antibody (which binds specifically to the phosphorylated, conserved phosphorylation region) by which the total levels of eIF2α and the ratio of phosphorylated eIF2α [eIF2α(P)] to total (phosphorylated and unphosphorylated) eIF2α can be determined. In *H. aspersa*, we have shown that the level of eIF2α mRNA expression is unchanged between the awake and estivating states. The amount of total eIF2α is the same in the estivating and awake states, and eIF2α(P) is undetectable and must represent ≤10% of total eIF2α in both states. Conversely, in *N. sutor* during estivation, the level of total eIF2α increases approximately 1.6-fold and the ratio of eIF2α(P)/eIF2α increases from 0.22±0.11 to 0.52±0.08, implicating eIF2α phosphorylation in the downregulation of protein synthesis during estivation in this animal. The differences in the amounts of eIF2α and the level of its phosphorylation between these two species also suggest possible differences either in the mechanism by which protein synthesis is downregulated during estivation or in the sensitivity of the initiation of translation to eIF2α(P) levels.

Key words: eIF2α, eukaryotic initiation factor, estivation, protein synthesis, metabolic depression, *Helix aspersa*, *Neobatrachus sutor*, phosphorylation.

Introduction

During adverse environmental conditions, many organisms are able to enter a state of quiescence and survive for extended periods on stored fuels. Associated with this quiescence there is typically a depression of basal metabolic rate by 60–100% (termed metabolic depression; Guppy and Withers, 1999), which necessitates a coordinated change in ATP production and utilization. Estivation is a form of metabolic depression that occurs in some animals in response to a lack of food and/or water or in response to otherwise potentially desiccating situations. It occurs without any change in ambient oxygen partial pressure (P_{O_2}) or temperature. The cellular signaling mechanisms that coordinate the change in energy metabolism during estivation, and indeed the initial cue that initiates estivation, remain unknown.

Given that ATP utilization decreases during metabolic depression, energy-consuming pathways must be downregulated. If their mode of regulation during estivation can be elucidated it may be possible to delineate the upstream signaling mechanisms that would then point to the initial cue for metabolic depression. Protein synthesis is a major energy-consuming pathway and, if maintained at a similar rate during metabolic depression, would become an impossibly costly process in terms of its contribution to total energy consumption. Accordingly, the downregulation of protein synthesis is a consistent phenomenon seen in metabolically depressed organisms. The extent of this downregulation has been well characterised in a number of both *in vivo* and *in vitro* systems and in a variety of different types of metabolic depression, including that associated with anoxia (Bailey and Driedzic, 1996; Hofmann and Hand, 1994), developmental arrest (Podrabsky and Hand, 2000), hibernation (Frerichs et al., 1998) and estivation (Fuery et al., 1998; Pakay et al., 2002).

Despite the often substantial decrease in the rate of protein synthesis during metabolic depression, mRNA pools appear to be maintained during the depressed state. For example, translatable mRNA can be detected in dormant *Artemia* embryos, and its *in vitro* translation demonstrates that there is no significant qualitative and/or quantitative differences in mRNA between anoxic and developing embryos (Hofmann and Hand, 1994). Also in *Artemia*, the addition of exogenous mRNA does not increase the translational capacity of lysates prepared from dormant embryos (Hofmann and Hand, 1994).

During anoxia in turtles, protein synthesis is undetectable in liver and white muscle (Fraser et al., 2001) while translatable mRNA concentrations increase by 38% in liver and remain constant in white muscle (Douglas et al., 1994). These data demonstrate that it is not message limitation that causes a downregulation in the rate of protein synthesis. The major site for the regulation of protein synthesis during metabolic depression must therefore be translation.

It is now generally accepted that the rate at which mRNA is translated into protein is limited by the rate of initiation of translation, and there are two specific processes in the initiation pathway that have been shown to be sites for physiological regulation (Pain, 1996). These are (1) the binding of the Met-$tRNA_i$ to the 43S pre-initiation complex, mediated by eIF2 (eukaryotic initiation factor 2), and (2) the initial binding of the 43S pre-initiation complex to the 5′ end of the mRNA (mediated by eIF4E and associated factors). eIF2 is involved in the binding of an initiation complex (eIF2–Met-$tRNA_i$–GTP) to the 43S ribosomal pre-initiation complex. The formation of this ternary complex is regulated by the phosphorylation of a conserved site on the alpha subunit of eIF2, which results in the formation of an inactive complex comprising eIF2α and eIF2B. This mechanism and the sequence of the phosphorylation site in eIF2α are conserved between yeast and man. An increase in the extent of eIF2α phosphorylation occurs concomitantly with a downregulation of the rate of protein synthesis in normal tissues responding to a diverse array of stresses, including heat shock (Hu et al., 1993), amino acid deprivation (Kimball et al., 1991), reperfusion following ischemia (Martin de la Vega et al., 2001) and anoxia (Tinton et al., 1997).

Data on the role of eIF2 in the regulation of protein synthesis during metabolic depression are limited to two systems, and, in both, the metabolic depression is confounded by extrinsic factors; changes either in temperature or ambient P_{O_2}. So decreased rates of protein synthesis and the concomitant accumulation of phosphorylated eIF2α have been observed in hibernating ground squirrels (Frerichs et al., 1998) but most of the metabolic depression in this system is due to decreased temperature. During short-term anoxia in the marine snail *Littorina littorea*, there is an accumulation of phosphorylated eIF2α that occurs concomitantly with a decrease in protein synthesis (Larade and Storey, 2002). But again, protein synthesis and metabolic rate depression in this system only occur as a result of large changes in ambient P_{O_2}.

The Australian desert frog *Neobatrachus sutor* and the land snail *Helix aspersa* both survive extended dry periods or potential desiccating environments by estivating. In *Neobatrachus*, there is a reduction in oxygen consumption of 50–70% after 8–12 weeks of estivation (Withers, 1993), and, likewise, within four weeks of the removal of food and water from *Helix aspersa* there is an 84% metabolic depression (Pedler et al., 1996). In contrast to the examples above, the metabolic depression in both of these animals occurs without any changes in ambient temperature or P_{O_2}. During metabolic depression, protein synthesis is downregulated, by 67% in liver slices from *Neobatrachus centralis* (Fuery et al., 1998) and by 78% and 48% *in vivo* in hepatopancreas and foot muscle, respectively, from *H. aspersa* (Pakay et al., 2002). The intrinsic nature (the absence of obvious, confounding, extrinsic effectors) of the metabolic depression in these animals makes them ideal models in which to study the regulation of protein synthesis and to search for intrinsic cues for metabolic depression.

The objective of this study was to determine whether eIF2α is involved in the downregulation of protein synthesis during metabolic depression associated with estivation. We have measured eIF2α mRNA levels in the snail *H. aspersa* and the levels and phosphorylation status of eIF2α in both the snail and the frog *N. sutor*. This comparative study highlights some fundamental differences in the expression and phosphorylation of eIF2α in two estivating species that may have regulatory significance and is the first study to provide evidence for a possible mechanism by which the rate of protein synthesis is downregulated during the metabolic depression associated with estivation.

Materials and methods

Materials

Rabbit anti-eIF2α(P) was purchased from Research Genetics (Huntsville, AL, USA). Sheep anti-rabbit IgG horseradish peroxidase was purchased from Silenus (Melbourne, Australia). SuperSignal West Femto Maximum Sensitivity Substrate was purchased from Pierce (Rockford, IL, USA). Hybond N, Hybond C+ extra, Hyperfilm MP, [γ-^{32}P]dCTP and [γ-^{32}P]ATP were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Reagents for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were from ICN Biomedicals (Aurora, OH, USA). PCR reagents, phenylmethanesulphonyl fluoride (PMSF), Leupeptin, E64 and ATP were from Boehringer (Mannheim, Germany). Benzamidine, aprotinin, Na_3VO_4, dithiothreitol (DTT), Hepes, EGTA, Tween 20, bovine serum albumin (BSA) and β-glycerophosphate were from Sigma Chemical Co. (St Louis, MO, USA). All other reagents were from BDH chemicals (distributed by MERCK, Victoria, Australia).

Experimental animals

Helix aspersa Müller were collected, maintained and sectioned as described in Pakay et al. (2002). *Neobatrachus sutor* Main 1957 were collected 80 km south of Newman, Western Australia. They were not fed but either sectioned or induced to estivate within a few days after arrival in the lab. They were kept at 20°C, individually, in 750 ml plastic containers with a sealed lid, which had a 4 mm-diameter hole for gas exchange. They were initially in approximately 500 ml of moist soil, which was allowed to dry out over time. All frogs cocooned as the soil dried out. Cocooned frogs were allowed to estivate for 5 months. Frogs were double pithed and sectioned by hand. Tissues were removed within 2–3 min and were immediately frozen in liquid nitrogen.

Sample preparation

Tissues were thawed on ice and homogenised in nine volumes of assay buffer using an Ultra-Turrax T25 homogeniser (Janke and Kunkel IKA Labortechnik) at 20 500 r.p.m. for 30 s on ice. Assay buffer consisted of 120 mmol l^{-1} KCl, 2 mmol l^{-1} DTT, 20 mmol l^{-1} Hepes pH 7.2, 12 mmol l^{-1} $MgCH_3COO$, 40 μmol l^{-1} ATP, 50 mmol l^{-1} NaF, 1 mmol l^{-1} benzamidine, 20 μg ml^{-1} leupeptin, 1 mmol l^{-1} PMSF, 10 μg ml^{-1} aprotinin and 5 μg ml^{-1} E64. This assay buffer was used because it is compatible with HCR (heme controlled repressor kinase), a specific eIF2α kinase (hence the ATP and Mg^{2+}), and contains a cocktail of protease inhibitors but no divalent cation chelators, kinase inhibitors or detergent. As it was a concern that the levels of phosphorylated eIF2α [eIF2α(P)] detected using this buffer could be affected by the action of kinases or metalloproteases or by the fact that not all membranes were disrupted, extraction was also performed using an alternate lysis buffer, which, in addition to the assay buffer described above, contained 4 mmol l^{-1} EDTA, 2 mmol l^{-1} EGTA, 0.1 mmol l^{-1} Na_3VO_4, 20 mmol l^{-1} β-glycerophosphate and 1% (v/v) Triton X-100 but no $MgCH_3COO$ and ATP. There were no differences in signal intensities on western blots between extracts prepared in the two buffers.

Post-mitochondrial supernatant was prepared by centrifuging homogenates at 13 800 ***g*** for 20 min at 4°C. The supernatant was removed and an aliquot stored for protein determination (DC protein assay kit; Bio-Rad, Hercules, CA, USA) using BSA as a standard.

Preparing recombinant rat eIF2α(P) for use as a positive control and standard

A positive control for western analysis of eIF2α(P) was prepared by phosphorylating recombinant rat eIF2α–FLAG (eIF2α expressed with the FLAG fusion octapeptide) using HCR partially purified to approximately 20% purity from rabbit reticulocytes (Jackson and Hunt, 1985). HCR was lyophilised after purification and redissolved in 20 mmol l^{-1} Tris-HCl pH 7.5, 100 mmol l^{-1} KCl, 0.1 mmol l^{-1} EDTA, 7 mmol l^{-1} β-mercaptoethanol, 30% (v/v) glycerol. Phosphorylation involved incubating eIF2α–FLAG with a sufficient amount of the HCR preparation to ensure complete phosphorylation (Kimball et al., 1998). The reaction was monitored by the addition of [γ-^{32}P]ATP, and aliquots were removed at 0 min, 5 min, 20 min and 40 min post addition of HCR. The aliquots were subjected to SDS–PAGE followed by autoradiography and densitometric analysis, which revealed that complete phosphorylation of eIF2α–FLAG occurred within 5 min.

Phosphorylation of endogenous eIF2α in frog and snail extracts

It was necessary to determine the total amount of eIF2α (phosphorylated and unphosphorylated) as well as the amount of eIF2α(P) in the post-mitochondrial supernatants. Since we were only using rabbit anti-eIF2α(P) (an antibody specific to the phosphorylated form of the protein only) in the western analysis, the total amount of eIF2α in post-mitochondrial supernatant was determined by converting (by phosphorylation) all of the endogenous eIF2α into eIF2α(P) and then determining the total amount of eIF2α(P). Phosphorylation was carried out using HCR. HCR (0.5 μl of the preparation described above) was added to a duplicate sample of each post-mitochondrial supernatant, containing 100 μg of protein in a total volume of 25 μl, and incubated at 37°C. Subsequently, the levels of total eIF2α and eIF2α(P) were determined by quantifying the amount of eIF2α(P) in both the phosphorylated sample (with HCR) and the unphosphorylated sample (without HCR) from each post-mitochondrial supernatant.

To ensure that all of the endogenous eIF2α was converted to eIF2α(P) by the HCR treatment, aliquots containing 25 μg of protein were removed at 20 min, 40 min and 60 min after the addition of HCR and the reaction stopped by adding SDS–PAGE loading buffer. The above reaction was also performed with 200 μmol l^{-1} ATP (*vs* 40 μmol l^{-1} ATP in the normal lysis buffer) in the reaction mix to ensure that ATP was not limiting in the reaction. In addition, to ensure that the HCR itself was not losing activity during the time course, additional HCR (0.5 μl) was added following the 40 min time point. The time courses were done using both awake and estivating samples.

Western analysis

The samples (equal amounts of protein) were subjected to SDS–PAGE and electrophoretically transferred using a Bio-Rad SD semi-dry trans-blot apparatus with Towbin buffer [25 mmol l^{-1} Tris-HCl pH 8.3, 150 mmol l^{-1} glycine, 20% (v/v) methanol] to Hybond C+ extra nitrocellulose membranes. After air drying for 30 min, the membranes were blocked for 1 h in 5% non-fat milk in TBST [20 mmol l^{-1} Tris-HCl pH 7.6, 137 mmol l^{-1} NaCl and 0.1% (v/v) Tween-20]. The membranes were then incubated for 2 h with a 1:5000 dilution of primary antibody [rabbit anti-eIF2α(P)] in 1% non-fat milk in TBST.

For western analysis of *H. aspersa* post-mitochondrial supernatant, the primary antibody was first pre-absorbed against *H. aspersa* hemolymph in order to remove keyhole limpet hemocyanin (KLH)-related non-specificity (Pakay et al., 2002). The membranes were then washed in TBST and incubated for 1 h with a 1:10 000 dilution of secondary antibody in 5% non-fat milk in TBST (sheep anti-rabbit IgG conjugated to horseradish peroxidase). All incubations and washes were performed at room temperature. Immunoreactive bands were detected by enhanced chemiluminescence (Supersignal West Femto Maximum Sensitivity Substrate) followed by exposure to autoradiography film. Densitometry was performed on scanned films using NIH Image 1.62. Immunoreactive bands were quantified by comparing their density to the density of known quantities of recombinant rat eIF2α(P)–FLAG. In order to verify equal loading and transfer, membranes were stained for total protein by Ponceau S staining.

Cloning and northern analysis of H. aspersa *eIF2α*

Total RNA was prepared from *H. aspersa* hepatopancreas using the acid guanidium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi, 1987). However, RNA prepared using this method contained a contaminant that inhibited any subsequent reverse transcription reactions. Therefore, to remove the contaminant, RNA from which the poly(A^+) RNA fraction was to be separated was first precipitated by salt, a procedure normally used when the RNA is contaminated by glycogen (Sambrook et al., 1989). Poly(A^+) RNA was prepared by chromatography of total RNA on an oligo-dT cellulose column with a single binding and elution cycle (Sambrook et al., 1989).

cDNA was synthesized from poly(A^+) RNA using the primer 5′-GCGGCCGCTTGAATTCCCAC$(T)_{17}$-3′ (Sambrook et al., 1989), and PCR was performed using a pair of degenerate primers, 5′-GC(AGCT)GA(AG)ATGGG(AGCT)GC(AGCT)-TA(TC)-3′ and 5′-AT(AG)TA(AGCT)CC(TC)TT(TC)TC-(TC)TT(AG)TC-3′ (Macromolecular Resources), based on the amino acid sequences conserved between yeast, *Drosophila melanogaster* and humans corresponding to residues 27–33 and 75–81 of human eIF2α. The PCR reaction yielded a 150 bp product that was directly cloned (pGEM®-T Easy Vector; Promega, Annandale, NSW, Australia) and sequenced (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems, Foster City, CA, USA). Analysis of sequencing reactions was carried out by the Department of Clinical Immunology at Royal Perth Hospital (Perth, Australia). A BLAST search revealed that the 150 bp fragment corresponded to eIF2α. A primer based on the 5′ end of this sequence and an oligo-dT primer were used to obtain a larger cDNA corresponding to eIF2α. This clone was sequenced and used to prepare a probe in the subsequent northern analysis.

Total RNA was analysed by northern analysis by hybridisation after formaldehyde gel electrophoresis (Sambrook et al., 1989). The probe was prepared by ^{32}P-labeling of the eIF2α message fragment by random priming (Sambrook et al., 1989). After transfer, hybridisation and washing, the membranes were exposed to a BAS-IIIs phosphorimaging plate (Fuji, Stamford, CT, USA), and the plates scanned using a BAS 1000 phosphorimager (Fuji). Densitometric analysis of the bands was performed using the program NIH Image 1.62. Equal loading of RNA for northern analysis was checked by densitometric analysis of the methylene-blue-stained rRNA band (Sambrook et al., 1989).

Statistics

All values are quoted as means ± S.E.M. (*N*). Comparisons between time points, tissues and/or treatments were made using unpaired *t*-tests. Statistical significance is quoted at the 5% level.

Results

Sequencing and northern analysis

The partial cDNA clone representing *H. aspersa* eIF2α mRNA is 2314 bp in length. Comparing the translation of the cDNA clone to the known amino acid sequences of yeast and human eIF2α (Fig. 1), there are approximately 32 amino acids missing from the amino terminus. *H. aspersa* eIF2α shows 62% and 48% identity at the amino acid level with human and yeast eIF2α, respectively, with a 19-amino-acid region surrounding the phosphorylation site completely conserved between the three species. The 3′ untranslated region is 1446 bp in length. The message is polyadenylated and the 3′ untranslated region (UTR) contains five putative polyadenylation signal sites at positions 988, 1068, 1123, 1943 and 2278.

Northern analysis of total RNA from *H. aspersa* tissues reveals that the cDNA hybridised with two messages (estimated to be 2.6 kb and 1.4 kb in length) in hepatopancreas. Northern analysis revealed that these messages were equally expressed in awake and estivating individuals. Methylene blue staining demonstrated that there was an equal amount of RNA loaded in each lane, as judged by the intensity of the ribosomal RNA band. There were no differences in the levels of expression between the different sized transcripts within each tissue type.

```
                ** ****  * ****************** ** ** * *   * *  *******
H. aspersa   1  YVKLLEYNDI EGMILLSELS RRRIRSINKL IRVGRNECVV VISVDKEKGY
Yeast       33  YVKLLEYDNI EGMILLSELS RRRIRSIQKL IRVGKNDVAV VLRVDKEKGY
Human       33  YVSLLEYNNI EGMILLSELS RRRIRSINKL IRIGRNECVV VIRVDKEKGY
                ********* *   *** *   * * * *** *  **       ** *
H. aspersa  51  IDLSKRRVSP EEVVKCEEKF AKAKAVNSIL RHVAELRGFK SSEKLEDLYE
Yeast       83  IDLSKRRVSS EDIIKCEEKY QKSKTVHSIL RYCAE----K FQIPLEELYK
Human       83  IDLSKRRVSP EEAIKCEDKF TKSKTVYSIL RHVAEVLEYT KDEQLESLFQ
                  **    *         * * *                          *
H. aspersa 101  RTAWFFDAKY GKAG-ASYEA FRLAVQNPNV LDDCDIDEET KSCLHDNICR
Yeast      129  TIAWPLSRKF GHA----YEA FKLSIIDETV WEGIEPPSKD VLDELKNYIS
Human      133  RTAWVFDDKY KRPGYGAYDA FKHAVSDPSI LDSLDLNEDE REVLINNINR
                  **** *** ***  * *   * * ** * * *      ***  *  *  * *
H. aspersa 151  --LTPQSVKI RADVDVACTY YDGVDAVKRA LKKGLELSTE SMPIKINLIA
Yeast      175  KRLTPQAVKI RADVEVSCFS YEGIDAIKDA LKSAEDMSTE QMQVKVKLVA
Human      183  -RLTPQAVKI RADIEVACYG YEGIDAVKEA LRAGLNCSTE NMPIKINLIA
                 * ** **   *    *    *      * *  *       *        *
H. aspersa 199  PPLYVVTTN TLERTEGLER LNKALQAIKE EITAAKGVFN IQQEARVVSDM
Yeast      225  APLYVLTTQ ALDKQKGIEQ LESAIEKITE VITKYGGVCN ITMPPKAVTAT
Human      232  PPRYVMTTT TLERTEGLSV LSQAMAVIKE KIEEKRGVFN VQMEPKVVTDT
                   **       *        **
H. aspersa 249  DEIELEKQL QKLEEAPQER AGDDDSDAEE EEEDEM
Yeast      275  EDAELQALL ESKELDNRSD SEDDEDESDD E
Human      282  DETELARQM ERLERENAEV DGDDDAEEME AKAED
```

Fig. 1. The partial predicted amino acid sequence of *Helix aspersa* eIF2α, deduced from translation of the *H. aspersa* eIF2α cDNA sequence, aligned with the predicted sequences of yeast (*Saccharomyces cerevisiae*) and human eIF2α. Conserved residues are indicated with an asterisk. The multiple alignment was performed manually after comparing each of the pairwise sequence alignments made using the program ALIGN by FASTA (University of Virginia, Charlottesville, VA, USA).

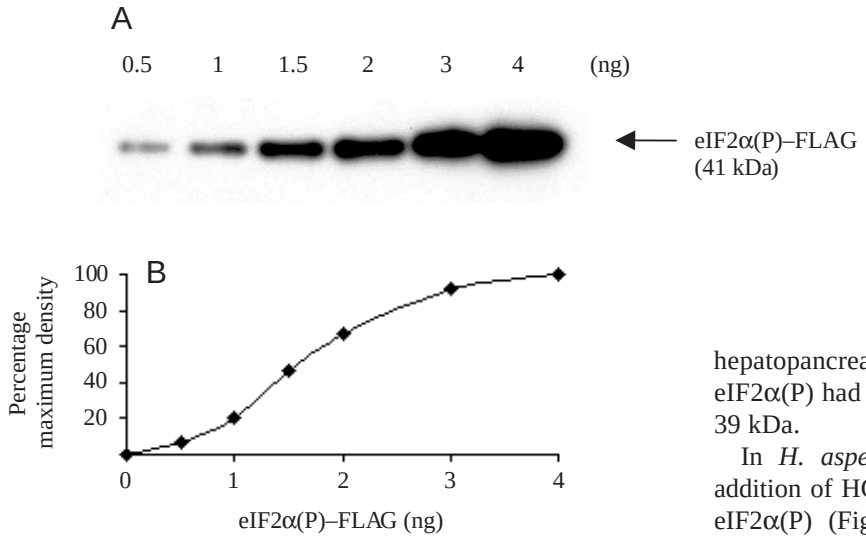

Fig. 2. (A) Representative western blot showing varying amounts of eIF2α–FLAG(P) using the immunoblot procedure described in the Materials and methods. The film of the immunoblot was scanned, and densitometric analysis was performed using the program NIH Image 1.62. (B) Representative standard curve of density *versus* amount of eIF2α–FLAG(P). Similar standards were run on all western blots to aid quantification.

Phosphorylating and detecting recombinant rat eIF2α–FLAG

The eIF2α–FLAG(P) was detected by western analysis, which indicated an apparent molecular mass of 41 kDa (Fig. 2A). The primary antibody was specific for the phosphorylated form of the protein only. There was a sigmoidal relationship between the density of the eIF2α–FLAG(P) band and the amount loaded (Fig. 2B). Using the described working concentration of the primary and secondary antibody, as little as 0.1 ng of eIF2α–FLAG(P) could be detected.

Phosphorylation and detection of eIF2α in snail and frog extracts

Endogenous eIF2α was phosphorylated by HCR in post-mitochondrial supernatant from both *H. aspersa* hepatopancreas and *N. sutor* liver. In both tissues, the eIF2α(P) had an apparent molecular mass by SDS–PAGE of 39 kDa.

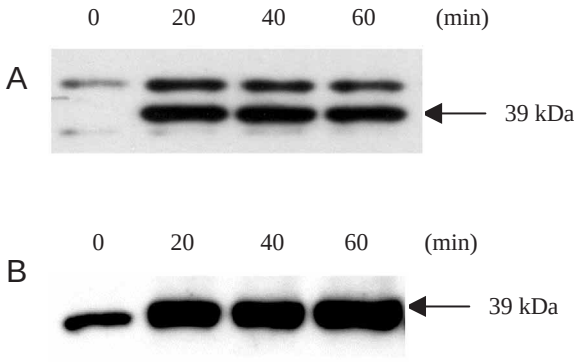

Fig. 3. Representative western immunoblots showing the time course of phosphorylation of endogenous eIF2α with HCR (heme controlled repressor kinase). Post-mitochondrial supernatant was isolated from *H. aspersa* hepatopancreas (A) or from *N. sutor* liver (B) and incubated with HCR in the presence of 40 μmol l^{-1} ATP. Aliquots were removed immediately prior to the addition of HCR (0 min) and at 20 min, 40 min and 60 min after the addition of HCR. Proteins were separated using SDS–PAGE, transferred to nitrocellulose membranes then probed with anti-eIF2α(P).

In *H. aspersa* post-mitochondrial supernatant, after the addition of HCR there was a rapid increase in the amount of eIF2α(P) (Fig. 3A). There was no apparent difference in quantity between the 20 min and 60 min time points. Increasing the concentration of ATP in the reaction from 40 μmol l^{-1} to 200 μmol l^{-1} had no effect on the density of the 39 kDa band after 60 min. Also there was no effect of adding additional HCR after 40 min. Note that *H. aspersa* extracts also showed an additional band at 42 kDa that could be detected in the absence of HCR. This band sometimes increased in intensity after the addition of HCR, but not consistently between samples (Fig. 3A).

Similarly, in *N. sutor* post-mitochondrial supernatant, after the addition of HCR there was a rapid increase in the amount of eIF2α(P) and there was no difference between the 20 min and 60 min time points (Fig. 3B). However, in *N. sutor* only the band corresponding to eIF2α(P) was detected. In both *N. sutor* and *H. aspersa*, there was no difference in the time course of phosphorylation with HCR between the awake and estivating states.

To calculate the ratio of eIF2α(P) to total eIF2α, the amount of eIF2α(P) before the addition of HCR was compared with that present after phosphorylation with HCR. These levels were quantified from eIF2α(P)–FLAG standards run on the same blot. In *H. aspersa* (Fig. 4A), there was no detectable endogenous eIF2α(P) in post-mitochondrial supernatant in either awake or estivating hepatopancreas but, since the minimum amount of eIF2α(P)–FLAG that could be detected was 0.1 ng, we can calculate that less than 12% of the eIF2α was phosphorylated in the snail lysates. The total amount of eIF2α was not significantly different between awake and estivating tissues (Table 1). The total amount of eIF2α represented approximately 0.002% of the total amount of protein in post-mitochondrial supernatant.

In *N. sutor*, there was more total and phosphorylated eIF2α in estivating *versus* awake tissues (Fig. 4B; Table 1). The ratio of phosphorylated to unphosphorylated eIF2α was significantly higher in estivating tissue compared with that in awake tissue (Table 1; $P<0.05$, $N=6$). The total amount of eIF2α represented approximately 0.005% and 0.008% of the total amount of protein in awake and estivating post-mitochondrial supernatant, respectively.

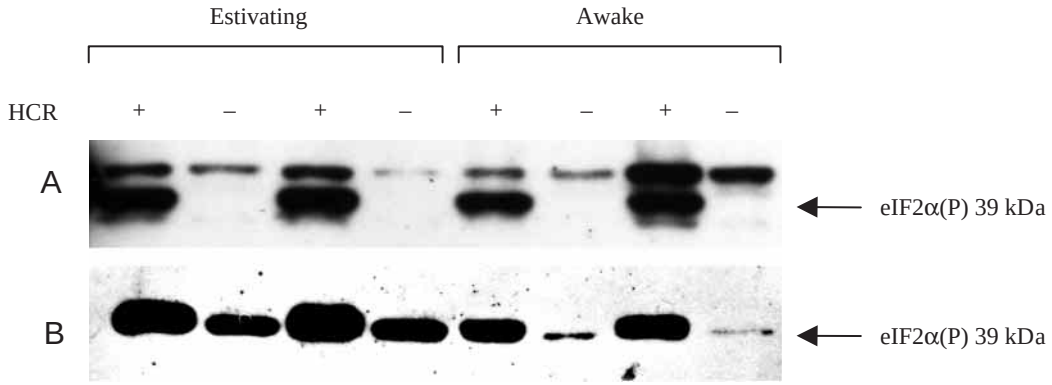

Fig. 4. Representative western immunoblots of post-mitochondrial supernatant either unphosphorylated or phosphorylated with HCR (heme controlled repressor kinase). (A) *Helix aspersa* hepatopancreas. (B) *Neobatrachus sutor* liver. Two identical samples from an individual were analysed, one sample was left untreated (–HCR) and the other was phosphorylated (+HCR). Samples from the same individual are shown as pairs on the figure. Proteins were separated using SDS–PAGE, transferred to nitrocellulose membranes and probed with anti-eIF2α(P). The comparison between samples incubated with HCR (+HCR) and without HCR (–HCR) was used to determine the ratio of phosphorylated to unphosphorylated eIF2α in both the awake and estivating states. The bands corresponding to eIF2α(P) have been quantified and are summarised in Table 1.

Discussion

Sequence and expression of H. aspersa *eIF2α mRNA*

As expected, the predicted partial sequence of *H. aspersa* eIF2α shares a high amino acid identity with other described eIF2αs. The most likely explanation for the different sized eIF2α mRNAs estimated from the northern analysis is that they differ in the size of their 3′ UTR. This corresponds to the sizes of messages predicted from the alternate polyadenylation sites in the cDNA sequence, assuming that *H. aspersa* eIF2α has a similar length coding region and 5′ UTR to other eIF2α sequences (Cigan et al., 1989; Ernst et al., 1987; Qu and Cavener, 1994). A similar pattern of eIF2α mRNA expression is seen in mouse and human tissues, where synthesis of various forms of eIF2α mRNA occurs in a tissue-specific manner. The biological significance of the different transcripts is unknown in *H. aspersa*, but it has been shown that there are differences in stability for the different transcripts in human T cells (Miyamoto et al., 1996).

There was no differential expression of the eIF2α mRNAs between the awake and estivating states of *H. aspersa*. The level of eIF2α protein per mg of total protein is the same in the awake and estivating states. The data cannot be used to draw conclusions about the rate of turnover of either eIF2α message or protein, but if eIF2α does play a role in regulating protein synthesis in *H. aspersa* during transitions between states, neither eIF2α mRNA nor protein levels are part of the mechanism. This leaves eIF2α phosphorylation as the only candidate mechanism by which eIF2α could be involved.

Detection of eIF2α phosphorylation – validation of the methods

The western analysis presented us with three initial problems. First, we needed to be able to detect eIF2α in two distantly related species. We therefore used an antibody based on a region conserved across all eukaryotes, the phosphorylated regulatory phosphorylation site. We were able to use this antibody to detect eIF2α(P) in both snail and frog extracts. The apparent size of the eIF2α(P) (39 kDa) was the same for both *H. aspersa* and *N. sutor* and was similar to the apparent size of mammalian eIF2α in SDS–PAGE (38 kDa). The 42 kDa band detected in *H. aspersa* extracts was phosphorylated by HCR in an individual-specific manner. Since all eIF2αs described so far have similar molecular masses (Deharo et al., 1996), it is unlikely that a 42 kDa protein is an eIF2α. It is unknown if it represents a physiological substrate for HCR, but it is likely that it shares sequence homology with the eIF2α phosphorylation site since it is recognised by both HCR and the primary antibody.

The use of this antibody led to the second problem, which was that this antibody measures the levels of eIF2α(P) but gives no information about the more physiologically relevant ratio of phosphorylated to unphosphorylated eIF2α. This is

Table 1. *Levels of total and phosphorylated eIF2α in* H. aspersa *hepatopancreas and* N. sutor *liver*

	Awake			Estivating		
	Total eIF2α	eIF2α(P)	Ratio	Total eIF2α	eIF2α(P)	Ratio
H. aspersa	0.82±0.12	n.d.<0.1	NA	0.97±0.09	n.d.<0.1	NA
N. sutor	2.40±0.35	0.55±0.15	0.22±0.11	4.00±0.55*	1.95±0.25*	0.52±0.08*

The values for total eIF2α and phosphorylated eIF2α [eIF2α(P)] are expressed as ng per 50 mg of post-mitochondrial supernatant protein (means ± S.E.M.; n=6). The ratio is the proportion of the total eIF2α that is phosphorylated (means ± S.E.M.; n=6). n.d., not detectable; NA, not applicable. An asterisk denotes that the value is significantly different from its comparative awake value (P<0.05).

especially true when long-term changes are involved, as changes in this ratio can occur without changes in the amount of eIF2α(P). We overcame this problem by totally phosphorylating all of the endogenous eIF2α in a sample, thereby enabling us to measure the total eIF2α as well as the phosphorylated form with the same antibody. The system we describe allows the use of SDS–PAGE with a single primary antibody, allowing simpler quantification of eIF2α and eIF2α(P) levels. This system relies on a single antibody specific for the conserved phosphorylation site and on phosphorylation by HCR, which has been shown to phosphorylate eIF2α from all eukaryotes tested so far (Mehta et al., 1986). Therefore, this system potentially enables the phosphorylation state of eIF2α to be determined in any eukaryotic system.

The third problem in this study was that we needed to quantify levels of eIF2α(P) in order to accurately estimate the ratio of eIF2α/eIF2α(P). To this end, we have:

(1) determined that the primary antibody is specific for only the phosphorylated form of eIF2α–FLAG;

(2) quantified the amounts of eIF2α(P) against known quantities of eIF2α(P)–FLAG (produced by phosphorylating recombinant rat eIF2α–FLAG with HCR); this was necessary as autoradiography film was used to obtain the required sensitivity and there is a sigmoidal relationship between the quantity of eIF2α(P) and the density of bands corresponding to eIF2α(P) on the film; and

(3) made the assumptions that the affinity of the primary antibody to endogenous *H. aspersa* and *N. sutor* eIF2α(P) is the same as the affinity to eIF2α–FLAG(P) and that the percentage recovery of eIF2α is the same from both tissues.

The absolute levels of eIF2α that we report here (in terms of the percentage of total protein present) in awake *H. aspersa* hepatopancreas (0.002%) and *N. sutor* liver (0.005%) are of a comparable magnitude to those reported for rat liver (0.004%; Everson et al., 1989) and rabbit tissues (0.003%; Oldfield et al., 1994) (assuming that these mammalian tissues contain 100 mg protein g^{-1} wet mass). Using the same system to determine the level of eIF2α in neonatal rat cardiomyoctes (Casey et al., 2002), we have found that approximately 0.006% of total protein is eIF2α, a value comparable with other mammalian systems.

Changes in phosphorylation state of eIF2α with estivation

The extent of eIF2α phosphorylation required to significantly suppress protein synthesis depends upon the eIF2/eIF2B ratio, as it is the high-affinity binding of eIF2α(P) to eIF2B that results in the inhibition of protein synthesis. This value is known to be somewhat variable among different animals and tissues. For example, the ratio is 5 in calf and rat brain (White et al., 2000), 2.5 in rabbit liver and 10 in rabbit brain (Oldfield et al., 1994). The variation appears to predominantly depend on eIF2B levels (Oldfield et al., 1994). The ratio of eIF2/eIF2B is a good predictor of the level of eIF2α phosphorylation required to completely inhibit protein synthesis (Everson et al., 1989). For example, in reticulocyte lysates the ratio of eIF2/eIF2B is approximately 5, and the level of eIF2α phosphorylation required to completely inhibit protein synthesis is approximately 25–30% (Pain, 1996). In Ehrlich cells, the ratio of eIF2/eIF2B is approximately 2, and the level of eIF2α phosphorylation required to completely inhibit protein synthesis is approximately 50–60% (Scorsone et al., 1987). The data for snails are inconclusive because endogenous levels of the phosphorylated form of eIF2α are below the limit of detection. However, the data are still useful as they define the limits of the eIF2α/eIF2α(P) ratio, which in turn (see above) can be used to predict the eIF2/eIF2B ratio. The eIF2/eIF2B ratio is not known for any non-mammalian species, but our data suggest (since we can calculate that less than 12% of the eIF2α was phosphorylated in the snail lysates) that in *H. aspersa* hepatopancreas the ratio would have to exceed 10 for phosphorylation of eIF2α to exert any control over the rate of protein synthesis.

The data from the frog liver are easier to interpret as both endogenous eIF2α and eIF2α(P) are detectable. The percentage of eIF2α(P) and, in contrast to the snail, the total amount of eIF2α increases from the awake to the estivating state. The increased percentage of phosphorylated eIF2α in the estivating state is consistent with the 67% decrease in the rate of protein synthesis seen in *N. sutor* liver in this state (Fuery et al., 1998). Due to the high proportion of eIF2α that is phosphorylated, for eIF2α phosphorylation to be involved in regulating the rate of protein synthesis in estivating *N. sutor* liver the ratio of eIF2/eIF2B would only need to be approximately 2. The function of increasing the level of eIF2α during estivation in the frog *versus* the snail is unknown but perhaps the difference reflects the degree of precision required to modulate the rate of protein synthesis (assuming that levels of eIF2B remain constant). For example, a low eIF2/eIF2B ratio under normal conditions would allow a high precision in controlling the rate of protein synthesis, as large changes in the percentage phosphorylation would result in relatively small changes in the rate of protein synthesis. However, with a low ratio it would be more difficult to completely downregulate protein synthesis, as a greater percentage of the eIF2α would need to be phosphorylated. Therefore, by upregulating the amount of eIF2α it would be easier to obtain the percentage of eIF2α(P) that is required to downregulate protein synthesis during estivation. Possibly, the snail needs less precision in its control over protein synthesis during normal conditions and therefore maintains a high eIF2/eIF2B ratio, which allows it to completely downregulate the rate of protein synthesis during estivation without needing to synthesise more eIF2α. This idea would seem consistent with the greater metabolic depression during estivation in the snail compared with the frog.

An alternative explanation is that the differences are not functional but obligatory. Possibly, the phosphorylated form of eIF2α is less susceptible to proteolytic breakdown; thus, upregulating the phosphorylated species [eIF2α(P)] is necessarily associated with an increase in the total level of eIF2α. There is evidence for a similar mechanism in apoptotic HeLa cells, where caspase cleaves eIF2α but shows a

preference for the unphosphorylated form and does not cleave eIF2α(P) complexed with eIF2B (Marissen et al., 2000). If this is a similar case, then the increased level of eIF2α expression seen in the estivating frog is not the result of an active upregulation of eIF2α levels but is due to a higher proportion of the more stable species [eIF2α(P)]. The data are consistent with this hypothesis, as the increase in the amount of eIF2α in *N. sutor* during estivation is equal to the increase in the amount of eIF2α(P).

Potential mechanisms for changes in eIF2α phosphorylation status in N. sutor

There are several possible mechanisms by which the percentage of eIF2α(P) could increase in the liver of estivating *N. sutor*. The downregulation of protein synthesis in other systems has been attributed to an increase in eIF2α kinase activity. Specific eIF2α kinases that are upregulated in activity in response to diverse stimuli, including heme deprivation, dsRNA, amino acid deprivation and endoplasmic reticulum stress, have been identified.

Alternatively, an increase in eIF2α(P) can occur due to a decrease in eIF2α phosphatase activity. For example, during hibernation in ground squirrels, the accumulation of eIF2α(P) has been attributed to a decrease in phosphatase (PP1) activity modulated by the stress-inducible growth arrest and DNA damage protein GADD34 (Connor et al., 2001). A similar mechanism may regulate protein synthesis in turtles that depress both metabolic rate and protein synthesis during anoxia, as during anoxia there is a decrease in PP1 activity in liver and brain (Mehrani and Storey, 1995).

Aside from modulation of kinase and/or phosphatase activity, the accumulation of eIF2α(P) in *N. sutor* could also be due to a change in accessibility of eIF2α to kinase or phosphatase activity. For example, changes in the affinity of p67 (a cellular glycoprotein found in reticulocyte lysate, which binds the γ-subunit of eIF2 and masks the regulatory phosphorylation site of eIF2α) could regulate changes in eIF2α phosphorylation state (Datta and Datta, 1999).

Summary

This is the first study to investigate the regulation of the initiation of translation by eIF2α in estivating organisms and provides evidence for a possible mechanism by which the rate of protein synthesis is downregulated during the metabolic depression associated with estivation. By finding a change in the phosphorylation status of a key translation regulatory protein (eIF2α) in *N. sutor* during estivation, we have provided the potential starting point for a link between the downregulation of an energy-consuming pathway and the initial intrinsic cue for metabolic depression associated with estivation. Future work will need to establish the ratio of eIF2α/eIF2B and also estimate changes in eIF2B activity in these tissues to determine if the changes in the percentage of eIF2α phosphorylation in *N. sutor* could account for the observed changes in the rate of protein synthesis. If the increase in phosphorylated eIF2α does lead to a decrease in guanine nucleotide exchange on eIF2, then identifying whether the increased phosphorylation is due to increased kinase or decreased phosphatase activity will be the next necessary step in delineating the mechanism of signal transduction between the intrinsic cue for estivation and the downregulation of protein synthesis. In the case of *H. aspersa*, future investigation is needed to determine whether there is a role for eIF2α phosphorylation in the downregulation of protein synthesis, possibly by directly assaying guanine nucleotide exchange on eIF2 in the awake and estivating states. Virtually nothing is known about the potential regulatory mechanisms in any estivating animal, but the data are accumulating and suggest that research in this direction will be an effective strategy to delineate the mechanisms of this common, but inscrutable, form of metabolic depression.

References

Bailey, J. R. and Driedzic, W. R. (1996). Decreased total ventricular and mitochondrial protein synthesis during extended anoxia in the turtle heart. *Am. J. Physiol.* **271**, R1660-R1667.

Casey, T. M., Pakay, J. L., Guppy, M. and Arthur, P. (2002). Hypoxia causes downregulation of protein and RNA synthesis in noncontracting mammalian cardiomyocytes. *Circ. Res.* **90**, 777-783.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

Cigan, A. M., Pabich, E. K., Feng, L. and Donahue, T. F. (1989). Yeast translation initiation suppressor sui2 encodes the alpha subunit of eukaryotic initiation factor 2 and shares sequence identity with the human alpha subunit. *Proc. Natl. Acad. Sci. USA* **86**, 2784-2788.

Connor, J. H., Weiser, D. C., Li, S., Hallenbeck, J. M. and Shenolikar, S. (2001). Growth arrest and DNA damage-inducible protein GADD34 assembles a novel signaling complex containing protein phosphatase 1 and inhibitor 1. *Mol. Cell. Biol.* **21**, 6841-6850.

Datta, B. and Datta, R. (1999). Induction of apoptosis due to lowering the level of eukaryotic initiation factor 2-associated protein, p67, from mammalian cells by antisense approach. *Exp. Cell Res.* **246**, 376-383.

Deharo, C., Mendez, R. and Santoyo, J. (1996). The eIF2-alpha kinases and the control of protein synthesis. *FEBS Lett.* **10**, 1378-1387.

Douglas, N., Giband, M., Altosaar, I. and Storey, K. B. (1994). Anoxia induces changes in translatable mRNA populations in turtle organs: a possible adaptive strategy for anaerobiosis. *J. Comp. Physiol. B* **164**, 405-414.

Ernst, H., Duncan, R. F. and Hershey, J. W. B. (1987). Cloning and sequencing of complementary DNAs encoding the α-subunit of translational initiation factor eIF-2. *J. Biol. Chem.* **262**, 1206-1212.

Everson, W. V., Flaim, K. E., Susco, D. M., Kimball, S. R. and Jefferson, L. S. (1989). Effect of amino acid deprivation on initiation of protein synthesis in rat hepatocytes. *Am. J. Physiol.* **256**, C18-C27.

Fraser, K. P. P., Houlihan, D. F., Lutz, P. L., Leone-Kabler, S., Manuel, L. and Brechin, J. G. (2001). Complete suppression of protein synthesis during anoxia with no post-anoxia protein synthesis debt in the red-eared slider turtle *Trachemys scripta elegans*. *J. Exp. Biol.* **204**, 4353-4360.

Frerichs, K. U., Smith, C. B., Brenner, M., Degracia, D. J., Krause, G. S., Marrone, L., Dever, T. E. and Hallenbeck, J. M. (1998). Suppression of protein synthesis in brain during hibernation involves inhibition of protein initiation and elongation. *Proc. Natl. Acad. Sci. USA* **95**, 14511-14516.

Fuery, C. J., Withers, P. C., Hobbs, A. A. and Guppy, M. (1998). The role of protein synthesis during metabolic depression in the australian desert frog *Neobatrachus centralis*. *Comp. Biochem. Physiol. A* **119**, 469-476.

Guppy, M. and Withers, P. (1999). Metabolic depression in animals: physiological perspectives and biochemical generalizations. *Biol. Rev. Camb. Philos. Soc.* **74**, 1-40.

Hofmann, G. E. and Hand, S. C. (1994). Global arrest of translation during invertebrate quiescence. *Proc. Natl. Acad. Sci. USA* **91**, 8492-8496.

Hu, B.-R., Ou Yang, Y.-B. and Wieloch, T. (1993). Heat-shock inhibits

protein synthesis and eIF-2 activity in cultured cortical neurons. *Neurochem. Res.* **18**, 1003-1007.

Jackson, R. J. and Hunt, T. (1985). A novel approach to the isolation of rabbit reticulocyte haem-controlled eIF2 alpha protein kinase. *Biochim. Biophys. Acta* **826**, 224-228.

Kimball, S. R., Antonetti, D. A., Brawley, R. M. and Jefferson, L. S. (1991). Mechanism of inhibition of peptide chain initiation by amino acid deprivation in perfused rat liver. Regulation involving inhibition of eukaryotic initiation factor 2 alpha phosphatase activity. *J. Biol. Chem.* **266**, 1969-1976.

Kimball, S. R., Horetsky, R. L., Jagus, R. and Jefferson, L. S. (1998). Expression and purification of the alpha-subunit of eukaryotic initiation factor eIF2 – use as a kinase substrate. *Prot. Express. Purif.* **12**, 415-419.

Kumar, R., Azam, S., Sullivan, J. M., Owen, C., Cavener, D. R., Zhang, P., Ron, D., Harding, H. P., Chen, J. J., Han, A. et al. (2001). Brain ischemia and reperfusion activates the eukaryotic initiation factor 2alpha kinase, PERK. *J. Neurochem.* **77**, 1418-1421.

Larade, K. and Storey, K. B. (2002). Reversible suppression of protein synthesis in concert with polysome disaggregation during anoxia exposure in *Littorina littorea*. *Mol. Cell. Biochem.* **232**, 121-127.

Marissen, W. E., Guo, Y. W., Thomas, A., Matts, R. L. and Lloyd, R. E. (2000). Identification of caspase 3-mediated cleavage and functional alteration of eukaryotic initiation factor 2 alpha in apoptosis. *J. Biol. Chem.* **275**, 9314-9323.

Martin de la Vega, C., Burda, J. and Salinas, M. (2001). Ischemia-induced inhibition of the intitiation factor 2α phosphatase activity in the rat brain. *Mem. Cell. Biophys. Biochem.* **12**, 1021-1025.

Mehrani, H. and Storey, K. B. (1995). Effects of anoxia on protein phosphatase in turtle organs: purification and properties of protein phosphatase type-1 from turtle liver. *Arch. Biochem. Biophys.* **316**, 836-843.

Mehta, H. B., Dholakia, J. N., Roth, W. W., Parekh, B. S., Montelaro, R. C., Woodley, C. L. and Wahba, A. J. (1986). Structural studies on the eukaryotic chain initiation factor 2 from rabbit reticulocytes and brine shrimp *Artemia* embryos. *J. Biol. Chem.* **261**, 6705-6711.

Miyamoto, S., Chiorini, J. A., Urcelay, E. and Safer, B. (1996). Regulation of gene expression for translation initiation factor elf-2-alpha – importance of the 3′ untranslated region. *Biochem. J.* **315**, 791-798.

Oldfield, S., Jones, B. L., Tanton, D. and Proud, C. G. (1994). Use of monoclonal antibodies to study the structure and function of eukaryotic protein synthesis initiation factor eIF-2B. *Eur. J. Biochem.* **221**, 399-410.

Pain, V. M. (1996). Initiation of protein synthesis in eukaryotic cells. *Eur. J. Biochem.* **236**, 747-771.

Pakay, J. L., Hobbs, A. A. and Guppy, M. (in press). Pre-absorption of a polyclonal antibody raised against a peptide – keyhole limpet haemocyanin conjugate to allow analysis of molluscan protein. *J. Moll. Stud.* **(**in press).

Pakay, J. L., Hobbs, A. A., Withers, P. C. and Guppy, M. (2002). The *in vivo* down-regulation of protein synthesis in the snail *Helix apersa* during estivation. *Am. J. Physiol.* **283**, R197-R204.

Pedler, S., Fuery, C. J., Withers, P. C., Flanigan, J. and Guppy, M. (1996). Effectors of metabolic depression in an estivating pulmonate snail (*Helix aspersa*): whole animal and *in vitro* tissue studies. *J. Comp. Physiol. B* **166**, 375-381.

Podrabsky, J. E. and Hand, S. C. (2000). Depression of protein synthesis during diapause in embryos of the annual killifish *Austrofundulus limnaeus*. *Physiol. Biochem. Zool.* **73**, 799-808.

Qu, S. M. and Cavener, D. R. (1994). Isolation and characterization of the drosophila-melanogaster eif-2-alpha gene encoding the alpha-subunit of translation initiation factor eif-2. *Gene* **140**, 239-242.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Press.

Scorsone, K. A., Panniers, R., Rowlands, A. G. and Henshaw, E. C. (1987). Phosphorylation of eukaryotic initiation factor 2 during physiological stresses which affect protein synthesis. *J. Biol. Chem.* **262**, 14538-14543.

Tinton, S., Tran, N. Q. and Buc, C. P. (1997). Role of protein-phosphorylation events in the anoxia signal-transduction pathway leading to the inhibition of total protein synthesis in isolated hepatocytes. *Eur. J. Biochem.* **249**, 121-126.

White, B. C., Sullivan, J. M., DeGracia, D. J., O'Neil, B. J., Neumar, R. W., Grossman, L. I., Rafols, J. A. and Krause, G. S. (2000). Brain ischemia and reperfusion: molecular mechanisms of neuronal injury. *J. Neurol. Sci.* **179**, 1-33.

Withers, P. C. (1993). Metabolic depression during aestivation in the Australian frogs, *Neobatrachus* and *Cyclorana*. *Aust. J. Zool.* **41**, 467-473.