

## Identification of the phosphorylation sites in elongation factor-2 from rabbit reticulocytes

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Received 14 January 1991; revised version received 13 February 1991

The sites in eukaryotic elongation factor eEF-2 phosphorylated by the  $\text{Ca}^{2+}$ /calmodulin-dependent eEF-2 kinase in vitro have been identified. The kinase catalysed the rapid incorporation of one mol of phosphate per mol eEF-2 and the slower incorporation of a second mol. All the phosphorylation sites in eEF-2 are contained in the CNBr fragment corresponding to residues 22–155. Tryptic digestion of phosphorylated eEF-2 yielded 3 phosphopeptides, one being unique to monophosphorylated eEF-2. The phosphorylation sites were identified as threonine residues 56 and 58, the former being more rapidly phosphorylated. Ala-Gly-Glu-Thr-Phe-Thr<sup>56</sup>-Asp-Thr<sup>58</sup>-Arg. The same sites are labelled in eEF-2 isolated from reticulocyte lysates.

Protein synthesis; Elongation factor-2; Protein phosphorylation; Protein kinase; Calcium/calmodulin; Rabbit reticulocyte

### 1. INTRODUCTION

It is now well established that translation in mammalian cells is regulated via several distinct mechanisms which include the phosphorylation of elongation factor-2 (eEF-2) [1–5]. eEF-2 (a monomeric protein of 100 kDa) mediates the translocation step of peptide-chain elongation. It is phosphorylated on threonine residues by a specific eEF-2 kinase, (previously termed  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase III [3,4]) and its phosphorylation is increased in intact cells in response to stimuli which increase intracellular  $\text{Ca}^{2+}$ -ion concentrations [6–13]. Phosphorylation of the endogenous eEF-2 impairs the translation of mRNA in the reticulocyte lysate cell-free system [5] and several other lines of evidence show that phosphorylated eEF-2 is inactive, or has only low activity [3,4,14,15].

There have been varied reports on the stoichiometry of eEF-2 phosphorylation in vitro. These range from about one mol phosphate per mol eEF-2 [3], through

two [16] to three mol phosphate per mol eEF-2 (reported in [1]). The work of Nairn and Palfrey [3] suggested that at least some of the phosphorylation sites in eEF-2 were located in the threonine-rich region between residues 50 and 60. We now show that in vitro two mol of phosphate can readily be incorporated into eEF-2, and that the residues labelled are threonines 56 and 58\*. We also demonstrate that the same sites are phosphorylated in reticulocyte lysates.

### 2. MATERIALS AND METHODS

#### 2.1. Materials

These were obtained as described previously [20]. eEF-2 and its kinase were prepared as described previously [21].

#### 2.2. Phosphorylation of eEF-2

eEF-2 (200  $\mu\text{g}$ ) was incubated with eEF-2 kinase in the presence of 20 mM (N-[2-hydroxyethyl]-piperazine-N'-[3-propane-sulphonic acid]) pH 7.6, 125  $\mu\text{M}$  ATP, 10 mM  $\text{MgCl}_2$ , 10  $\mu\text{g} \cdot \text{ml}^{-1}$  of calmodulin, 0.15 mM  $\text{CaCl}_2$ , and 10  $\mu\text{Ci}$  of [ $\gamma$ - $^{32}\text{P}$ ]ATP in a final volume of 250  $\mu\text{l}$  at 30°C. The amount of kinase used in preparative experiments incorporated 1 mol of phosphate per mol of eEF-2 within 1 min. Lower amounts of kinase were used in some other experiments (see section 3). Proportionately smaller scale phosphorylations were performed for other analyses.

Samples were taken at various time points for SDS-PAGE, for isoelectric focusing (IEF) (see below), or for other analyses where phosphorylation was terminated by addition of an equal volume of ice-cold 20% trichloroacetic acid. The pellet was recovered by centrifugation and washed with ice-cold 10% trichloroacetic acid, followed by ice-cold acetone (eEF-2 had previously been determined to be the only phosphoprotein present).

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Abbreviations: eEF-2, eukaryotic elongation factor-2; IEF, isoelectric focusing; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis

\*The numbering is based on the sequence of eEF-2 itself rather than the open reading frame: the initiator methionine residue is absent from the mature protein [17–19]

### 2.3. SDS-polyacrylamide gel electrophoresis and isoelectric focusing

SDS-PAGE was performed as described [20]. One-dimensional slab IEF was performed essentially as described [22] using gels (0.75 mm thick) containing 9 M urea (enzyme grade, Bethesda Research Labs., MD, U.S.A.), 4.8% (w/v) acrylamide, 0.2% (w/v) bis-acrylamide and 3.5% (v/v) ampholytes (pH ranges 3-10 and 7-9 mixed in a ratio of 1:4, Pharmacia-LKB, Uppsala, Sweden).

### 2.4. CNBr digestion, reduction and carboxidomethylation

Precipitated eEF-2 or eEF-2 in chips excised from IEF gels was digested with CNBr [23]. The eluant was removed and the chips were extracted several times with 70% formic acid. The samples were then dried down in a Speedvac centrifuged evaporator (Savant, Hicksville, NY) and extensively washed with water. The gel derived digests were redissolved in 0.1% (w/v) SDS and the Coomassie blue stain and SDS removed by acetone precipitation of the peptides [20]. The peptides were redissolved in 50  $\mu$ l of 0.4 M ammonium bicarbonate (pH 8.2) containing 8 M urea and reduced and carboxidomethylated as described [24]. The products were analysed by tricine SDS-PAGE [25] adapted for a mini-gel system [20]. Alternatively, the reaction was terminated by direct injection onto a reverse-phase HPLC column (see below). After washing with solvent A to remove the urea, the radiolabelled peptide(s) were eluted with 100% solvent B and dried down.

### 2.5. Proteinase digestion and two-dimensional peptide mapping

Digestions were carried out at 30°C in a final volume of 25  $\mu$ l 0.1 M ammonium bicarbonate, pH 8.3 for 2 h, using 10  $\mu$ g of trypsin, followed by overnight incubation with a second identical dose of trypsin. Digestions with Asp-N proteinase were performed as described previously [23]. Two-dimensional peptide mapping comprising electrophoresis at pH 3.6 and thin-layer chromatography was performed as described earlier [20].

### 2.6. Reverse-phase chromatography

Reverse-phase HPLC was performed as previously described [23] using a linear gradient of 0 to 10% solvent B over 50 min (solvent A: 0.1% trifluoroacetic acid in water, solvent B: 0.1% trifluoroacetic acid in acetonitrile). Phosphopeptides for sequencing were rechromatographed under the same conditions with a shallower gradient (0 to 5% B over 50 min), or using 10 mM ammonium acetate in place of 0.1% trifluoroacetic acid as solvent A.

### 2.7. Amino acid sequence analysis and identification of labelled phosphothreonine residues

Peptides were sequenced using an Applied Biosystems 470 or 477A gas-phase sequencer with an on-line phenylthiohydantoin analyser. Phosphorylated residues were identified essentially as described [26], except that samples were loaded onto 1 mm<sup>2</sup> pieces of precycled glass-fibre disc, and these were in turn placed over an intact disc, such that reagent flow first encountered the disc pieces. Pieces were removed after the appropriate degradation cycle and the radioactivity was eluted with 50% formic acid. The presence of <sup>32</sup>P label in phosphate or peptides was distinguished by electrophoresis at pH 3.6 [20] or at pH 1.9 [23].

## 3. RESULTS AND DISCUSSION

### 3.1. Stoichiometry and time course of phosphorylation

The chips from an SDS-polyacrylamide gel corresponding to eEF-2 were excised and counted for <sup>32</sup>P-radioactivity (Fig. 1). One mol of phosphate was rapidly incorporated (within 4 min under these conditions) and phosphorylation more slowly reached a value of 2 mol phosphate per mol of eEF-2 after about one hour. The precise reaction kinetics clearly depend on the

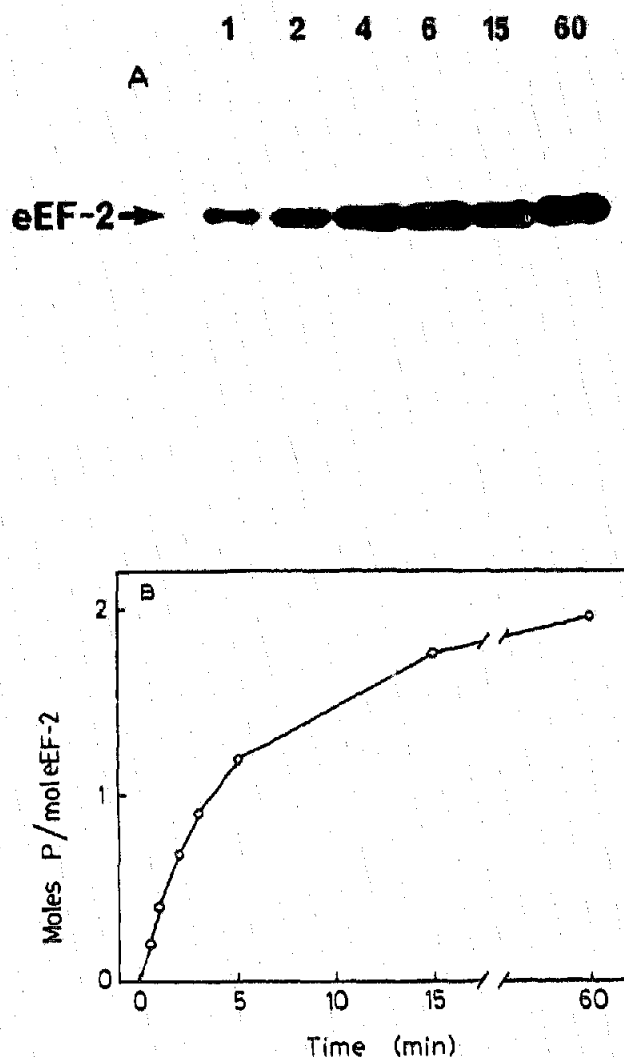


Fig. 1. Time course of phosphorylation of eEF-2 in vitro as analysed by SDS-PAGE. Panel A: autoradiograph of an SDS-polyacrylamide gel of eEF-2 incubated with eEF-2 kinase and [ $\gamma$ -<sup>32</sup>P]ATP for the times shown (in minutes). The incubation conditions are given in section 2. The labelled arrow shows the position of eEF-2. Panel B: the chips corresponding to eEF-2 from a similar experiment to that shown in Panel A were excised from the gel and counted for <sup>32</sup>P radioactivity. The results are expressed as mol P incorporated per mol eEF-2.

amount of kinase added, but in all cases the first mol of phosphate was incorporated 10–20 times faster than the second, and longer incubation produced no significant further increase in phosphorylation.

These findings were independently verified by examining the time course of phosphorylation on an IEF gel to resolve forms of eEF-2 which differ in their phosphorylation state (Fig. 2). Two species more acidic than untreated eEF-2 appeared within 15 min. By 60 min, nearly all the eEF-2 had been converted to the most acidic species. In preparative experiments using more kinase (see section 2), complete conversion to the most acidic species occurred within 10 min. The three species were evenly spaced on the IEF gel and

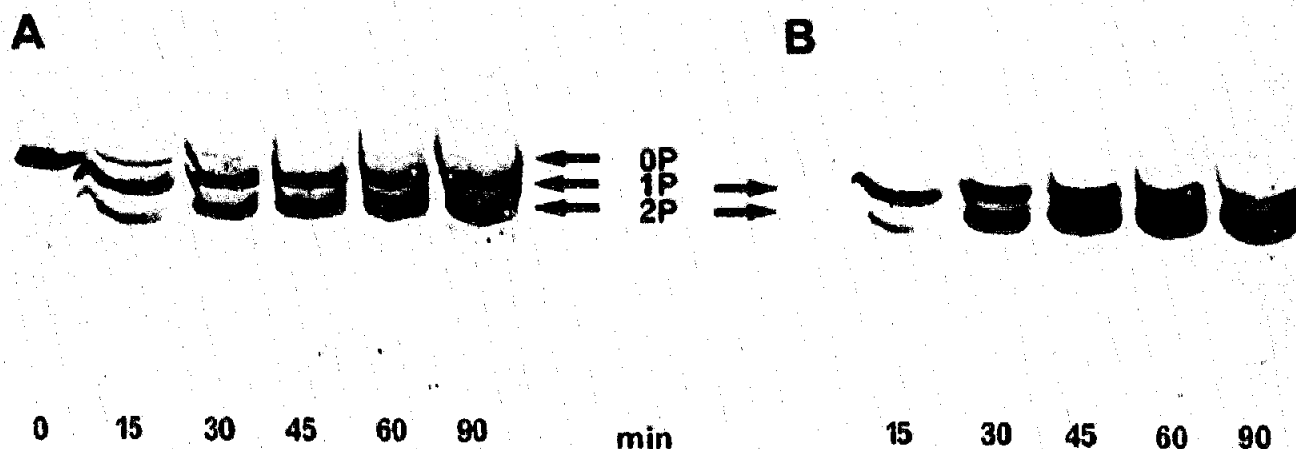


Fig. 2. Time course of phosphorylation of eEF-2 *in vitro* as analysed by isoelectric focusing. A similar incubation to that in Fig. 1 was analysed by isoelectric focusing. Samples of  $^{32}\text{P}$ -labelled eEF-2 were taken at the times indicated and subjected to IEF. The figure shows an autoradiograph of the resulting gel. The positions of unphosphorylated eEF-2 (OP) and mono- and bisphosphorylated forms of eEF-2 (1P and 2P, respectively) are indicated. Panels A and B show, respectively, the Coomassie blue-stained gel and the corresponding autoradiograph.

presumably represented the non-phosphorylated and mono- and bisphosphorylated forms of eEF-2. These findings confirm the data in Fig. 1.

These findings are in disagreement with Nairn and Palfrey [3] who reported a value of  $1 \text{ mol} \cdot \text{mol}^{-1}$ , and with Ovchinnikov et al. (reported in [1]) who found  $3 \text{ mol} \cdot \text{mol}^{-1}$ .

### 3.2. Identification of phosphopeptides

Phosphoamino acid analysis revealed that only phosphothreonine was present in bisphosphorylated eEF-2, consistent with all the previous work on this protein (not shown). A CNBr digest of phosphorylated eEF-2 gave a single radiolabelled fragment with an apparent  $M_r$  of 14.5 kDa on tricine SDS-PAGE (Fig. 3A). This must correspond to the largest CNBr fragment (residues 22–155), since no other fragment is larger than about 11.5 kDa (residues 497–603). It should be noted that although the sequence of rabbit eEF-2 used here, is not known, the three known mammalian eEF-2 sequences contain methionines at the same positions [17–19]. Thus, all the label in bisphosphorylated eEF-2 is located within residues 23–156 of its sequence.

To locate the phosphorylation sites, this CNBr fragment was subjected to tryptic digestion, after carboxyiodomethylation to block cysteine residues. Two-dimensional maps of tryptic digests of monophosphorylated eEF-2 obtained from an IEF gel gave a single radiolabelled peptide (Fig. 3B) (peptide C, nomenclature based on the order of elution from reverse-phase HPLC). Bisphosphorylated eEF-2 gave two phosphopeptides A and B (Fig. 3C), although the relative proportions of these species varied in different digests. Higher doses of trypsin gave a higher proportion of A (not shown).

The tryptic phosphopeptides were also analysed by reverse-phase chromatography on a  $\text{C}_{18}$  column.

Monophosphorylated eEF-2 gave a single major phosphopeptide corresponding to peptide C on two-dimensional maps (Fig. 3E). Bisphosphorylated eEF-2 gave two peaks of radioactivity, eluting slightly earlier than peptide C and corresponding to peptides A and B seen on two-dimensional maps (Fig. 3F).

### 3.3. Identification of phosphorylated residues

Peptide C was found to have the sequence Phe-Thr-Asp-Thr-Arg- on gas-phase sequencing. All the radioactivity was released after the third cycle of the degradation (not shown). Also, treatment of peptide C with the Asp-N proteinase resulted in its complete conversion to a phosphopeptide which, unlike peptide C itself, migrated towards the anode at pH 3.6 (Fig. 3D). This confirmed that all the radioactivity was associated with the first threonine in the sequence. Peptide C therefore has the sequence shown in Table I.

Since digests of bisphosphorylated eEF-2 contain only peptides A and B, and no peptide C, the sequences of these peptides must contain that of peptide C, the only phosphopeptide obtained from monophosphorylated eEF-2. Thus, A and B must represent variants containing the sequence -Phe-Thr(P) $^{56}$ -Asp-Thr $^{58}$ -Arg-. The additional phosphothreonine residue could then be threonine-58 (present in peptide C). Alternatively A and B may correspond to peptides extended at the C- and/or N-terminus compared with peptide C. In the latter case they might start, for example, at alanine-50 (resulting from tryptic cleavage at arginine-49), and thus include threonine-53. However, during the Edman degradation of peptides A and B, 55% and 52%, respectively, of the  $^{32}\text{P}$ -radioactivity was released after 3 cycles thus excluding the possibility that A and B were N-terminal extensions of peptide C starting at alanine-50 (or beyond) and phosphorylated at threonine-53 (since no radioactivity would then have been released after cycle three).

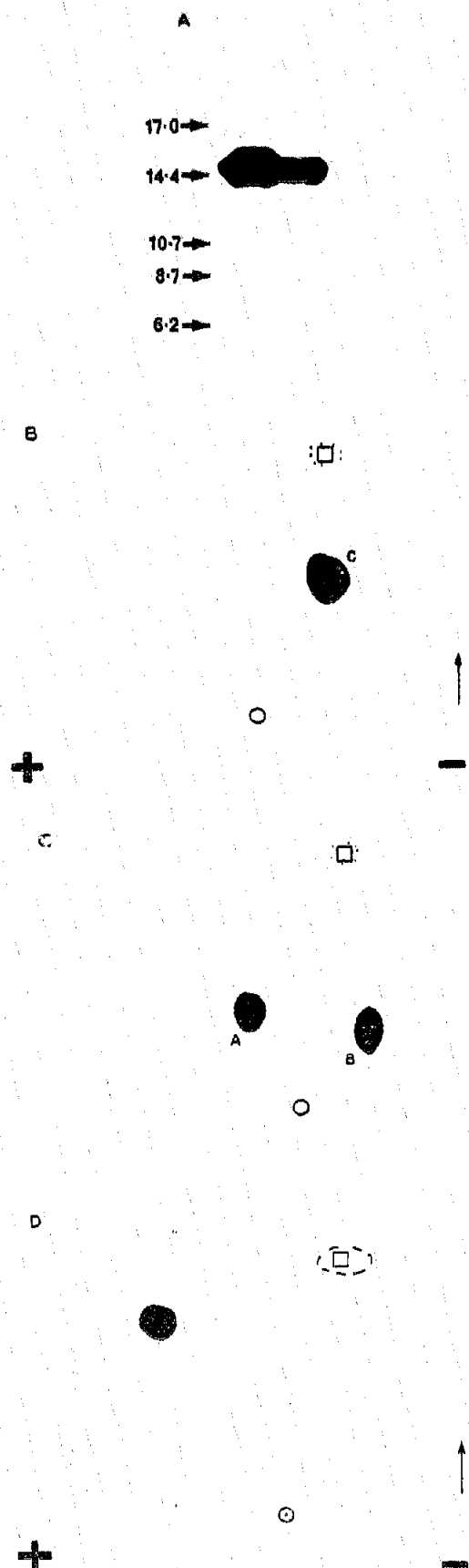


Fig. 3. Analysis of phosphopeptides from phosphorylated eEF-2. Panel A: bisphosphorylated eEF-2 was subjected to digestion with CNBr and the resulting fragments were analysed on a Tricine SDS-polyacrylamide gel. The positions of molecular mass markers are shown (values in kDa). The figure shows an autoradiograph of the fixed gel. Panels B and C: the products of tryptic digestion of eEF-2 previously cleaved by CNBr were analysed by two-dimensional mapping (electrophoresis at pH 3.6 and chromatography, see section 2 for details). The positions of peptides A, B and C are indicated where appropriate and the origin is marked by an open circle, and the migration of the dinitrophenyllysine marker by the open square. The directions of electrophoresis and chromatography (arrow) are also indicated. The figures are autoradiographs of the resulting maps. Panels B and C show peptides from monophosphorylated and bisphosphorylated eEF-2 respectively. Panel D: peptide C was digested with the Asp-N proteinase from *P. fragi* and the products were analysed by two-dimensional mapping. Panels E and F: Analysis of tryptic fragments from phosphorylated eEF-2 by reverse-phase chromatography. Tryptic fragments from a mixture of mono- (Panel E) and bisphosphorylated (Panel F) eEF-2 were applied to the  $\text{C}_{18}$  column which was developed with a gradient of increasing concentrations of acetonitrile as indicated (flow rate  $1 \text{ ml} \cdot \text{min}^{-1}$ ). The fractions (0.5 ml) were counted for  $^{32}\text{P}$  radioactivity. The positions of peaks referred to as A, B and C are indicated.

Essentially all the radioactivity (93% and 96% for peptides A and B respectively) was released after 5 cycles indicating that they must represent the two possible bisphosphorylated variants of peptide C, phosphorylated at threonines-56 and -58. They must presumably differ at their C-termini. It is likely that B contains an additional lysine which would be consistent

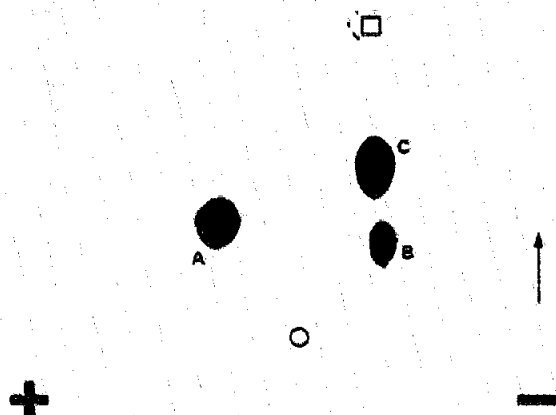


Fig. 4. Mapping of phosphopeptides from rabbit reticulocyte eEF-2 phosphorylated in situ. Tryptic phosphopeptides were analysed by two-dimensional mapping. The positions of peptides corresponding to peaks A, B and C (Fig. 3) are indicated. The figure is annotated as for Fig. 3, Panels B and C.

with the electrophoretic behaviour. It is known that the presence of adjacent phosphoamino acids can impede the cleavage of peptide bonds by trypsin [27,28] yielding variants of this kind and this would account for the variable proportions of A and B observed in different preparations. Their changes, and the fact that further tryptic digestion generated no other species, are consistent with the structures shown in Table I.

Threonines-56 and -58 are therefore the residues phosphorylated by eEF-2 kinase in vitro, and since monophosphorylated eEF-2 is labelled only at threonine-56, phosphorylation of 56 must precede that of 58. These residues lie within the probable guanine nucleotide-binding region of eEF-2 [18] and near its putative 'effector domain' [29].

### 3.4. Sites in eEF-2 phosphorylated in reticulocyte lysates

Reticulocyte lysate incubations containing [ $\gamma$ - $^{32}$ P]ATP were subjected to SDS-PAGE and the band corresponding to eEF-2 was excised, and digested successively with CNBr and trypsin. Two-dimensional phosphopeptide maps (Fig. 4) contained only the species corresponding to peptides A, B and C indicating that both mono- and bisphosphorylated eEF-2 is pre-

sent in the lysate, and that phosphorylation occurs at the same sites as those identified in vitro. After completion of our work, Ovchinnikov et al. [30] reported that, in vitro, threonine 53 was phosphorylated in addition to residues 56 and 58. However, we have no evidence for significant phosphorylation of more than the two sites described here either in vitro or in reticulocyte lysates under any condition tested.

**Acknowledgements:** We are grateful to Professor Philip Cohen for allowing us to use the gas-phase sequencing and ancillary facilities in the Department of Biochemistry, University of Dundee. This work was supported by grants from the Science and Engineering Research Council and the Medical Research Council to CGP. K.S. was the recipient of a Scholarship from the British Council, and performed this work during this visit to Bristol. We are grateful to Dr. G.R. Drapeau (Montreal) for Asp-N proteinase, and to Guy Rutter (Bristol) for calmodulin.

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Table I

Structures of Phosphopeptides derived from eEF-2

Peptide	Sequence
eEF-2	$^{49}$ Arg-Ala-Gly-Glu-Thr-Arg-Phe-Thr-Asp-Thr-Arg-Lys $^{60}$
A	Phe-Thr(P)-Asp-Thr(P)-Arg
B	Phe-Thr(P)-Asp-Thr(P)-Arg-Lys
C	Phe-Thr(P)-Asp-Thr-(Arg) <sup>a</sup>

<sup>a</sup>based on automated sequence analysis

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