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The brain isoform of a key ATP-regulating enzyme, creatine kinase, is a phosphoprotein

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Two-dimensional electrophoretic analysis of crude microtubule preparations from the rat brain revealed the presence of three polypeptides in positions corresponding to those of the isovariants of purified rat brain creatine kinase (CK-BB). By the use of [γ-^{32}P]ATP, the two more acidic forms of these polypeptides were shown to be phosphorylated. Their identity as phosphorylated forms of CK-BB was established by using various peptide mapping techniques. Thus CK-BB is a phosphoprotein and its isoelectric variation may be attributed to phosphorylation.

The enzyme creatine kinase (EC 2.7.3.2) plays a major role in the energy metabolism of the cell by utilizing the phosphagen creatine phosphate to phosphorylate ADP, thus regenerating ATP in conditions of high energy demand (Watts, 1973). Although widely regarded as a soluble enzyme, recent work in this and other laboratories has shown that the brain form of creatine (CK-BB) may be found associated with ATP-consuming supramolecular structures such as the synaptic plasma membrane (Lim *et al.*, 1983), with acetylcholine-receptor-rich membranes from *Torpedo* electrocytes (Barrantes *et al.*, 1983) and with the slow component B of axonal transport (Brady & Lasek, 1981). When CK-BB from these sources or that from rat (Reiss & Kaye, 1981) or human (Jackson & Thompson, 1981) brain is analysed by two-dimensional isoelectric-focusing–polyacrylamide-gel electrophoresis, the enzyme is resolved into a number of closely spaced species (approx. pI 5.9) of molecular mass 42000 Da. These isoelectric variants of CK-BB are also present in preparations of crude microtubules from the rat brain. We now report that the observed isoelectric variation of CK-BB is due to its phosphorylation, and that a protein kinase that phosphorylates this enzyme is present in preparations of crude microtubules from the rat brain. The extremely specific occurrence of CK-BB may be related to the accessibility of this isoform of the enzyme to modulation by phosphorylation.

Abbreviation used: CK-BB, creatine kinase brain form.

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Experimental

Materials

Adult Wistar rats (55–60 days old) from our animal colony were used in these experiments. Rat brain CK-BB was purified by the method of Reiss & Kaye (1981). The same preparation of purified CK-BB was used throughout these experiments. Analysis on sodium dodecyl sulphate-containing as well as two-dimensional gels showed that CK-BB was the major protein constituent of these preparations, although some minor contaminants were present. Crude microtubule fractions from the rat brain were prepared by the method of Fellous *et al.* (1977), with the use of one cycle of polymerization to pellet these proteins. Pellets were resuspended in 100mM-Mes (4-morpholine-ethanesulphonic acid)/NaOH buffer, pH 6.75, containing 1mM-EGTA and 5mM-$MgSO_4$ at a protein concentration of 5mg/ml and stored at −80°C.

Phosphorylation of crude microtubule fractions

Phosphorylation was performed at a protein concentration of 2mg/ml in the buffer described above containing, in addition, 50μM-diadenosine pentaphosphate (adenylate kinase inhibitor). This mixture was preincubated at 37°C for 30min before phosphorylation for 2min (Fig. 1) or for 5min (Figs. 2, 3 and 4) with the use of 30μM-[γ^{32}P]-ATP (>1Ci/mmol; Amersham International). The reaction was terminated by addition of an equal volume of sodium dodecyl sulphate-containing lysis buffer (see below). Purified rat brain CK-BB, when present, was at a concentration of 0.2mg/ml.

Two-dimensional electrophoresis

Two-dimensional isoelectric-focusing–polyacrylamide-gel-electrophoretic analysis was performed essentially as described by O'Farrell (1975), but with 2% pH 3.5–10 Ampholines in place of the mixture recommended and with the inclusion of 0.5% sodium dodecyl sulphate in the lysis buffer. Gels were stained with Coomassie Blue and subjected to autoradiography (48–60h) to locate the ^{32}P-labelled proteins. The migration of CK-BB, actin, tubulin and 70kDa microtubule-associated protein under these conditions has been the subject of previous papers from our laboratory (Lim *et al.*, 1983; Hall *et al.*, 1984; Whatley *et al.*, 1984).

Two-dimensional tryptic fingerprinting of CK-BB

The spots corresponding to 0, 1 and 2 (Fig. 1) on two-dimensional analyses were each excised from the gel and subjected to radioiodination followed by tryptic fingerprinting analysis as described by Elder *et al.* (1977). The forms 1 and 2 of CK-BB from two-dimensional gels of crude microtubules were excised together and subjected to similar analysis.

[^{32}P]Phosphopeptide maps of CK-BB

Crude microtubules were phosphorylated in the presence of added purified rat brain CK-BB as described above. Spots corresponding to forms 1 and 2 of CK-BB on two-dimensional gels were each excised from the gels, crushed and the resultant slurry was incubated at room temperature for 30min with 0.2mg of *Staphylococcus aureus* V8 proteinase/ml. Peptides were separated on 10–20%-linear-gradient sodium dodecyl sulphate-containing gels, silver-stained, dried and subjected to autoradiography to locate the ^{32}P-labelled peptides. For two-dimensional phosphopeptide fingerprinting analysis, spots 1 and 2 were excised together from the gels and subjected to the procedure described by Elder *et al.* (1977), but with the omission of the radioiodination steps.

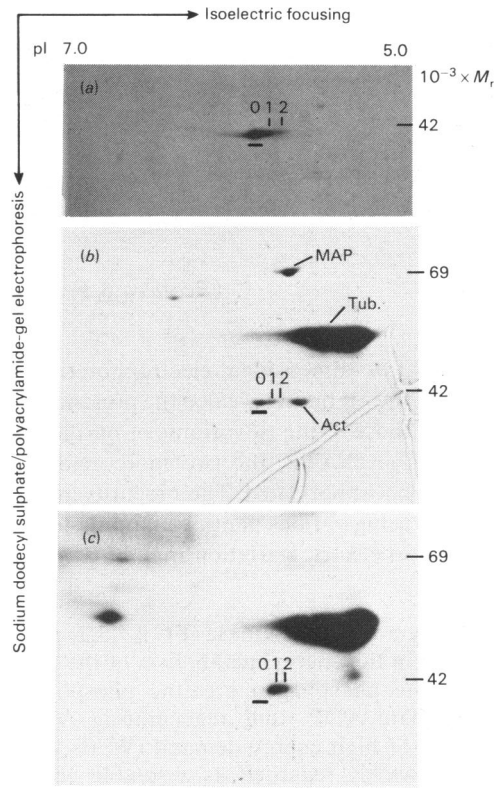

Fig. 1. *Isoelectric variants of CK-BB resolved by two-dimensional isoelectric focusing–polyacrylamide-gel electrophoresis*

The isoelectric variants are labelled 0, 1 and 2 in the Figure. The bar marks the position of the non-phosphorylated form (labelled 0) and phosphorylated forms are labelled 1 and 2. (*a*) Coomassie Blue stained gel of CK-BB purified from the rat brain. (*b*) Coomassie Blue-stained gel of crude microtubule preparation from the rat brain after phosphorylation as described below. The positions of CK-BB isovariants, actin (Act.), tubulin (Tub.) and the 70kDa microtubule-associated protein (MAP) are indicated. (*c*) Autoradiograph of (*b*), showing the incorporation of [^{32}P]phosphate at positions on the gel corresponding to forms 1 and 2 of CK-BB when crude microtubules are incubated with [γ-^{32}P]ATP. Tubulin is also phosphorylated. The isoelectric gradient and positions of molecular-mass markers are as indicated.

Results and discussion

On two-dimensional isoelectric-focusing–polyacrylamide-gel electrophoretic analysis CK-BB purified from the rat brain is resolved into a number of isoelectric variants (approx. pI 5.9) of molecular mass 42000Da, as shown labelled 0, 1 and 2 in Fig. 1(*a*). It has been suggested that this may be due to the existence of isoforms of this enzyme (Barrantes *et al.*, 1983), but there is no evidence to support this view. When preparations of crude microtubules are subjected to two-dimensional electrophoretic analysis, the presence of a series of spots corresponding in position to CK-BB is observed adjacent to actin (Fig. 1*b*). We have previously reported the presence and characterization of these spots in two-dimensional electrophoretic analyses of synaptic plasma membranes from the rat brain (Lim *et al.*, 1983). Preparations of crude microtubules also contain a number of protein kinase activities that phos-

phorylate microtubule proteins such as tubulin (DeLorenzo *et al.*, 1982), microtubule-associated proteins (Pallas & Solomon, 1982), Tau factors (Pierre & Nunez, 1983) and other proteins. Thus, when these preparations are incubated with [γ-^{32}P]ATP, extensive protein phosphorylation is observed. Preliminary experiments showed that the amount of protein-bound [^{32}P]phosphate peaked between 5 and 10min after addition of the [γ-^{32}P]ATP, and then decreased, suggesting that the preparation contained protein phosphatase activity. Two-dimensional electrophoretic analysis followed by autoradiography to locate the ^{32}P-labelled proteins in these preparations (Fig. 1*c*) show that phosphorylated proteins include tubulin and two spots corresponding in migration to the more acidic forms of CK-BB, indicating that the isoelectric variance of CK-BB may be due to its phosphorylation.

This observation was substantiated by performing phosphorylations of crude microtubule preparations as described above with the addition of exogenous CK-BB purified (Reiss & Kaye, 1981) from rat brain (Fig. 2). The addition of CK-BB to these preparations resulted in the increased Coomassie Blue-staining intensity of the relevant spots adjacent to actin (Fig. 2*a*, −CK-BB, and Fig. 2*c*, +CK-BB). Autoradiography of these gels showed that the incorporation of [^{32}P]phosphate observed at this position was also increased when exogenous CK-BB was present during phosphorylation (Fig. 2*b*, −CK-BB and Fig. 2*d*, +CK-BB). Thus it appeared that a protein kinase activity present in preparations of crude microtubules from rat brain was capable of phosphorylating both endogenous CK-BB as well as the exogenously added purified CK-BB. However, it was not possible to obtain complete phosphorylation of CK-BB owing to the protein phosphatase activity present in the crude microtubule preparation.

To prove that the enhanced phosphorylation observed with the addition of exogenous CK-BB was not due to a contaminating protein co-purifying with the enzyme, each of the spots 0, 1 and 2 observed in two-dimensional analyses of purified CK-BB (see Fig. 1*a*) was excised from the

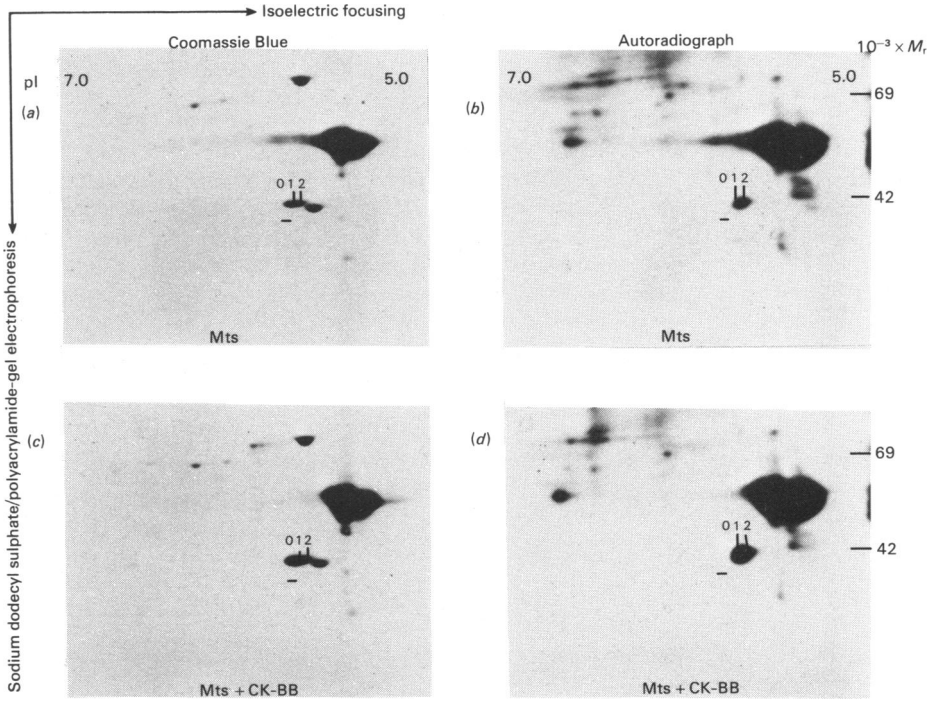

Fig. 2. *Phosphorylation of endogenous and exogenously added CK-88 in brain microtubule preparations*
Crude microtubule preparations (Mts) were phosphorylated in the absence (*a* and *b*) and in the presence (*c* and *d*) of added CK-BB purified from rat brain (see Fig. 1*a*). Bar represents non-phosphorylated CK-BB, and phosphorylated forms are labelled 1 and 2. Addition of purified CK-BB to crude microtubule preparations resulted in an increased Coomassie Blue-staining intensity of the relevant spots adjacent to actin (*c*). The incorporation of [^{32}P]phosphate at this position was also increased (*d*). Identities of other proteins are as indicated in Fig. 1(*b*). The isoelectric gradient and positions of molecular-mass markers are as indicated.

gel and subjected to radioiodination followed by tryptic fingerprinting (Elder *et al.*, 1977). The results (Figs. 3*a*, 3*b* and 3*c*) showed that these spots give rise to similar if not identical radioiodinated tryptic peptides. When the phosphoprotein spot adjacent to actin in two-dimensional gels of crude microtubules was subjected to tryptic fingerprinting analysis (Fig. 3*d*), the radioiodinated peptides obtained were similar to those of the CK-BB isovariants (Figs. 3*a*, 3*b* and 3*c*). The identity of actin in preparations of crude microtubules from the rat brain was also established by using two-dimensional tryptic fingerprinting (results not shown). Thus it was established that the three spots

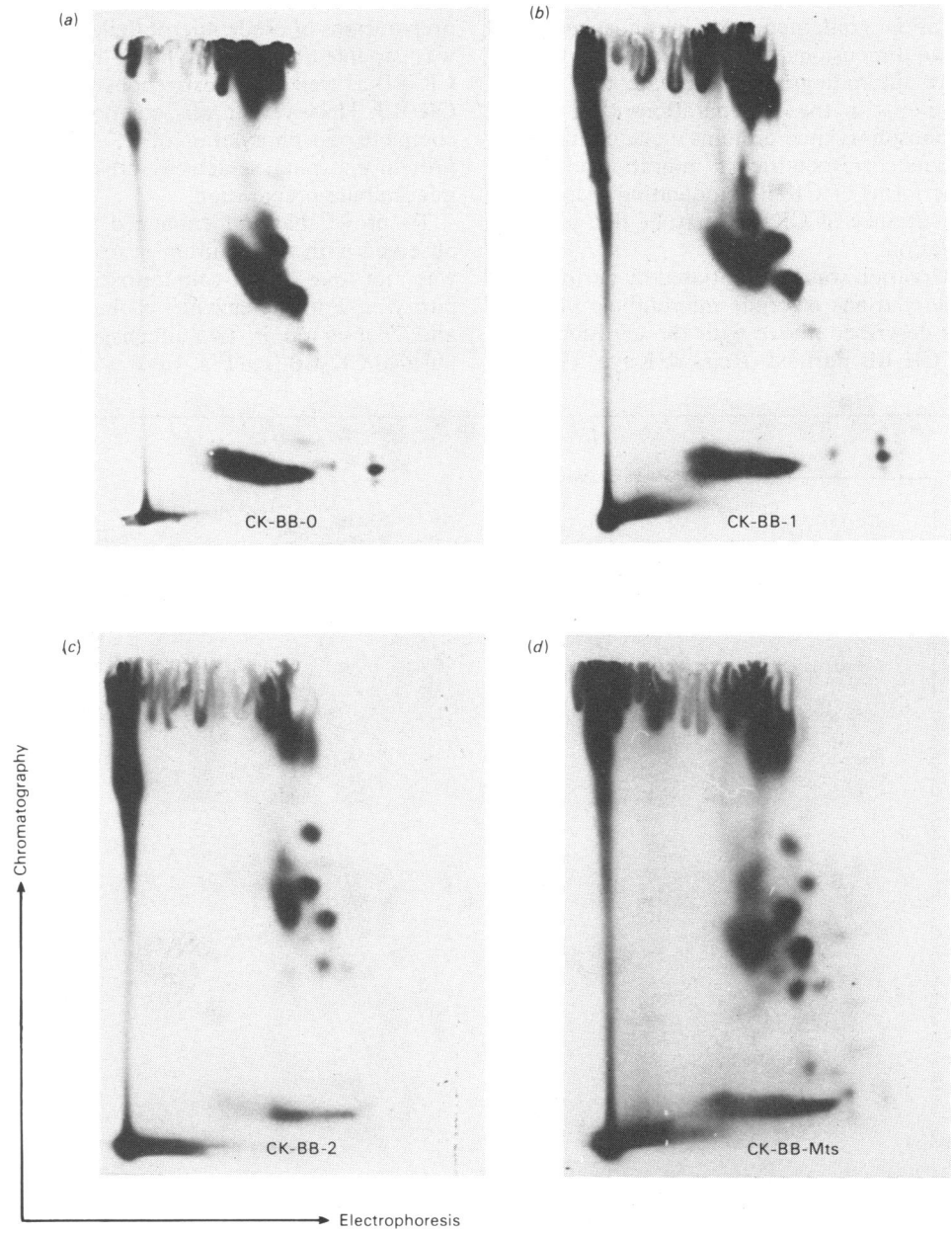

Fig. 3. *Two-dimensional tryptic fingerprinting of the isoelectric variants of purified CK-BB labelled, 0, 1 and 2 in Fig. 1(a)* These three forms yield similar if not identical radioiodinated tryptic fragments (*a*, *b* and *c*). (*d*) shows the similarity of tryptic fragments derived from phosphorylated CK-BB (spots 1 and 2) present in two-dimensional analyses of crude microtubules (Mts).

obtained in two-dimensional electrophoretic analysis of purified CK-BB were due to isoelectric variance of this enzyme and not to the co-purification of contaminant proteins. The phosphoprotein migrating adjacent to actin in two-dimensional analysis of crude microtubules was also identified as CK-BB.

The spots corresponding to CK-BB were excised from two-dimensional gels and subjected to various proteolytic digestion procedures to confirm that the ^{32}P-labelled phosphoprotein and the stained protein migrating at this position were identical, and to characterize further the binding between the [^{32}P]phosphate and the enzyme molecule. The correspondence on sodium dodecyl sulphate/polyacrylamide-gel electrophoretograms (Fig. 4*a*) of ^{32}P-labelled and silver-stained peptides generated by the limited digestion of spots 1 and 2 excised from two-dimensional gels excludes the possibility that the radioactivity was being incorporated into minor phosphoproteins co-migrating with CK-BB. More-exhaustive digestion of phosphorylated CK-BB with the use of the two-dimensional phosphopeptide fingerprinting method revealed that most of the [^{32}P]phosphate was incorporated into one tryptic fragment (Fig. 4*b*, labelled 1), although two other fragments (labelled 2 and 3) are weakly labelled. Further degradation of these peptides by acid hydrolysis followed by co-electrophoresis with the phosphorylated forms of serine, threonine and tyrosine showed that [^{32}P]-phosphate was incorporated at serine residues on the CK-BB molecule (results not shown). The observation that the association of radioactivity with the peptides and the serine residues was preserved throughout the various electrophoretic and chromatographic stages incurred in these procedures confirms that the radioactive label is covalently bound to the CK-BB molecules and is not due to enzyme–substrate interactions between CK-BB and [γ-^{32}P]ATP.

In conclusion, we have demonstrated that the isoelectric variance observed in two-dimensional analyses of CK-BB is attributable to its phosphorylation. Recent studies show that the brain form of creatine kinase differs from its other isoforms in many significant aspects. Thus this isoform, which is the form of creatine kinase that exists in the

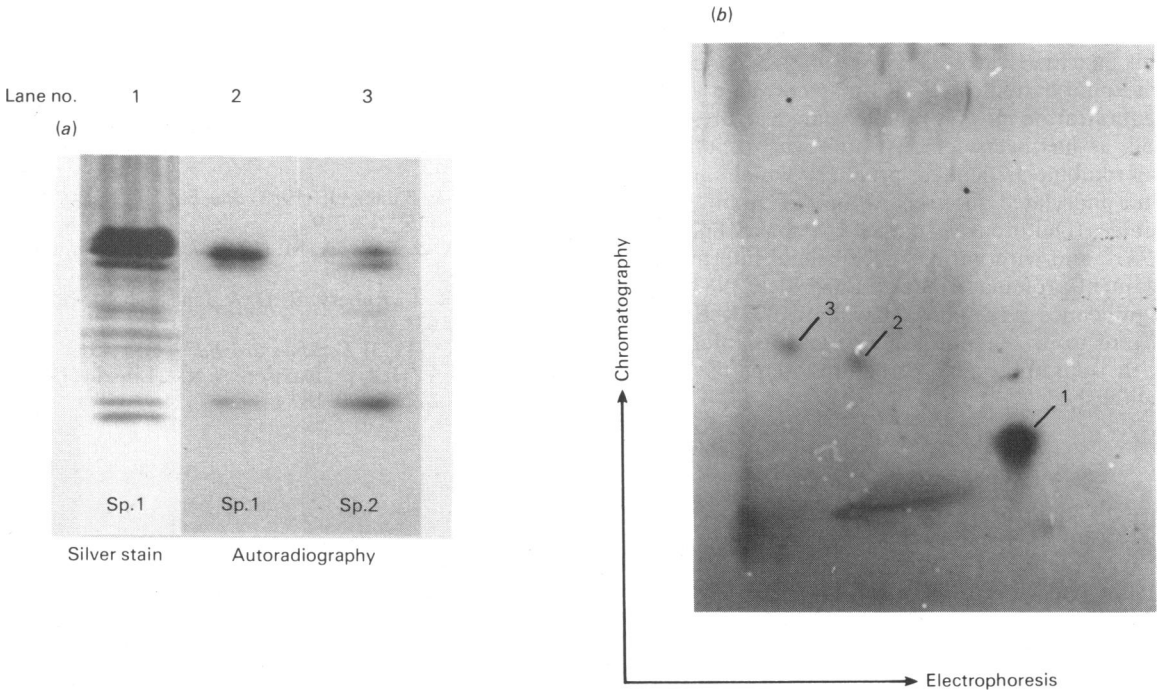

Fig. 4. [^{32}*P*]*Phosphopeptides generated by limited* (*a*) *and exhaustive* (*b*) *digestion of CK-BB*
(*a*) Phosphorylated forms 1 (Sp. 1) and 2 (Sp. 2) of CK-BB were subjected to limited proteolysis with *Staphylococcus aureus* V8 proteinase. The phosphopeptides generated from both forms are identical (*a*, lanes 2 and 3) and correspond to silver-stained peptides generated by this procedure (*a*, lane 1). (*b*) Two-dimensional tryptic phosphopeptide fingerprinting of phosphorylated CK-BB, showing one strongly (*b*, 1) and two weakly (*b*, 2 and 3) labelled radioactive peptides.

embryo (Iyengar *et al.*, 1982*a*) and foetus (Watts, 1973), is able to interact with the lower concentrations of ADP and phosphocreatine that obtain in non-muscle cells (Iyengar *et al.*, 1982*b*). In the uterus, the synthesis of CK-BB is induced in response to treatment with oestrogens (Reiss & Kaye, 1981), and raised amounts of this isoform of the enzyme have also been detected in the serum of patients with metastatic breast cancer (Thomson *et al.*, 1980). In *Torpedo* electrocytes, both the brain (BB) and the muscle (MM) isoforms of the enzyme have been detected, but two-dimensional immunoblotting analysis has shown that only the BB form is associated with acetylcholine-receptor-rich membranes (Barrantes *et al.*, 1983). These observations on CK-BB suggest that this isoform of the enzyme possesses unique characteristics relevant to the maintenance of ATP concentrations in specialized instances of high energy demand. Since isoelectric variants corresponding to phosphorylated and non-phosphorylated forms of this enzyme can be identified from diverse sources (Brady & Lasek, 1981; Jackson & Thompson, 1981; Reiss & Kaye, 1981; Barrantes *et al.*, 1983; Lim *et al.*, 1983), it is likely that the phosphorylation of this enzyme is physiologically relevant. Two effects of the phosphorylation of CK-BB are conceivable. Firstly, phosphorylation may affect kinetic parameters of the ATP-regulating reaction catalysed by this enzyme (Hardie & Guy, 1982). Secondly, the phosphorylation of CK-BB may correlate with its sequestration into supramolecular structures in the cell, as has been demonstrated with tubulin and microtubule-associated proteins, which are also phosphorylated in preparations of brain microtubules (DeLorenzo *et al.*, 1982; Pallas & Solomon, 1982), and with various cytoskeletal intermediate filament proteins (Moon & Lazarides, 1983). The significance of the phosphorylation of CK-BB with regard to its reaction kinetics and its interaction with supramolecular structures remains to be established.

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References

Barrantes, F. J., Mieskes, G. & Walliman, T. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5440–5444

Brady, S. T. & Lasek, R. J. (1981) *Cell (Cambridge, Mass.)* **23**, 515–523

DeLorenzo, R. J., Conzalez, B., Goldenring, J., Bowling, A. & Jacobson, R. (1982) *Prog. Brain Res.* **56**, 255–286

Elder, J. H., Pickett, R. A., Hampton, J. & Lerner, R. A. (1977) *J. Biol. Chem.* **252**, 6510–6515

Fellous, A., Francon, J., Lennon, A. & Nunez, J. (1977) *Eur. J. Biochem.* **78**, 167–174

Hall, C., Mahadevan, L. C., Whatley, S. A., Biswas, G. & Lim, L. (1984) *Biochem. J.* **219**, 751–761

Hardie, D. G. & Guy, P. S. (1982) *Prog. Brain Res.* **56**, 145–161

Iyengar, M. R., Chung Wha, L., Iyengar, C. W. L., Chen, H. Y., Brinster, R. L., Bornslaeger, E. & Schultz, R. M. (1982*a*) *Dev. Biol.* **96**, 263–268

Iyengar, M. R., Fluellen, C. E. & Iyengar, C. W. L. (1982*b*) *J. Muscle Res. Cell Motil.* **3**, 231–246

Jackson, P. & Thompson, R. J. (1981) *J. Neurol. Sci.* **49**, 429–438

Lim, L., Hall, C., Leung, T., Mahadevan, L. & Whatley, S. (1983) *J. Neurochem.* **41**, 1177–1181

Moon, R. T. & Lazarides, E. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5495–5499

O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021

Pallas, D. & Solomon, F. (1982) *Cell (Cambridge, Mass.)* **30**, 407–414

Pierre, M. & Nunez, J. (1983) *Biochem. Biophys. Res. Commun.* **115**, 212–219

Reiss, N. A. & Kaye, A. M. (1981) *J. Biol. Chem.* **256**, 5741–5749

Thomson, R. J., Rubery, E. D. & Jones, H. M. (1980) *Lancet* **ii**, 673–675

Watts, D. C. (1973) *Enzymes 3rd Ed.* **3**, 334–451

Whatley, S. A., Hall, C., Davison, A.N. & Lim, L. (1984) *Biochem. J.* **220**, 179–187