MULTIPLE PHOSPHORYLATION SITES OF MICROTUBULE-ASSOCIATED PROTEIN (MAP_2) OBSERVED AT HIGH ATP CONCENTRATIONS

Khalid ISLAM and Roy BURNS

Biophysics Section, Department of Physics, Imperial College of Science and Technology, London, SW7 2BZ, England

Received 10 November 1980; revised version received 1 December 1980

1. Introduction

Microtubule protein, purified by cycles of assembly and disassembly, contains associated non-tubulin proteins, which include two high-molecular weight proteins termed MAP_1 and MAP_2 [1–4]. MAP_2 is of particular interest, as it is the preferred substrate for phosphorylation by an endogenous cAMP-dependent protein kinase [5–9]. In [3–5] the MAP_2 protein was reported to be phosphorylated to the extent of 2 mol/mol protein in the presence of cAMP. In [7, 10,11] a phosphoprotein phosphatase has been reported and the protein kinase/phosphoprotein phosphatase suggested to determine the final extent of phosphorylation [7]. The existence of such an equilibrium hinders any calculation of the maximum number of phosphorylation sites. Here, we examine the pattern and stoichiometry of phosphorylation of microtubule protein as a function of ATP concentration, and observe that the stoichiometry of MAP_2 phosphorylation is significantly higher than in earlier reports.

2. Materials and methods

2.1. *Preparation of microtubule protein*

Microtubule protein was purified by cycles of assembly and disassembly from day-old chick brains in the absence of glycerol. Chick brains were homogenised in an equal volume (w/v) of MAB buffer (0.1 M 2-(*N*-morpholino)ethane sulphonic acid–2.5 mM ethyleneglycol-bis-(β-aminoethylether) *N*, *N'*-tetraacetic acid–0.5 mM $MgSO_4$–0.1 mM ethylene dinitrilo-tetraacetic acid–0.1 mM dithiothreitol, pH 6.4). The homogenate was centrifuged for 30 min at 20 000 × *g* at 4°C. The supernatant was adjusted to 1 mM GTP and incubated for 10 min at 37°C, then centrifuged for 30 min at 20 000 × *g* at 30°C. The microtubule pellet was then homogenised in MAB, using 10% of the initial homogenisation volume, and cold dissociated for 30 min at 4°C. After centrifugation at 65 000 × *g* at 4°C for 30 min to remove the cold-stable material, the supernatant was readjusted to 1 mM GTP, incubated at 37°C for 25 min, and centrifuged for 30 min at 30°C at 65 000 × *g*. The pellets were homogenised in an appropriate volume of MAB buffer, and cold-dissociated for 30 min. The protein was centrifuged for 30 min at 65 000 × *g* at 4°C, and the 2 × cold-dissociated protein in the supernatant (7–10 mg/ml) was stored in liquid nitrogen.

2.2. *Phosphorylation of microtubule protein*

The 2 × cold-dissociated microtubule protein was thawed, and used immediately withhout further purification. Typically, the assay volume was 100 μl: 50 μl MAB–2 μM cAMP–4 mM $MgSO_4$–200 μM podophyllotoxin (to inhibit microtubule polymerisation [12])–40 μl microtubule protein in MAB and 10 μl of the appropriate concentration of [γ-^{32}P]ATP and unlabelled ATP, added immediately before the incubation period. The assays were incubated at 37°C for 25 min, at which time the reaction was terminated by either trichloroacetic acid precipitation or by the addition of SDS, depending upon the subsequent treatment of the samples. For determination of the total trichloroacetic acid-precipitable counts, a 20 μl aliquot was added to 250 μl 10% trichloroacetic acid and 50 μg bovine serum albumin was then added to aid the precipitation of the protein. The protein was collected onto glass fibre discs (Whatman GF/C, 21 mm), which were washed with sufficient (10 × 9 ml) 5%

trichloroacetic acid to ensure complete removal of any free [γ-^{32}P]ATP. The discs were then washed with ethanol to remove the residual trichloroacetic acid and counted in a liquid scintillation counter (Beckman LS-150) in a scintillant of 0.5% 2-(4′-*tert*-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole/toluene. A background of either an assay mixture lacking microtubule protein or one containing protein, but stopped at t_0 was routinely subtracted. Inputs were determined by drying appropriate aliquots of the reaction mixture onto glass fibre discs.

2.3. *SDS–polyacrylamide gel electrophoresis*

SDS–polyacrylamide gel electrophoresis was performed essentially as in [13]. The reaction in 50 μl phosphorylation assay was terminated by the addition of an equal volume of Laemmli tracking buffer, and boiled for 2 min. The gels were stained with 0.025% Coomassie brilliant blue–10% acetic acid–25% *iso*-propanol, and destained in 10% acetic acid [14]. As free ATP migrated with the solvent front, the gels were extensively destained with frequent changes of the acetic acid to ensure that the background of unincorporated [γ-^{32}P]ATP was low. The gels were scanned with a Gelman gel scanner, equipped with a digital peak area printout. To measure incorporated ^{32}P, the gels were sliced into 0.5 mm slices, using a Mickle gel

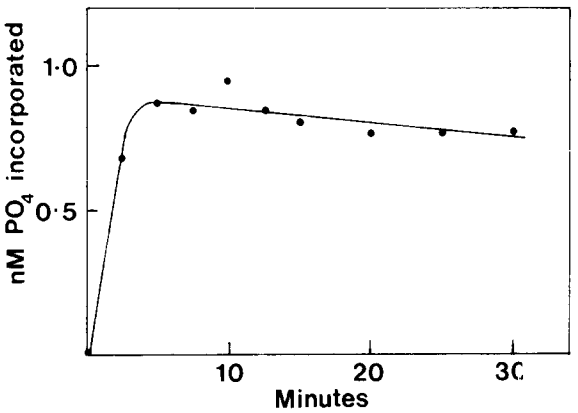

Fig.1. Phosphorylation of microtubule protein by an endogenous cAMP-dependent protein kinase. Time course of incorporation of ^{32}P into trichloroacetic acid-precipitable protein. Microtubule protein (2.94 mg/ml) was incubated at 37°C with 500 μM ATP.

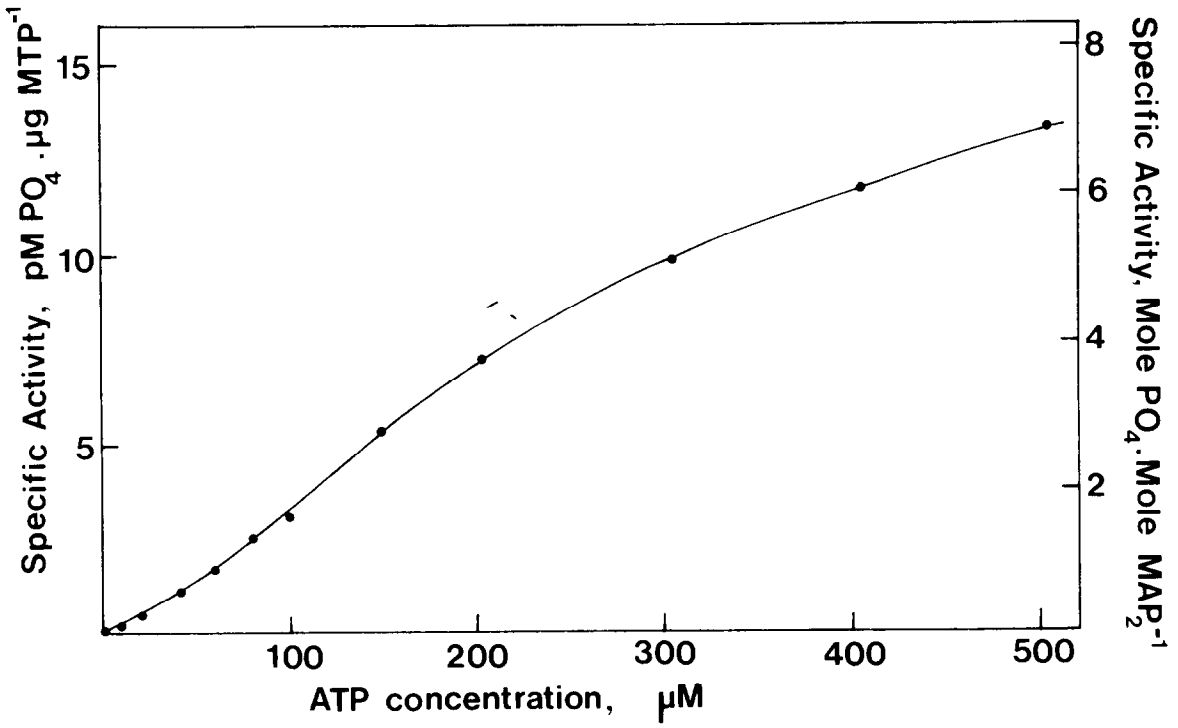

Fig.2. Endogenous phosphorylation of microtubule protein as a function of [ATP]. Incorporation of ^{32}P into trichloroacetic acid-precipitable protein as a function of [ATP] (1–500 μM). The protein was incubated under standard reaction conditions. The mol/mol ^{32}P/MAP$_2$ ratios were calculated as in section 3.

slicer, dried onto filter paper (Whatman no. 1), and counted in a liquid scintillation counter.

2.4. *Protein estimation*

Protein concentrations were determined as in [15], using bovine serum albumin as the calibration standard.

2.5. *Materials*

All biochemical reagents were purchased from Sigma (London) Chemical Co. Other reagents were of 'Analar' grade. [γ-^{32}P]ATP (15.3 Ci/mmol) was purchased from the Radiochemical Centre, Amersham.

3. Results and discussion

When 2 × cold-dissociated microtubule protein is incubated with 500 μM [γ-^{32}P]ATP, there is a rapid incorporation during the first 5 min ^{32}P into trichloroacetic acid-precipitable protein (fig.1). There is no apparent lag phase, the half-maximal is reached in 1–2 min and, although there is a slight fall with time, the plateau value is maintained for at least 30 min. The inclusion of 20 mM NaF reduced the fall with time, but had negligible effect on the plateau value. NaF was not routinely included, as it exerts side effects on the properties of the microtubule protein (unpublished) and as there was only a slight decrease of the plateau value with time.

Incubation of microtubule protein with an increasing concentration of [γ-^{32}P]ATP (1–500 μM) shows a concentration-dependent increase in the amount of ^{32}P incorporated into trichloroacetic acid-precipitable protein (fig.2), with the plateau value being attained at >500 μM ATP. The protein was fractionated on SDS–polyacrylamide gels (fig.3). The absorbance scan (fig.3a) reveals two major protein bands, one at 300 000 M_r which constitutes 19% of the total protein and is identified as MAP_2, and another at app. M_r 55 000, which constitutes 65% of the total protein and which is tubulin [1]. Although various minor bands appearing on the gel are phosphorylated (fig.3b–d), 29% of the total incorporated ^{32}P comigrates with MAP_2. The pattern of incorporation is not significantly different on increasing the concentration of ATP (e.g.,

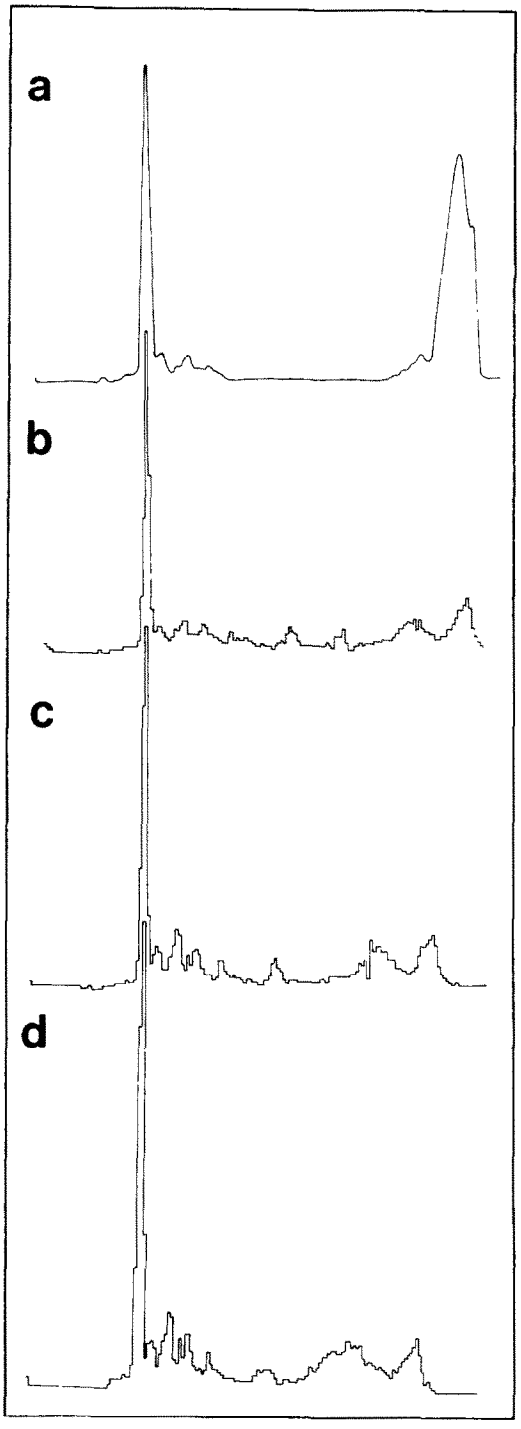

Fig.3. Pattern of phosphorylation of microtubule protein as a function of the ATP concentration. Microtubule protein (4.83 mg/ml) was incubated at 37°C for 25 min with (b) 11 μM ATP, (c) 81 μM ATP, (d) 504 μM ATP and fractionated on 6% polyacrylamide gels. The gels were stained with Coomassie brilliant blue, scanned at 500 nm (typical example: (a)), and sliced into 0.5 mm sections (b–d). The radioactivity was determined by scintillation counting. The peak slices of gels (b–d) contain 6, 6 and 16 × 10^3 cpm/slice. The same amount of protein was loaded onto each gel.

fig.3b–d). No single component is selectively labelled at high ATP concentrations.

The stoichiometry of ^{32}P incorporation into MAP_2 (fig.2) was calculated by applying the following corrections and assumptions to the total trichloroacetic acid-precipitable counts:

(i) The residual background [γ-^{32}P]ATP in the gel slices after destaining was calculated from the mean of 5 slices taken immediately ahead of the solvent front. The total counts recovered from the gels after correction for background and decay average 110% of the trichloroacetic acid-precipitable counts, indicating that the two methods are comparable.
(ii) Coomassie brilliant blue stains the tubulins and the microtubule-associated proteins quantitatively and with the same colour development over the ranges applied.
(iii) Bovine serum albumin and the microtubule proteins show the same degree of colour development in the Hartree reaction.
(iv) The app. M_r MAP_2 is 300 000 [5].
(v) MAP_2 constitutes 19.3% of the total protein (e.g., fig.3, mean of 8 det.).
(vi) The incorporation of ^{32}P into MAP_2 constitutes 29% of the total incorporated counts (e.g., fig.3, mean 8 det.).

A curve similar to that derived from the trichloroacetic acid-precipitable counts is observed when the stoichiometry is calculated from the counts comigrating with the MAP_2 peak, the MAP_2 content determined from densitometric tracings of the individual stained gels, and the application of the corrections and assumptions (i)–(iv) above.

Maximal phosphorylation is not observed (fig.2), even at high ATP concentrations, but the maximal value can be determined from a double reciprocal plot of mol/mol $^{32}P/MAP_2$ and the ATP concentration (fig.4). Such a double reciprocal plot diverges from linearity at low ATP concentrations, possibly due to the presence of various ATPases [6,9], lowering the effective ATP concentration. We have confirmed, by thin-layer chromatography, that extensive ATP hydrolysis has occurred at low ATP concentrations by the end of the 25 min incubation period. This hydrolysis reflects in part dephosphorylation of labelled MAPs and can be reduced by the inclusion of NaF. However, even under these conditions, the maximal extent of

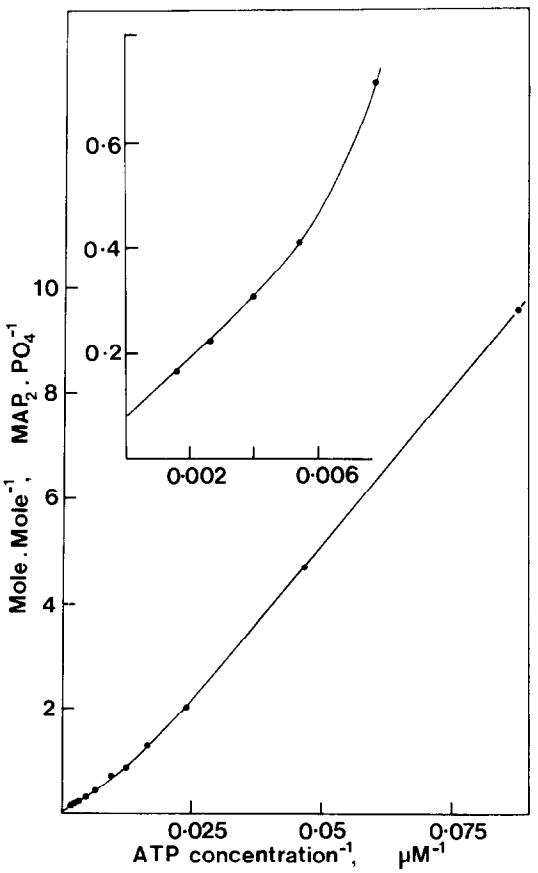

Fig.4. Double reciprocal plot of the mol/mol $^{32}P/MAP_2$ *vs* 1/[ATP] of the data shown in fig.2. Insert: Expansion of the intersect.

phosphorylation, compared with the use of high ATP concentrations, is not observed. Hydrolysis and turnover are minimal at high ATP concentrations and the double reciprocal plot of this portion of the curve shows an intercept of 10–12 mol/mol $^{32}P/MAP_2$ (fig.4 insert).

This value contrasts with the reported stoichiometry of 2 mol/mol [5] or the more recent value of 3–4 mol/mol [7], both of which were determined using low ATP concentrations. The use of such suboptimal concentrations raises a number of difficulties. Preparations of microtubule protein contain a number of enzymatic activities, including nucleoside diphosphate kinase [16], the GTPase intrinsic to tubulin [17], and the phospho protein phosphatase, which cumulatively reduce the effective ATP concentration. ADP has been shown to inhibit the protein kinase of bovine brain at low ATP concentrations [18], and this

necessitates the use of ATP concentrations considerably above the K_m for the protein kinase [19]. It is also possible that different workers are using preparations of microtubule protein containing different protein kinases. For instance, optimal concentrations of cAMP have been shown to double the number of phosphorylated residues [5,6] and that the heat-stable protein inhibitor abolishes this stimulation [6]. By contrast, other preparations exhibit a 5-fold stimulation and all the protein kinase activity is sensitive to this inhibitor [8].

MAP_2 is the major protein phosphorylated, although a number of minor components are also labelled, having M_r-values of 280, 240, 220, 190, 130 and $70–80 \times 10^3$ (fig.3). The pattern is independent of the ATP concentration (fig.3a–c) and, as the observed kinetics for the phosphorylation of these proteins is the same as for MAP_2 (unpublished), it is possible that they are breakdown products of MAP_2, as suggested on the basis of immunological cross-reaction [20].

The function of the observed phosphorylation of these microtubule-associated proteins by an endogenous cAMP-dependent protein kinase is unknown. MAP_2 is known to form regular projections from the outer surface of the microtubule [20,21] with a periodicity of 1 MAP_2 projection/32 nm, equivalent to 1 MAP_2 subunit/9 tubulin dimers at saturating MAP levels [22,23]. Consequently, the multiple phosphorylation sites of MAP (10–12 mol/mol) may be involved in the interaction between MAP and tubulin, with ATP as one of several factors which modulate the interaction.

Acknowledgements

We wish to thank the Medical Research Council for their support, and Mr Lutrario of Ross Breeders Limited for generously donating the chicks.

References

[1] Borisy, G. G., Marcum, J. M., Olmsted, J. B., Murphy, D. B. and Johnson, K. A. (1975) Ann. NY Acad. Sci. 253, 107–132.
[2] Dentler, W. L., Granett, S. and Rosenbaum, J. L. (1975) J. Cell Biol. 65, 237–241.
[3] Sloboda, R. D., Dentler, W. L. and Rosenbaum, J. L. (1976) Biochemistry 15, 4497–4505.
[4] Sloboda, R. D. and Rosenbaum, J. L. (1979) Biochemistry 18, 48–55.
[5] Sloboda, R. D., Rudolf, S. A., Rosenbaum, J. L. and Greengard, P. (1975) Proc. Natl. Acad. Sci. USA 72, 177–181.
[6] Sheterline, P. (1977) Biochem. J. 168, 533–539.
[7] Coughlin, B. A., White, H. D. and Purich, D. L. (1980) Biochem. Biophys. Res. Commun. 92, 89–94.
[8] Rappaport, L., Leterrier, J. F., Virion, A. and Nunez, J. (1976) Eur. J. Biochem. 62, 539–549.
[9] Sheterline, P. (1976) Biochem. Soc. Trans. 4, 789–791.
[10] Sheterline, P. and Schofield, J. G. (1975) FEBS Lett. 56, 297–302.
[11] Sandoval, I. V. and Cuatrecasas, P. (1976) Nature 262, 511-514.
[12] Wilson, L., Bamburg, J. R., Mizel, S. B., Grisham, L. M. and Creswell, K. M. (1974) Fed. Proc. FASEB 33, 158–166.
[13] Laemmli, U. K. (1970) Nature 227, 680–685.
[14] Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2617.
[15] Hartree, E. F. (1972) Anal. Biochem. 48, 422–426.
[16] Burns, R. G. and Islam, K. (1980) Cell Biol. Int. Rep. 4, 788.
[17] David-Pfeuty, T., Erickson, H. and Pantaloni, D. (1977) Proc. Natl. Acad. Sci. USA 74, 5372–5376.
[18] Miyamoto, E., Kuo, J. F. and Greengard, P. (1969) J. Biol. Chem. 244, 6395–6402.
[19] Corbin, J. A. and Reimann, E. M. (1974) Methods Enzymol. 38/c, 287–290.
[20] Sloboda, R. D., Dentler, W. L. and Rosenbaum, J. L. (1976) Biochemistry 15, 4497–4505.
[21] Vallee, R. B. and Borisy, G. G. (1977) J. Biol. Chem. 252, 377–382.
[22] Amos, L. A. (1977) J. Cell Sci. 72, 642–654.
[23] Kim, H., Binder, L. I. and Rosenbaum, J. L. (1979) J. Cell Biol. 80, 266–276.