Phosphorylation of Myelin Basic Protein by an Adenosine 3':5'-Cyclic Monophosphate-Dependent Protein Kinase

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(Received 20 August 1973)

Myelin basic protein was shown to be a substrate for protein kinase from rabbit muscle. One of the major sites of phosphorylation was the serine residue in the sequence Gly-Arg-Gly-Leu-Ser-Leu. The arginine residue in this sequence is known to be a substrate for a protein methylase.

Cyclic AMP-dependent protein kinases are extensively distributed in animal tissues (Krebs, 1972). These enzymes are frequently assayed by using histone as an exogenous substrate, although phosphorylation of a limited number of other proteins has been reported (Krebs, 1972; Johnson *et al.*, 1971; Traugh *et al.*, 1973; Weller & Rodnight, 1973).

Myelin contains a protein that has a number of properties in common with histones. This protein is small and highly basic, is acetylated at the *N*-terminal residue, is methylated at an arginine residue and appears to lack tertiary structure *in vitro* (Carnegie, 1971). In the present paper myelin basic protein is shown to be a substrate for a rabbit muscle protein kinase.

Materials and methods

Rabbit muscle protein kinase was prepared by the procedure of Walsh *et al.* (1968). Myelin basic proteins were isolated from rat and human brains (Dunkley & Carnegie, 1973; Deibler *et al.*, 1972). [γ - 32 P]ATP was prepared as described by Glynn & Chappell (1964). Other materials and methods were as described by Kemp *et al.* (1973) or Carnegie (1971).

Phosphorylation of the human myelin basic protein. Standard assay mixtures contained 5 μ mol of Hepes buffer [2-(*N*-2-hydroxyethyl)piperazin-*N'*-yl]ethanesulphonic acid, potassium salt, pH 7.5], 1 μ mol of MgCl₂, 52.03 nmol of [γ - 32 P]ATP (specific radioactivity 63.5 d.p.m./pmol) and 100 μ g of protein in a final volume of 100 μ l. In assays containing cyclic AMP a final concentration of 2 μ M was used. Assays, done in duplicate, were carried out at 37°C for 10 min. Termination of the reaction, acid washing and liquid-scintillation counting were all as described by Kemp *et al.* (1973).

Results and discussion

There was no detectable phosphorylation of the myelin basic protein in the absence of protein kinase from rabbit muscle.

In reaction mixtures containing protein kinase and myelin basic protein most of the radioactivity of the phosphorylated product was alkali-labile (96%; 0.097 M-KOH, 100°C, 15 min) and stable in acid (100%; 0.1 M-HCl, 100°C, 15 min). After partial acid hydrolysis (2 M-HCl, 100°C, 2 h) and electrophoresis in formic acid-acetic acid-water (1:4:45, by vol.; pH 2), 61% of the phosphorylated product was not hydrolysed and remained at the origin and 16% migrated with the *O*-phosphoserine marker. Only a small amount of radioactivity (3%) was detected in the *O*-phosphothreonine position. The remaining (20%) radioactivity migrated with the *P*_i marker. No correction was made for hydrolysis of *O*-phosphoserine or *O*-phosphothreonine. The phosphorylated product bound tightly to Sephadex CM-25 and was not eluted with 0.1 M-NaHCO₃. The phosphorylated product migrated towards the cathode during electrophoresis in polyacrylamide gel (18% acrylamide, pH 2.9). The radioactivity, in the gel, associated with the basic protein accounted for 91% of the total radioactivity precipitated with trichloroacetic acid. Under these electrophoresis conditions none of the proteins derived from the rabbit muscle protein kinase migrated towards the cathode.

Dephosphorylated casein (Kemp *et al.*, 1973), whole calf thymus histone type IIA (Sigma Chemical Co., St. Louis, Mo., U.S.A.) and basic protein of human myelin were compared as substrates for the rabbit muscle protein kinase. The concentrations of dephosphorylated casein, histone and myelin basic protein (mg/ml) required to give half-maximum velocities, in the presence of 2 μ M-cyclic AMP, were 3.7 $\pm$ 0.5, 1.4 $\pm$ 0.1 and 2.7 $\pm$ 0.5 respectively. The corresponding maximum velocities (pmol of

Fig. 1. Peptide 'map' of tryptic digest of phosphorylated basic protein of human myelin

Electrophoresis was at 3 kV for 33 min at pH 6.5 and chromatography was in butan-1-ol-pyridine-acetic acid-water (15:10:3:12, by vol.). Conditions and numbering of the peptides 1-30 were as described by Carnegie (1971). Spots (major, -----; minor,) detected by radioautography are labelled A to P; of those spots only A, B and C showed a distinct ninhydrin colour. Lysine and leucine were used as markers for the electrophoresis and chromatography.

P_i transferred/min per mg of enzyme protein) obtained with these substrates were 31 ± 2 , 956 ± 53 and 540 ± 75 . Thus the myelin basic protein was an effective substrate for the kinase. Cyclic AMP caused a 2.4-fold increase in the maximum velocity of rabbit muscle protein kinase with this protein as substrate.

Tryptic digestion of the phosphorylated basic protein. Preparation of phosphorylated protein for sequence studies was done in a solution containing 125 μ mol of Hepes buffer (potassium salt, pH 7.5), 25 μ mol of $MgCl_2$, 426.33 nmol of [γ - ^{32}P]ATP (specific radioactivity 64.13 d.p.m./pmol), 5 μ mol of cyclic AMP, 10 mg of human basic protein and rabbit muscle protein kinase (1.32 mg) in a final volume of 2.55 ml. The reaction mixture was incubated at 37°C for 5 h. The phosphorylated basic protein was purified by fractionation on Sephadex CM-25 (Carnegie, 1971; Dunkley & Carnegie, 1973), and examined by gel electrophoresis in formic acid-acetic acid-urea. The stained gel was sliced into 2 mm sections and of the radioactivity measured

74% was recovered in a single band corresponding to the myelin basic protein. The remainder was associated with minor components which were possibly breakdown products produced by traces of proteinases present during the prolonged incubation. The phosphorylated basic protein (0.28 μ mol; 0.37 mol of P_i /mol) was digested with trypsin (100 μ g, 2 h) and 15% of the digest was fractionated by paper electrophoresis and chromatography (Carnegie, 1971).

The peptide 'map' of the digest is shown in Fig. 1. Three spots A, B and C, not normally observed in tryptic 'maps' of the basic protein, were present. Spot A was predominant and spot C had a yellow colour with ninhydrin. Phosphorylated peptides were located by radioautography for 24 h and the radioactivity of the peptides was determined by using a Nuclear-Chicago gas-flow counter (efficiency 30.8%) (Table 1). The peptide spots were washed with acetone, eluted with 6M-HCl and heated in sealed tubes in a boiling-water bath for 1½ h. The

Table 1. *Recovery of radioactivity from a tryptic digest of phosphorylated myelin basic protein*

For position of spots A to H see Fig. 1. Spots I to P from the human protein had a total of 8% of the radioactivity of the digest. Two additional spots, which were present only in the digest of the rat protein, had 1.8 and 6.9% of the radioactivity. 'Unknown' denotes an unidentified spot with an electrophoretic mobility, at pH 3.5, 1.61 times that of *O*-phosphoserine.

Spot	Recovery of radioactivity (%)		³² P-labelled amino acid in hydrolysate of human and rat proteins
	Human	Rat	
A	27.4	22.5	Ser
B	11.8	10.1	Thr
C	15.2	27.8	Ser
D	7.6	9.8	Ser
E	4.4	Absent	Ser and unknown
F	5.2	3.9	Ser
G	8.5	3.9	Ser
H	2.6	1.2	Ser
Total recovery	90	84.6	
Total d.p.m. taken	0.47×10^6	1.52×10^6	

partial hydrolysates were examined for *O*-phosphoserine by electrophoresis at pH 3.5 and radioautography (Table 1) (Murray & Milstein, 1967).

Rat myelin contains two basic proteins, one which is similar to the human protein and one which has, when compared with the human protein, a linear deletion of 40 amino acids in the C-terminal half of the molecule (P. R. Dunkley & P. R. Carnegie, unpublished work). The smaller protein from rats was phosphorylated as described above and a tryptic digest examined (Table 1). Most of the spots on the peptide 'map' were identical with those found in the digest of the human protein, which would suggest that phosphorylation was occurring in the regions of invariant amino acid sequence.

Identification of spots. Spot A material was isolated from the remainder of the digest of the human protein. It had the composition His (0.9), Gly (0.9), Ser (1.0) and Lys (1.2). Histidine was shown to be *N*-terminal by Edman degradation followed by amino acid analysis. This peptide has a unique composition in the protein, and is derived from peptide T28, His-Gly-Ser-Lys, which represents amino acid residues 10 to 13 (Carnegie, 1971).

Spot C material was more heavily phosphorylated in the rat protein than in the human protein. From its yellow colour with ninhydrin, high R_F value and amino acid composition spot C material was identified as being derived from Gly-Leu-Ser-Leu-Ser-Arg. Electrophoretic analysis of a thermolysin digest of spot C material showed that the first serine residue (i.e. Leu-Ser-Leu) rather than the second was phosphorylated. This peptide results from tryptic

digestion at arginine-107 and arginine-113 in the human protein. Spot F material also appears to be derived from this region of the protein, as it contained the monomethylarginine residue (no. 197), which is resistant to tryptic digestion (Baldwin & Carnegie, 1971). The myelin basic proteins are heterogeneous with regard to methylation of arginine-107. For example the rat protein has 12 times as much unmethylated arginine at this position as has the human protein (P. R. Dunkley & P. R. Carnegie, unpublished work). It was surprising to find no trace of dimethylarginine in spot F, as there is 1.6 times as much of the dimethyl form in the human protein. The mono- and di-methyl forms would be expected to be in the same position on the peptide 'map'. Perhaps the extent of methylation influences the degree of phosphorylation.

The remaining spots were present in too low a yield to enable definitive identification and the minor spots may have come from degradation products of the protein. Spot E contained, in addition to a small amount of phosphoserine, an unidentified acidic phosphate which was still present after 3 h hydrolysis with acid.

Thus, although the myelin basic proteins have a high content of serine and threonine, only a few residues appear to be phosphorylated to any marked extent by the rabbit muscle protein kinase. There is no obvious similarity in amino acid sequence in the regions of the protein containing peptides A and C. This would suggest that either the kinase recognizes some feature of ordered structure in the protein, or the kinase preparation is a mixture of enzymes with

subtle differences in their substrate specificities, as has been suggested by Bitensky & Gorman (1973).

Johnson *et al.* (1971) found that myelin had some endogenous protein kinase activity and also had a large proportion of the total substrate in brain for a cyclic AMP-dependent protein kinase. In preliminary experiments we have found that, when myelin was incubated with [γ - 32 P]ATP, the endogenous kinase phosphorylated the basic protein but not the proteolipid, which is the major protein in myelin. Since phosphorylation of cerebral proteins can be readily demonstrated *in vivo* (Rodnight, 1971) it will be important to assess the extent of phosphorylation of the myelin basic protein, in different physiological and behavioural states.

We thank the Australian Research Grants Committee and the National Multiple Sclerosis Society, New York, U.S.A., for financial support and Mr. G. Luxford for preparing the basic proteins. This work was presented in part at the 4th Meeting of the International Society for Neurochemistry, August 1973.

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