Endogenous cyclic AMP-stimulated phosphorylation of a Wolfgram protein component in rabbit central-nervous-system myelin

Jane M. BRADBURY,* R. Stewart CAMPBELL and R. J. THOMPSON

Department of Clinical Biochemistry, School of Clinical Medicine, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QR, U.K.

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1. Cyclic AMP-stimulated phosphorylation of membrane proteins in central-nervous-system myelin was investigated, with rabbit brain myelin. 2. Subfractionation of a myelin membrane preparation by sucrose-density-gradient centrifugation produced a rapidly sedimenting population of membrane vesicles containing 5'-nucleotidase and acetylcholinesterase, a light membrane fraction containing myelin basic protein and 2',3'-cyclic nucleotide 3'-phosphodiesterase, and an intermediate membrane fraction containing the highest specific activity of 2',3'-cyclic nucleotide 3'-phosphodiesterase and a small proportion of myelin basic protein. 3. Cyclic AMP stimulation of protein phosphorylation was confined to a protein of M_r 49 700, which co-electrophoresed with the upper component of the Wolfgram protein doublet. Cyclic AMP did not affect the phosphorylation of myelin basic protein. 4. Cyclic AMP-stimulated phosphorylation of this protein followed 2',3'-cyclic nucleotide 3'-phosphodiesterase activity on subcellular fractionation and was correspondingly high in the intermediate or 'myelin-like' fraction on sucrose-density-gradient centrifugation.

The protein composition of central-nervous-system myelin is relatively simple, consisting largely of myelin basic protein of M_r 18 400 (Eylar, 1970; Carnegie, 1971), proteolipid protein (Folch & Lees, 1951; Autilio, 1966) and the Wolfgram doublet of proteins of M_r 44 000 and 47 000 (Wolfgram & Kotorii, 1968). In addition, at least one enzyme, CNPase, is considered to be intrinsic to the myelin membrane (Kurihara & Tsukada, 1967; Sims & Carnegie, 1979) and probably constitutes part of the Wolfgram doublet itself (Drummond & Dean, 1980; Sprinkle *et al.*, 1980). There has also been considerable interest in another myelin-associated enzyme activity (protein kinase, EC 2.7.1.37) and its role especially in the phosphorylation of myelin basic protein (Sulakhe *et al.*, 1980a,b; Turner *et al.*, 1982). The initial demonstration of phosphorylation of myelin membranes by exogenous cyclic AMP-dependent protein kinase (Johnson *et al.*, 1971; Carnegie *et al.*, 1973; Miyamoto & Kakiuchi, 1974) was followed by evidence for an endogenous cyclic AMP-independent protein kinase acting on myelin

basic protein (Carnegie *et al.*, 1973; Miyamoto & Kakiuchi, 1974; Miyamoto *et al.*, 1974; Steck & Appel, 1974), and also for an endogenous cyclic AMP-dependent protein kinase that appeared only to phosphorylate exogenous substrates (Miyamoto, 1975). The major protein kinase phosphorylating myelin basic protein was shown to be Ca^{2+} - and calmodulin-dependent by Sulakhe *et al.* (1980a,b) and Endo & Hidaka (1980), but more recently the major Ca^{2+} -dependent kinase in myelin has been reclassified by Turner *et al.* (1982) as a phospholipid-sensitive protein kinase C-type enzyme. Endogenous myelin membrane protein kinases involved in the phosphorylation of myelin basic protein are therefore currently believed to be (1) a basal (cyclic nucleotide- and Ca^{2+} -independent) kinase, (2) a phospholipid- and Ca^{2+} -dependent kinase, and (3) possibly a Ca^{2+} - and calmodulin-dependent kinase. Cyclic AMP-dependent kinases are thought to be of little importance in the phosphorylation of myelin basic protein (Sulakhe *et al.*, 1980b; Turner *et al.*, 1982). However, in both rat and rabbit myelin membranes cyclic AMP-stimulated phosphorylation of a protein of a higher molecular mass has been observed which on electrophoresis migrates in the region of the Wolfgram doublet (Sulakhe *et al.*,

Abbreviations used: CNPase, 2',3'-cyclic nucleotide 3'-phosphodiesterase; 5'NT, 5'-nucleotidase. AChE, acetylcholinesterase; SDS, sodium dodecyl sulphate.

* To whom correspondence should be addressed.

1980a; Endo & Hidaka, 1980; Turner *et al.*, 1982). *In vivo*, phosphorylation of proteins migrating in this region has also been shown in rat brain myelin (Agrawal *et al.*, 1982). Sulakhe *et al.* (1980a) showed in rat brain that this endogenous cyclic AMP-stimulated phosphorylation was characteristic of medium-heavy or 'myelin-like' membrane fractions rather than of light, multilamellar or 'mature' myelin, but did not discount the possibility of contamination from non-myelin membranes.

The present study shows that rabbit central-nervous-system myelin contains an endogenous cyclic AMP-stimulated kinase that specifically phosphorylates the upper component of the Wolfgram protein doublet. Cyclic AMP-stimulated phosphorylation of this protein closely follows CNPase activity during subcellular fractionation.

Materials and methods

Materials

All chemicals were of AnalaR grade and obtained from BDH Chemicals, Poole, Dorset, U.K., unless otherwise stated.

Isolation and subfractionation of myelin

This was based on the methods of Whittaker (1965). Adult New Zealand White rabbits (2½ kg) were stunned and then killed by decapitation. The brain was immediately removed into ice-cold 0.32 M-sucrose. After removal of the meninges, the brain was coarsely chopped and then homogenized in 10 vol. of ice-cold 0.32 M-sucrose with 12 passes of an Aldridge homogenizer. The homogenate was filtered through muslin and then centrifuged as detailed in Scheme 1. The final pellet, P₄, was resuspended in 5 ml of 0.32 M-sucrose. For subfractionation 2 ml of fraction P₄ was layered on to two continuous linear gradients of 0.32–1.2 M-sucrose (total volume of gradient 20 ml). The gradients were centrifuged at 25 000 rev./min in an MSE 3 × 25 ml swing-out rotor (65 000 *g*_{av.}) for 3.5 h at 4°C. The same profile was obtained if the gradients were centrifuged for 16 h at a similar speed. Fractions (1 ml) were collected by using a cannula passed gently to the bottom of the gradient and a peristaltic pump. Generally the fractions from two gradients were pooled. Sucrose concentrations in the fractions were measured by using a refractometer.

In some experiments the fractions were concentrated by centrifugation at 40 000 rev./min for 30 min at 4°C in an MSE 6 × 5.5 ml swing-out rotor (146 000 *g*_{av.}). The final pellets were resuspended in 0.2 ml of 0.32 M-sucrose.

Electron microscopy

Samples for electron microscopy were fixed

Scheme 1. Subcellular fractionation of rabbit brain

Fresh rabbit brain was homogenized as described in the Materials and methods section and then subjected to the protocol shown here. All centrifugation steps were done at 4°C. Fractions were harvested from the discontinuous gradient with a Pasteur pipette. P₂A and P₂B were collected from the interfaces of the discontinuous gradient. The region of the gradient between these fractions was collected as P₂A–P₂B. The region of the gradient below P₂B was collected as P₂B–P₂C. P₂C consisted of the mitochondrial pellet resuspended in a small volume of 0.32 M-sucrose.

overnight at 4°C by mixing 1 ml of fraction with 4 ml of ice-cold 2.5% (v/v) glutaraldehyde (electron-microscopy grade) in 0.1 M-sodium phosphate buffer, pH 7.4. The tissue pellets were collected by spinning at 30 000 rev./min for 30 min at 4°C in a Beckman SW50.1 rotor (75 000 *g*_{av.}). After the supernatants had been decanted off, the pellets were rinsed in 0.1 M-sodium phosphate buffer, pH 7.4, before the addition of 1 ml of 0.1% osmic acid in the same buffer, after which they were left for 3 h. The pellets were then dehydrated through graded ethanol, and embedded in Spurr's resin.

Sections were cut on an LKB Ultratome III and examined in an AEI EM6B electron microscope.

Enzyme assays

CNPase (EC 3.1.4.37) activity was measured with 2',3'-cyclic nicotinamide-adenine dinucleotide phosphate as the substrate as described by Sogin (1976). Lactate dehydrogenase (EC 1.1.1.27), citrate synthase (EC 4.1.3.7), AChE (EC 3.1.1.7) and cyclic AMP phosphodiesterase (EC 3.1.4.17) activities were assayed as described by Johnson (1960), Srere (1969), Ellman *et al.* (1961) and Hutton *et al.* (1981) respectively. 5'-NT (EC 3.1.3.5) activity was measured by using the method of Stanley *et al.* (1980) with 0.1% Triton X-100 in the reaction mixture. Protein concentrations were measured by using the method of Lowry *et al.* (1951), with bovine serum albumin (Armour Pharmaceutical Co., Eastbourne, East Sussex, U.K.) as a standard.

Electrophoresis

Laemmli-type SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) with an apparatus similar to that described by Studier (1973) was used to examine the polypeptides present in myelin. Gels were stained by a modification of the method of Fairbanks *et al.* (1971) in which the gels were stained overnight in 0.03% Kenacid Blue R in 25% (v/v) propan-2-ol/7.5% (v/v) acetic acid and then destained in the same solution minus the dye. All staining steps were done at 4°C with continuous shaking. When they were dry, radioactively labelled gels were autoradiographed at -70°C in a

Cronex X-ray cassette with intensifying screen with the use of Cronex 4 X-ray film (Dupont, St. Neots, Cambs., U.K.).

Phosphorylation

Phosphorylation of myelin and myelin sub-fractions was performed in a total volume of 40 μ l containing 10 μ g of protein. The reaction mixture contained 30 mM-Tris/HCl, pH 7.4, 1 mM-MgCl₂, 0.05% Triton X-100, 0.05 mM-ATP (Sigma Chemical Co.) and 0.005 mM-[³²P]ATP (0.5–2.0 Ci/mmol) prepared as described by Walsh *et al.* (1971) by using [³²P]orthophosphate (Amersham International, Amersham, Bucks., U.K.). Reactions were done with or without 5 μ M-cyclic AMP (Sigma Chemical Co.). Incubations were carried out for 5 min at 37°C and were stopped by adding 40 μ l of SDS-sample buffer (containing 200 mM-Tris/HCl, pH 8.8, 2% SDS, 5 mM-EDTA, 1 M-sucrose, 10 mM-dithiothreitol and 0.01% Bromophenol Blue) and boiling for 3 min. Further treatment of the samples was as described above under 'Electrophoresis'. Incorporation of label was linear with time for up to 10 min. To quantify the incorporation of phosphate into various proteins in myelin, bands were cut out of dried gels and their radioactivities counted by Čerenkov radiation.

Results

Subcellular fractionation

Scheme 1 summarizes the subcellular fractionation scheme used in the present study, and the marker enzyme values obtained during this procedure are shown in Table 1. The final myelin (P₄)

Table 1. Marker enzyme activities in the subcellular fractionation procedure for the preparation of myelin fraction P₄. Only the enzyme activities in 'end fractions' (i.e. fractions that are not further processed; see Scheme 1) are shown. '% of total activity' refers to the percentage of total activity in the initial homogenate recovered in each fraction; total activities in the homogenate from a 6.5 g rabbit brain were 57.93 units, 77 996 units, 16.97 units, 393.6 units and 5808 units for AChE, 5'-NT, citrate synthase, lactate dehydrogenase (LDH) and CNPase respectively (where units are expressed as μ mol/min in each case). Total recoveries of enzyme activities were 87%, 101%, 113%, 78% and 93% respectively. Approx. 3% (23 mg) of the total initial homogenate protein (781 mg) appeared in the final myelin P₄ fraction. Although 5'-NT and CNPase activities were detectable in SN₄, the protein content was below the limit of detection. It is assumed that the high proportion of CNPase sedimenting in P₁ represents large myelin fragments and myelinated axons. 'Specific activities' are expressed as units/mg of protein.

Fraction	AChE		5'-NT		Citrate synthase		LDH		CNPase	
	% of total activity	Specific activity	% of total activity	10 ³ × Specific activity	% of total activity	Specific activity	% of total activity	Specific activity	% of total activity	Specific activity
H	100	0.074	100	7.05	100	0.021	100	0.50	100	7.44
P ₁	23	0.044	41	7.54	6	0.013	19	0.245	53	10.3
SN ₂	22	0.065	21	6.46	25	0.023	42	0.907	7	2.28
P ₂ A–P ₂ B	11	0.237	10	20.67	3	0.018	4	0.52	5	11.5
P ₂ B	15	0.137	16	13.18	5	0.013	8	0.49	3	2.8
P ₂ B–P ₂ C	1	0.068	2	9.55	11	0.16	2	0.16	1	3.02
P ₂ C	10	0.210	4	7.19	59	0.364	2	0.32	1	0.71
SN ₄	0	—	1	?	N.D.	—	0	—	5	?
P ₄	5	0.142	6	14.52	3.4	0.03	1	0.226	19	50.5

fraction contained 19% of the initial homogenate CNPase activity, with a 6.78-fold increase in specific activity (Table 1). Only 1% of the initial lactate dehydrogenase and 3.4% of the initial citrate synthase activity appeared in the P_4 fraction. The citrate synthase specific activity in P_4 was less than 10% of that found in the mitochondrial fraction. However, 5% of the total AChE and 6% of the total 5'NT activity appeared in P_4 , both at about twice the specific activity in the initial homogenate and both at approx. 50–70% of the specific activity found in P_2A – P_2B , the fraction containing the highest specific activity for these two enzymes (Table 1).

Phosphorylation of myelin fraction P_4

Fig. 1 shows the pattern of phosphorylation of a P_4 sample prepared as described in the Materials

Fig. 1. Phosphorylation of myelin

A 10 μ g portion of P_4 was phosphorylated and prepared for SDS/polyacrylamide-gel electrophoresis as described in the Materials and methods section. Then 5 μ g of protein per track was applied to an SDS/12.5%-polyacrylamide gel. Track 1 shows Kenacid Blue-stained molecular-mass markers (phosphorylase b, M_r 94000; bovine serum albumin, M_r 67000; ovalbumin, M_r 43000; carbonic anhydrase, M_r 30000). Tracks 2 and 5 show the Kenacid Blue staining of the autoradiograms shown in tracks 3 and 4 respectively. Track 3 is myelin phosphorylated in the presence of 5 μ M-cyclic AMP; track 4 is myelin phosphorylated in the absence of cyclic AMP. W, Wolfgram protein doublet; MBP, myelin basic protein.

and methods section. Tracks 2 and 5 clearly show the limited number of Kenacid Blue-staining moieties present in myelin. Tracks 3 and 4 show the even simpler phosphorylation pattern: only two proteins were phosphorylated to any great extent. Myelin basic protein was phosphorylated in a cyclic AMP-independent manner, and an unknown protein of apparent M_r 49700 (49700 ± 1800 , ten determinations) incorporated phosphate in a cyclic AMP-stimulated manner. Inclusion of 5 μ M-cyclic AMP in the incubation medium increased phosphorylation of the band by up to 100%. Superimposition of the autoradiogram on to the stained and dried gel showed that the phosphorylated 49700- M_r protein co-electrophoresed with the upper band of the Wolfgram protein doublet (labelled W in Fig. 1). [For the purposes of the present study we have adopted the definition of Wolfgram proteins proposed by Sprinkle *et al.* (1980) as being the doublet running close to an ovalbumin standard on SDS/polyacrylamide-gel electrophoresis.] In order to establish whether the cyclic AMP-stimulated phosphorylation was characteristic of the myelin fraction P_4 rather than of other subcellular fractions, 10 μ g of each fraction from the subcellular fractionation procedure (Scheme 1) was incubated in the phosphorylation medium with and without cyclic AMP and then subjected to SDS/polyacrylamide-gel electrophoresis as described in the Materials and methods section. The area of the gel corresponding to the Wolfgram protein doublet (which was clearly visible as such in fractions P_2 , P_2A , P_3 , SN_3 , P_4 and SN_4 , but not the remaining fractions) was then excised and its radioactivity counted by Čerenkov radiation. The results are shown in Fig. 2: cyclic AMP-stimulated protein phosphorylation of this area of the gel segregated with the P_4 myelin fraction rather than any other subcellular fraction.

Subfractionation of the myelin P_4 fraction

The enzyme activity and protein profiles obtained when P_4 was further fractionated on a linear sucrose gradient are shown in Fig. 3(a). The peptide compositions of individual sucrose-gradient fractions are shown in Fig. 3(b). The protein peak (fractions 9–13) corresponded to the peak of myelin basic protein as seen on SDS/polyacrylamide-gel electrophoresis (Fig. 3b). The peak of 5'NT activity (Fig. 3a) co-sedimented with the peak of AChE activity (not shown) and contained little or no myelin basic protein (Fig. 3b). The myelin specific marker CNPase occurred before the protein and myelin basic protein peak with a smaller peak of activity lying under the protein peak (Fig. 3a). This pattern was normally seen, although occasionally it appeared more as a single broad peak of activity just before, but overlapping

Fig. 2. Phosphorylation of the 49700- M_r band in subcellular fractions of rabbit brain

A 10 μ g portion of each subcellular fraction shown in Scheme 1 was phosphorylated in triplicate in the presence ($\square$) and in the absence ($\square$) of 5 μ M-cyclic AMP, 5 μ g of labelled protein was run per track of a SDS/12.5%-polyacrylamide gel, and the region of the gel corresponding to the upper band of the Wolfgram protein doublet was excised and its radioactivity counted by Cerenkov radiation.

with, the myelin basic protein peak. This region of the gradient showed the highest concentration of Wolfgram doublet proteins (Fig. 3b). Under the electron microscope the region containing the bulk of myelin basic protein showed mainly large multilamellar structures typical of myelin (Fig. 4c). The region of the gradient with the highest 5'NT and AChE activities (fractions 1–7) consisted of small unilamellar structures (Fig. 4a), and the intermediate region (the CNPase peak) contained a mixture of the two sorts of structure (Fig. 4b) except that the larger vesicles contained fewer lamellae than those seen in the myelin basic protein peak.

Phosphorylation of sucrose-gradient fractions

Individual gradient fractions were phosphorylated in the presence and in the absence of 5 μ M-cyclic AMP and the incorporation of radioactive phosphate into the upper band of the Wolfgram doublet and myelin basic protein was determined by excising the relevant regions of SDS/polyacrylamide gels and counting the radioactivity of the gel slices by Cerenkov radiation (Fig. 5). The amount of phosphate incorporated into myelin basic protein was unaffected by the addition of cyclic AMP, whereas incorporation of phosphate into the Wolfgram region was stimulated by cyclic AMP. The peak of cyclic AMP-stimulated phosphorylation corresponded to the CNPase and Wolfgram protein doublet peak rather than to the

5'NT and AChE peak or to the myelin basic protein peak (Fig. 5). No cyclic AMP phosphodiesterase activity could be detected in any of the fractions from the sucrose gradient.

Discussion

The purpose of the present study is to establish whether the cyclic AMP-stimulated phosphorylation seen in myelin membrane preparations by previous workers (Sulakhe *et al.*, 1980a; Endo & Hidaka, 1980; Turner *et al.*, 1982) is intrinsic to the myelin membrane or whether it represents a cyclic AMP-stimulated phosphorylation in a contaminating non-myelin membrane. The virtual absence of cytoplasmic and mitochondrial enzyme markers and the high recovery of a myelin-membrane marker enzyme (CNPase; Sims & Carnegie, 1979) indicate that the myelin fraction P₄ used in the present work represents myelin as defined by current biochemical criteria. Phosphorylation of myelin fraction P₄ in the presence and in the absence of cyclic AMP shows a cyclic AMP-stimulated phosphorylation of a protein of M_r 49700, in agreement with previous workers who used different subcellular fractionation methods (Sulakhe *et al.*, 1980a; Turner *et al.*, 1982). In the present study the phosphorylated band co-migrated exactly with the upper component of the Wolfgram protein doublet (Fig. 1). Phosphorylation of other subcellular fractions under similar conditions

Fig. 3. *Linear-sucrose-density-gradient centrifugation of myelin fraction P₄*

Myelin was subfractionated on a linear sucrose density gradient as described in the Materials and methods section. (a) shows the enzyme specific-activity profiles and protein profile of the fractions obtained from a typical separation. ●, Protein concentration; ○, CNPase specific activity; ■, 5'NT specific activity; □, concn. of sucrose in the fractions. (b) shows an SDS/12.5%-polyacrylamide-gel electrophoretogram of the same fractions stained with Kenacid Blue (10 μg of protein/track). Molecular-mass markers were as in Fig. 1.

Fig. 4. *Electron microscopy of myelin subfractions*

Typical electron-microscopy profiles from the three types of subfractions described in the text are shown here. Magnification $\times 45000$. (a) 5'NT and AChE peak (fraction 5); (b) CNPase peak (fraction 8); (c) myelin basic protein peak (fraction 11).

shows that cyclic AMP-stimulated phosphorylation of proteins of this apparent molecular mass cosegregates with the preparation of myelin fraction P_4 (Fig. 2), i.e. cyclic AMP-stimulated phos-

phorylation in this fraction cannot be explained by contamination with any of the other fractions shown in Scheme 1.

Further subfractionation of myelin fraction P_4

Fig. 5. *Phosphorylation of myelin subfractions*
 Subfractions ($10\mu\text{g}$ of protein) of myelin for which enzyme and protein data are shown in Fig. 3 were phosphorylated in triplicate in the absence ($\circ$) and in the presence ($\bullet$) of $5\mu\text{M}$ -cyclic AMP. The incorporation of $[^{32}\text{P}]$ phosphate into myelin basic protein (a) and the $49700\text{-}M_r$ band (b) was determined after SDS/polyacrylamide-gel electrophoresis ($5\mu\text{g}$ of protein/track) by excising the relevant region of the gel and counting its radioactivity by Čerenkov radiation.

on a continuous sucrose gradient produces three populations of membrane fragments definable by enzyme markers, protein content and electron microscopy (Figs. 3 and 4). The lightest membrane fraction contains most of the protein applied to the gradient, the highest content of myelin basic protein, little or no 5'NT or AChE activity and a moderate specific activity of CNPase. This fraction morphologically appears as large vesicles of multilamellar myelin (Fig. 4c). The heaviest and

most rapidly sedimenting membrane population consists morphologically of a relatively homogeneous population of smaller vesicles (Fig. 4a) that contains virtually all the 5'NT and AChE activity applied to the gradient (Fig. 3a) but is devoid of myelin basic protein (Fig. 3b) and of CNPase activity. As a high specific activity of 5'NT and AChE was observed in the fraction sedimenting between the myelin and synaptosome fractions on the initial discontinuous $0.8\text{--}1.2\text{M}$ -sucrose gradient (fraction $\text{P}_2\text{A--P}_2\text{B}$, Scheme 1), the rapidly sedimenting vesicles obtained from the linear sucrose gradient (Fig. 3a) could represent contamination from a population of membrane fragments intermediate in density between myelin and synaptosomes. Although the origin of these vesicles is unclear, the dissociation of 5'NT and AChE activity from CNPase activity and myelin basic protein seen in Fig. 3(a) does not provide support for 5'NT being an integral component of rabbit brain myelin membrane, as has been proposed for rat brain myelin (Cammer *et al.*, 1980). The intermediate fraction on sucrose-density-gradient centrifugation contains the highest specific activity of CNPase, low activities of 5'NT and AChE (Fig. 3a), a relatively low content of myelin basic protein but a relatively high proportion of the Wolfram doublet proteins (Fig. 3b). Morphologically, this fraction appears as a mixture of the previous two fractions (Fig. 4b) with the exception that the larger vesicles have fewer lamellae than have those of the light myelin fraction (Fig. 4c). It is assumed that this fraction is equivalent to 'myelin-like' membranes (Banik & Davison, 1969) or 'SN₄ fraction' (Waehneltd *et al.*, 1977), a myelin-related membrane fraction that is widely believed to represent a transition state between the plasmalemma of the oligodendrocyte and compact multilamellar myelin (Banik & Davison, 1969; Waehneltd *et al.*, 1977; Quarles, 1980).

Phosphorylation of fractions obtained from sucrose-density-gradient centrifugation clearly shows that cyclic AMP-stimulated phosphorylation co-sediments with the intermediate peak of CNPase activity (Fig. 5). No cyclic AMP stimulation of the phosphorylation of myelin basic protein was seen, in agreement with previous workers (Sulakhe *et al.*, 1980b). The cyclic AMP-stimulated phosphorylation, which co-migrates with the upper component of the Wolfram doublet in unfractionated myelin P_4 (Fig. 1), is correspondingly greatest in those fractions with the greatest amount of Wolfram doublet proteins (Figs. 3b and 5). The present studies therefore show that rabbit central-nervous-system myelin contains a cyclic AMP-stimulated phosphorylation system involving the higher-molecular-mass component of the Wolfram doublet. It has been

proposed that this phosphorylated protein represents the R-subunit of a membrane-bound type II cyclic AMP-dependent kinase undergoing auto-phosphorylation (Sulakhe *et al.*, 1980a). However, since there is now considerable evidence that Wolfram proteins may represent the CNPase enzyme itself (Drummond & Dean, 1980; Sprinkle *et al.*, 1980), the possibility that cyclic AMP-stimulated phosphorylation of this enzyme is occurring needs further investigation.

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