Photoaffinity labelling of central-nervous-system myelin

Evidence for an endogenous type I cyclic AMP-dependent kinase phosphorylating the larger subunit of 2',3'-cyclic nucleotide 3'-phosphodiesterase

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1. Endogenous cyclic AMP-stimulated phosphorylation of a 49 700- M_r Wolfgram protein component in rabbit central nervous system was investigated by using photoaffinity labelling and 2',3'-cyclic nucleotide 3'-phosphodiesterase activity staining after electroblotting on to nitrocellulose paper. 2. Photoaffinity labelling with 8'-azidoadenosine 3',5'-cyclic monophosphate showed a cyclic AMP-binding protein that appeared to be intrinsic to the myelin membrane and appeared to represent the R-subunit of a type I cyclic AMP-dependent protein kinase. This photoaffinity-labelled protein was of larger apparent M_r than the protein showing cyclic AMP-stimulated phosphorylation. 3. Blotting of one-dimensional sodium dodecyl sulphate/polyacrylamide-gel electrophoretograms followed by staining for 2',3'-cyclic nucleotide 3'-phosphodiesterase activity showed two activity bands corresponding to the two components of the Wolfgram protein doublet. Cyclic AMP-stimulated protein phosphorylation corresponded to the upper component of this doublet. 4. Electroblotting of two-dimensional non-equilibrium pH-gradient electrophoretograms also showed co-migration of cyclic AMP-stimulated protein phosphorylation with enzyme activity. 5. It is proposed that central-nervous-system myelin contains an endogenous type I cyclic-AMP dependent protein kinase that phosphorylates the larger subunit of 2',3'-cyclic nucleotide 3'-phosphodiesterase.

Phosphorylation of proteins is generally believed to be of great importance in the regulation of biological activities (Greengard, 1978). There is now considerable evidence for phosphorylation systems being intrinsic to the multilamellar myelin sheath surrounding central-nervous-system axons (Carnegie *et al.*, 1973; Miyamoto & Kakiuchi, 1974; Miyamoto *et al.*, 1974; Steck & Appel, 1974; Miyamoto, 1975; Sulakhe *et al.*, 1980a,b; Endo & Hidaka, 1980; Turner *et al.*, 1982). The major protein being phosphorylated in the myelin membrane is myelin basic protein, which appears to be phosphorylated via a basal protein kinase (cyclic nucleotide- and Ca^{2+} -independent; Steck & Appel, 1974; Miyamoto, 1975; Sulakhe *et al.*,

1980a), a phospholipid- and Ca^{2+} -dependent kinase (Turner *et al.*, 1982) and possibly a Ca^{2+} - and calmodulin-dependent kinase (Sulakhe *et al.*, 1980a,b; Endo & Hidaka, 1980). Endogenous cyclic AMP-dependent protein kinases seem to have little or no effect on the phosphorylation of myelin basic protein (Sulakhe *et al.*, 1980b). However, cyclic AMP-stimulated phosphorylation of an unidentified protein, of higher molecular mass than myelin basic protein, that migrates in the region of the Wolfgram protein doublet (Wolfgram & Kotorii, 1968) has been observed by several groups (Sulakhe *et al.*, 1980a; Endo & Hidaka, 1980; Turner *et al.*, 1982). Sulakhe *et al.* (1980a) proposed that the labelling of this protein represented the autophosphorylation of the regulatory subunit of a type II protein kinase (EC 2.7.1.37) that possibly was not intrinsic to the myelin membrane. We have shown that there is a cyclic AMP-stimulated phosphorylation of a 49 700- M_r ,

Abbreviations used: CNPase, 2',3'-cyclic nucleotide 3'-phosphodiesterase; SDS, sodium dodecyl sulphate; cyclic 8-azido-AMP, 8-azidoadenosine 3',5'-cyclic monophosphate.

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protein in myelin that co-migrates with the upper component of the Wolfgram protein doublet (Wolfgram & Kotorii, 1968) and that appears to be intrinsic to the myelin membrane, as it closely follows the established myelin marker enzyme CNPase on subcellular fractionation (Bradbury *et al.*, 1984). Since there is now evidence that the Wolfgram protein doublet may represent CNPase (Drummond & Dean, 1980; Sprinkle *et al.*, 1980), an alternative possibility would be that the cyclic AMP-stimulated phosphorylation seen in the myelin membrane (Bradbury *et al.*, 1984) represents modification of the phosphodiesterase enzyme itself (Turner *et al.*, 1982). The present paper presents evidence for a type I cyclic AMP-dependent protein kinase in myelin that specifically phosphorylates the larger subunit of CNPase.

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

The method used for the isolation of myelin was based on the methods of Whittaker (1965) as described in Bradbury *et al.* (1984). Further subfractionation of the myelin on linear sucrose density gradients was as described in Bradbury *et al.* (1984).

Enzyme assays

CNPase (EC 3.1.4.37) was assayed with 2',3'-cyclic NADP as the substrate (Sogin, 1976). Lactate dehydrogenase (EC 1.1.1.27), citrate synthase (EC 4.1.3.7) and acetylcholinesterase (EC 3.1.1.7) were assayed as described by Johnson (1960), Srere (1969) and Ellman *et al.* (1961) respectively, in order to monitor the progress of subcellular fractionation and subfractionation of myelin. The fractionations used in the present paper gave essentially the same results as those described in Bradbury *et al.* (1984). 5'-Nucleotidase (EC 3.1.3.5) was assayed as described by Stanley *et al.* (1980). Cyclic AMP phosphodiesterase (EC 3.1.4.17) was assayed by the method described by Hutton *et al.* (1981).

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 standard.

Phosphorylation

Myelin samples were phosphorylated exactly as described in Bradbury *et al.* (1984).

Photoaffinity labelling

To determine whether any of the bands being phosphorylated in myelin were R-subunits of cyclic AMP-dependent protein kinases, cyclic 8-azido-[³²P]AMP, triethylamine salt (0.5 Ci/mmol; ICN Chemical and Radioisotope Division, Irvine, CA, U.S.A.), was used to photoaffinity-label myelin. Samples were labelled in a medium similar to that described by Pomerantz *et al.* (1975) but with the addition of Triton X-100. Samples (25 μ l) containing 10–15 μ g of protein were incubated with 25 μ l of double-strength reaction mixture (10 mM-Tris/HCl, pH 7.4, 40 mM-MgCl₂, 0.05% Triton X-100, 0.375 μ M-cyclic 8-azido-AMP) in the presence and in the absence of cyclic AMP at a final concentration of 0.1 mM. The reactions were done in plastic flat-bottomed microtitre well plates resting on ice to maintain the temperature at 4°C. Incubations were done in the dark for 30 min before exposure of the samples to u.v. light for 10 min with the u.v. source held at a distance of 10 cm (Gallenkamp portable u.v. light, 125 W bulb, peak output 365 nm; Gallenkamp and Co., London E.C.2, U.K.). A 40 μ l sample was taken from each well and boiled for 3 min with 40 μ l of SDS-sample buffer (Bradbury *et al.*, 1984) to prepare the samples for electrophoresis.

Preparation of soluble fractions from rabbit heart and brain

Soluble fractions were made from fresh rabbit heart and brain by Polytron-homogenization of the organs in 0.32 M-sucrose, centrifuging at 3000 rev./min in a Sorvall SS34 rotor (830 g_{av}) for 15 min at 4°C and re-spinning the supernatant at 40000 rev./min in a Beckman SW50.1 rotor (140000 g_{av}) for 1 h at 4°C. The supernatants were stored at –20°C until needed.

Electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli (1970).

Two-dimensional gel electrophoresis with non-equilibrium pH-gradient electrophoresis in the first dimension and SDS/polyacrylamide-gel electrophoresis in the second was used to separate basic proteins (O'Farrell *et al.*, 1977). Sample preparation was as described by Ames & Nikaido (1976). The 11 cm-long tube gels (1.5 mm internal diameter) were prepared as described by O'Farrell *et al.* (1977) and contained 2% pH 3.5–10 Ampholines (LKB Instruments, Croydon, Surrey, U.K.). The second dimension consisted of a 10% polyacrylamide resolving gel with a 5% polyacrylamide stacking gel.

Polyacrylamide gels were either stained for protein by using Kenacid Blue R as described in

Bradbury *et al.* (1984) or used to determine CNPase migration in the gel system.

Activity staining of CNPase after gel electrophoresis

Proteins separated by one-dimensional or two-dimensional gel electrophoresis were transferred to nitrocellulose paper (0.45 μ m; Anderman and Co., East Molesey, Surrey, U.K.) by electroblotting (Towbin *et al.*, 1979; Burnette, 1981) in a Bio-Rad Trans-Blot Electrophoretic Transfer cell for 16 h at 5 V/cm (Thompson *et al.*, 1983) with 20 mM-Tris base/150 mM-glycine/20% (v/v) methanol as the tank buffer (Towbin *et al.*, 1979). The nitrocellulose sheets were then preincubated for 1 h in 50 mM-Mes (4-morpholine-ethanesulphonic acid; Sigma Chemical Co., Poole, Dorset, U.K.) buffer, pH 6.1, containing 30 mM-MgCl₂, 0.1% Triton X-100, 3 M-guanidinium chloride (grade 1; Sigma Chemical Co.), 1 mM-EDTA, 1 mM-dithiothreitol and 5% (v/v) glycerol, then for 1 h in the same buffer without guanidinium chloride. The pre-incubation steps were essential for recovery of enzyme activity after electrophoresis in SDS (Hager & Burgess, 1980). The blots were then stained for activity by using Nitro Blue Tetrazolium [3,3'-(3,3'-dimethoxy-4,4'-diphenylene)-2,2'-di-*p*-nitrophenyl-5,5'-diphenylditetrazolium chloride (grade III; Sigma Chemical Co.)] and phenazine methosulphate (Sigma Chemical Co.) to detect the NADPH produced in the normal enzyme-linked reaction for CNPase (Sogin, 1976). It was found necessary to immobilize the coloured reaction product with the use of dilute agarose. Equal volumes of 1% agarose (electrophoresis grade; Fisons Scientific Apparatus, Loughborough, Leics., U.K.) in water at 55°C and reaction mixture [200 mM-Mes buffer, pH 6.1, containing 60 mM-MgCl₂, 0.2% Triton X-100 and 0.1 mM-2',3'-cyclic NADP, and in addition 4 mg of glucose 6-phosphate (Boehringer Mannheim, Lewes, East Sussex, U.K.)/ml, 0.4 mg of Nitro Blue Tetrazolium/ml, 0.04 mg of phenazine methosulphate/ml and 0.7 unit of glucose-6-phosphate dehydrogenase (grade II from yeast; Boehringer Mannheim)] were rapidly mixed and poured on top of the nitrocellulose filter. Generally a colour reaction was visible within 15 min. The reaction was stopped by swamping the agarose overlay with 10% acetic acid.

Results

Photoaffinity labelling with cyclic 8'-azido-AMP

The results of photoaffinity labelling of myelin fraction P₄ (Bradbury *et al.*, 1984) in parallel with supernatant fractions from rabbit heart and rabbit brain homogenates are shown in Fig. 1. Two proteins were labelled in rabbit heart supernatant

with apparent M_r values of approx. 51 000 and 55 000 (Fig. 1b); these presumably represented the regulatory (R-) subunits of a type I and a type II cyclic AMP-dependent protein kinase respectively (Flockhart & Corbin, 1982). Rabbit brain supernatant showed prominent labelling of the R_{II}-subunit compared with the R_I-subunit, and also faint labelling of two lower-molecular-mass proteins, which were assumed to be proteolytic breakdown products (Fig. 1b). Myelin fraction P₄ showed labelling of a protein with the same electrophoretic mobility as the R_I-subunit from heart and brain, with faint labelling of a protein with the same mobility as the R_{II}-subunit from these organs (Fig. 1b). The labelling of this upper R_{II}-subunit in myelin P₄ fractions was somewhat variable and in some experiments approached the intensity of the labelling in the lower- M_r R_I-type subunit. The inclusion of 0.1 mM-cyclic AMP in the incubation medium virtually abolished the photoaffinity labelling in the myelin fraction and heart supernatant but produced only a moderate decrease in labelling in the brain supernatant, presumably because of high cyclic AMP phosphodiesterase activity in these preparations (Walter *et al.*, 1978). Unlike brain and heart supernatants, which were photoaffinity-labelled satisfactorily in the absence of detergents, the presence of Triton X-100 was essential to produce photoaffinity labelling in the myelin fraction (Fig. 1b). Extensive washing of myelin fraction P₄ by centrifugation in 2 M-NaCl (three washes) did not diminish photoaffinity labelling compared with unwashed P₄ membranes (results not shown). The lower- M_r photoaffinity-labelled R_I-type subunit migrated in all three samples with a slightly higher apparent M_r than the upper band of the Wolfgram protein doublet (Figs. 1a and 1b).

Fig. 2 shows the effect of photoaffinity-labelling fractions produced by sucrose-density-gradient centrifugation of myelin fraction P₄ (Bradbury *et al.*, 1984). Labelling of higher- M_r R_{II}-type subunit was most prominent in the most rapidly sedimenting fractions from the gradient (fractions 2–8, Fig. 2), whereas labelling of a lower- M_r R_I-type subunit was most prominent in the intermediate fractions from the gradient (fractions 6–11, Fig. 2). Both types of subunits became less prominent in fractions from the upper part of the gradient containing multilamellar myelin (Bradbury *et al.*, 1984). Thus photoaffinity labelling of these gradient fractions indicated that the presence of an R_{II}-type subunit was associated with membrane fractions containing 5'-nucleotidase and acetylcholinesterase activity, whereas the presence of an R_I-type subunit was more characteristic of membrane fractions containing CNPase activity (Bradbury *et al.*, 1984). The labelling shown in Fig. 2

Fig. 1. Photoaffinity labelling of myelin membranes

Portions (10 μ g of protein) of soluble heart and brain fractions and purified myelin (fraction P₄; Bradbury *et al.*, 1984) were photoaffinity-labelled as described in the Materials and methods section in the presence and in the absence of 0.1 mM-cyclic AMP. Apart from the myelin fraction, none of the fractions required Triton X-100 for labelling. A 5 μ g portion of labelled protein was applied per track of an SDS/10%-polyacrylamide gel. After electrophoresis the gel was stained with Kenacid Blue R, dried and autoradiographed. (a) Kenacid Blue-stained gel. The Wolfgram doublet of proteins shows clearly in the four myelin tracks, about one half of the way down the gel. (b) Autoradiogram of the gel shown in (a). The lower of the two bands labelled in the heart soluble fraction in the absence of cyclic AMP was designated as an R_I-type subunit of a cyclic AMP-dependent protein kinase. The upper band was designated as an R_{II}-type subunit.

could be abolished by including 0.1 mM-cyclic AMP in the medium (not shown; cf. Fig. 1). Myelin basic protein also showed some photoaffinity labelling with cyclic 8'-azido-AMP (Fig. 2); however, this was unaffected by the inclusion of 0.1 mM-cyclic AMP and was therefore presumably a non-specific phenomenon.

Double-labelling of myelin fractions with cyclic 8'-azido-AMP and [³²P]ATP

Since alterations in mobility on SDS/polyacrylamide-gel electrophoresis can be produced by different phosphorylation states (Hofmann *et al.*, 1975; Uno *et al.*, 1977), double-labelling experiments were performed in which a myelin sample was first phosphorylated and then photoaffinity-labelled with cyclic 8'-azido-AMP (Fig. 3). Phosphorylation of the myelin sample with non-radioactive ATP followed by photoaffinity labelling did

not alter the mobility of the affinity-labelled protein compared with that seen with photoaffinity labelling without prior phosphorylation (compare tracks 1 and 2, Fig. 3). Phosphorylation with [³²P]ATP alone produced a radioactive protein with a lower apparent M_r than that of the radioactive protein seen with photoaffinity labelling (compare tracks 1 and 2 with track 4, Fig. 3). Phosphorylation with [³²P]ATP followed by radioactive photoaffinity labelling produced an accentuated radioactive band (track 3, Fig. 3), which appeared to be more prominent than the sum of the individual higher- M_r photoaffinity-labelled protein (tracks 1 and 2, Fig. 3) and the lower- M_r phosphorylated protein (track 4, Fig. 3), presumably because of the cyclic AMP-like action of the cyclic 8'-azido-AMP analogue. It was concluded from these experiments that the photoaffinity-labelled protein could be distinguished from the cyclic

Fig. 2. Photoaffinity labelling of myelin subfractions

A myelin (P_4) preparation was subfractionated by linear-sucrose-density-gradient centrifugation (0.32–1.2M-sucrose gradient, 3½ h spin, 65000g_{av}; Bradbury *et al.*, 1984). The fractions obtained had enzyme profiles similar to those reported previously (Bradbury *et al.*, 1984), with the protein peak at fraction 11, CNPase activity peak at fraction 8 and 5'-nucleotidase peak at fraction 5. Portions (15 µg) of fractions 1–18 were photoaffinity-labelled as described in the Materials and methods section in the absence of cyclic AMP. Then 8 µg of protein per track was electrophoresed on an SDS/12.5%-polyacrylamide gel, and the stained and dried gel autoradiographed as shown here. The final track in the Figure (P) shows the phosphorylation pattern of fraction 11.

AMP-stimulated phosphorylated protein by a greater apparent M_r (Fig. 3).

Activity staining of CNPase on SDS/polyacrylamide-gel electrophoresis and two-dimensional non-equilibrium pH-gradient electrophoresis

Fig. 4 shows the results of staining for CNPase activity on nitrocellulose 'blots' of one-dimensional SDS/polyacrylamide-gel electrophoretograms of a myelin P_4 fraction. This showed two closely spaced bands of enzyme activity, at a migration position corresponding exactly to that of the Wolfram protein doublet. Two lower- M_r bands, not visible on Kenacid Blue staining of SDS/polyacrylamide-gel electrophoretograms, also showed CNPase activity. These lower- M_r bands were virtually absent when freshly prepared myelin P_4 samples were used and became more prominent with increased freezing and thawing or storage at 4°C; for this reason it is assumed that they represent proteolytic cleavage products of the intact enzyme. Addition of 10mM-2',3'-cyclic AMP (a competing substrate with 2',3'-cyclic nicotinamide-adenine dinucleotide phosphate) abolished activity staining (not shown). When the stained blot was autoradiographed, cyclic AMP-stimulated phosphorylation co-migrated exactly with the upper

band of CNPase activity (Fig. 4). In order to exclude co-migration of a phosphorylated non-CNPase protein with a non-phosphorylated CNPase protein, a phosphorylated myelin P_4 sample was analysed by two-dimensional gel electrophoresis as described in the Materials and methods section, and stained for enzyme activity as shown in Fig. 5. Two areas of enzyme activity were seen after staining, with the higher- M_r band migrating more rapidly towards the cathode (Fig. 5a). Autoradiography of the same nitrocellulose blot showed that the radioactivity in the phosphorylated myelin sample co-migrated exactly with the CNPase activity (Fig. 5b). Distinct radioactive species could be seen, suggesting multiple phosphorylated forms of the enzyme.

Discussion

Previous work has shown that the cyclic AMP-stimulated phosphorylation of a 49700- M_r protein co-segregates with myelin and the myelin marker enzyme CNPase on subcellular fractionation and appears to be intrinsic to the myelin membrane itself (Bradbury *et al.*, 1984). Furthermore the phosphorylated protein co-migrates with the upper component of the Wolfram protein doublet

Fig. 3. Double-labelling of myelin with cyclic 8'-azido-AMP and [^{32}P]ATP

Portions (20 μg of protein) of fraction 12 from the sucrose-density-gradient subfractionation of myelin for which photoaffinity labelling is shown in Fig. 2 were treated as below, and 10 μg was loaded per track of an SDS/12.5%-polyacrylamide gel. Track 1: myelin photoaffinity-labelled as described in the Materials and methods section. Track 2: the myelin sample was first incubated with the normal phosphorylation mixture containing unlabelled ATP only (30 mM-Tris/HCl buffer, pH 7.4, containing 1 mM-MgCl₂, 0.05% Triton X-100 and 0.055 mM-ATP; Bradbury *et al.*, 1984) for 5 min at 37°C and then photoaffinity-labelled as described in the present study; the final concentrations of reagents in the reaction mixture were kept as indicated in the Materials and methods section by adjusting the stock solutions to allow for reagents already present from the phosphorylation reaction. Track 3: as track 2, but [^{32}P]ATP was included in the phosphorylation mixture (0.005 mM-[^{32}P]ATP, of specific radioactivity 0.5–2.0 Ci/mmol). Track 4: myelin phosphorylated with [^{32}P]ATP only.

(Bradbury *et al.*, 1984). It has been suggested that this protein represents the R-subunit of a type II cyclic AMP-dependent protein kinase undergoing autophosphorylation (Sulakhe *et al.*, 1980a). With the use of cyclic 8'-azido-AMP as a photoaffinity label the present studies show that the cyclic AMP-binding protein within myelin membrane fractions electrophoreses, not with an R_{II}-subunit mobility, but rather with the mobility characteristic of an R_I-subunit (Fig. 1). A similar finding has been previously reported by Walter *et al.* (1978). Further fractionation of myelin P₄ preparations on linear-

Fig. 4. Activity staining for CNPase after SDS/polyacrylamide-gel electrophoresis

Portions (10 μg of protein) of a myelin sample (P₄) were phosphorylated for 5 min at 37°C in the absence and in the presence of 5 μM -cyclic AMP with the use of the reaction mixture described in Fig. 3. The samples were boiled in an equal volume of SDS-sample buffer, and 5 μg of protein was applied per track of an SDS/12.5%-polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose paper by electroblotting. The 'blot' was then preincubated and stained for CNPase activity as described in the Materials and methods section. Tracks 1 and 2 show CNPase activity in a myelin sample phosphorylated in the absence and in the presence of 5 μM -cyclic AMP. There is no obvious difference in the intensity of staining in the two tracks. Tracks 3 and 4 show a short autoradiographic exposure (1 day) of the blot shown in tracks 1 and 2. As myelin basic protein transfers relatively poorly to nitrocellulose filters under the conditions used, the intensities of the 49 700- M_r band and myelin basic protein are similar on the autoradiograph. Tracks 5 and 6 show a longer autoradiographic exposure of the blot (6 days). No labelling is visible in the region of the apparent proteolysis products of CNPase (see the text).

sucrose-density-gradient centrifugation (Fig. 2) shows that the cyclic AMP-binding R_I-type subunit is primarily located in those fractions with the highest specific activity of CNPase, i.e. in those fractions in which cyclic AMP-stimulated phosphorylation is greatest and that are thought to represent 'myelin-like' membrane fragments (Bradbury *et al.*, 1984). Although cyclic AMP binding by an apparent R_{II}-type subunit (as judged by electrophoretic mobility; Fig. 1) can be shown in myelin P₄ fractions (Fig. 1), this primarily sedi-

ments with heavier membrane fragments (Fig. 2), which possibly do not represent true myelin membrane and which characteristically do not show cyclic AMP-stimulated phosphorylation of the 49700- M_r protein (Bradbury *et al.*, 1984). The phosphorylation of the upper component of the Wolfgram protein doublet can be shown by double-labelling experiments to be distinct from the labelling produced by cyclic 8'-azido-AMP (Fig. 3). The evidence presented here therefore indicates that the cyclic AMP-stimulated phosphorylation of the 49700- M_r protein seen in myelin membrane fractions does not represent autophosphorylation of an R_{II} -type subunit, although an R_I -subunit is present in the membrane closely associated with the peak activity of cyclic AMP-stimulated phosphorylation of the 49700- M_r protein and with maximum CNPase activity (Fig. 2; Bradbury *et al.*, 1984). Type I protein kinases characteristically do not show autophosphorylation of the R-subunit (Flockhart & Corbin, 1982). The type I cyclic AMP-dependent kinase present in the myelin membrane appears to be an intrinsic activity, as it cannot be removed by repeated centrifugation in high-salt solutions.

The 49700- M_r phosphorylated protein co-migrates with the upper component of the Wolfgram protein doublet (Bradbury *et al.*, 1984). Previous workers have presented evidence that the Wolfgram protein doublet represents the CNPase enzyme itself (Drummond & Dean, 1980; Sprinkle *et al.*, 1980). Purified CNPase appears to be a dimer of M_r 100000 with slightly dissimilar M_r subunits, both of which are catalytically active

(Müller, 1982). Purified CNPase from several sources co-migrates with the Wolfgram protein doublet in the same species, and both immunological and structural similarities between purified Wolfgram proteins and CNPase have been clearly demonstrated (Drummond & Dean, 1980; Sprinkle *et al.*, 1980). However, the purification procedure for the production of Wolfgram protein results in complete loss of CNPase activity (Drummond & Dean, 1980). CNPase activity on urea/polyacrylamide-gel electrophoretograms was demonstrated by Braun & Barchi (1972), but the resolution of the system only allowed localization of the activity in the Wolfgram protein region and not to specific bands. The blotting and enzyme activity staining procedure described here allows a direct demonstration of CNPase activity co-migrating exactly with the Wolfgram protein doublet (Fig. 4). This procedure also demonstrates that the cyclic AMP-stimulated protein phosphorylation coincides with the higher- M_r subunit of CNPase (Fig. 4). Lower- M_r bands of enzyme activity, which become more prominent with aging of the myelin sample, presumably represent proteolytic breakdown products of the higher- M_r CNPase doublet (Fig. 4). It is noteworthy that these lower- M_r species, although possessing enzyme activity, do not appear to be phosphorylated, thus suggesting that they are derived from the lower- M_r non-phosphorylated subunit of CNPase (Fig. 4). Finally, the present work demonstrates that activity staining after two-dimensional non-equilibrium pH-gradient electrophoresis also shows co-migration of CNPase enzyme activity

Fig. 5. CNPase activity staining of two-dimensional gels

Portions (150 μ g) of labelled myelin (P_4) prepared as described in the Materials and methods section were applied to the anodic end of a gel tube, prepared as described by O'Farrell *et al.* (1977), and electrophoresed for a total of 1650 V·h with 0.01 M- H_3PO_4 as anode buffer and 0.02 M-NaOH as cathode buffer. The tube gels were then equilibrated in SDS-sample buffer for 30 min and applied to an SDS/10% polyacrylamide gel with 5% stacking gel. (a) CNPase-activity-stained blot of two-dimensional gel. The small amount of activity at the positive end of the gel is presumably due to some of the enzyme entering the first dimension poorly. (b) Autoradiogram of the blot shown in (a). Only the higher- M_r species of CNPase was labelled. Again some streaking towards the anode was observed.

and cyclic AMP-stimulated protein phosphorylation (Fig. 5). It is therefore concluded from the present and the accompanying paper (Bradbury *et al.*, 1984) that central-nervous-system myelin contains an intrinsic type I cyclic AMP-dependent protein kinase that specifically phosphorylates the larger subunit of CNPase.

The physiological significance of the present findings is not yet clear. Preliminary work has not shown any apparent effect on K_m or V_{max} of CNPase, at least towards 2',3'-cyclic nicotinamide-adenine dinucleotide phosphate as substrate; however, this is presumably not the substrate *in vivo* for the enzyme (Sims & Carnegie, 1979). It is also uncertain how accessible the enzyme is *in vivo* to soluble cytoplasmic signals such as a rise in cyclic AMP concentration, since *in vitro* the presence of detergent is necessary to demonstrate the phosphorylation effect. However, studies performed *in vivo* have shown that proteins in the region of the Wolfgram protein doublet (and myelin basic protein itself) are readily phosphorylated (Agrawal *et al.*, 1982). Finally, the appearance of CNPase on two-dimensional electrophoresis suggests multiple phosphorylation sites on the larger subunit (Fig. 5), and these could possibly be influenced by other controlling factors.

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