Calcium Ion Stimulated Endogenous Protein Kinase Catalyzed Phosphorylation of Basic Proteins in Myelin Subfractions and Myelin-like Membrane Fraction from Rat Brain†

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ABSTRACT: Polypeptide composition and endogenous phosphorylation were investigated in the subfractions of rat brain myelin isolated by either discontinuous or continuous sucrose density gradient centrifugation of myelin. Similarly, a myelin-like membrane fraction (SN_4) was also studied. Observations were made that strongly indicated the presence of a calcium-stimulated protein kinase in a highly purified myelin preparation and which exclusively phosphorylated myelin basic proteins of the membrane preparation. Adenosine cyclic 3′,5′-phosphate (cAMP) stimulated kinase on the other hand was found to be considerably enriched in the myelin-like membrane fraction. Although this latter enzyme is also capable of phosphorylating the basic proteins, its effect was at least 5 times weaker compared to the calcium-stimulated myelin protein kinase. Within the gradient subfractions there appeared a close relation between the amount of basic proteins and their calcium-stimulated phosphorylation; a similar relationship, however, was not obtained in the case of cAMP-dependent phosphorylation of myelin basic proteins. The former (i.e., Ca^{2+}-stimulated phosphorylation) was found to require a protein factor that functionally resembled calmodulin. The results thus raise an interesting possibility of the presence of calmodulin-like proteins and a calcium-stimulated protein kinase in adult myelin membrane from mammalian brain, both of which have been hitherto unrecognized constituents of myelin membranes.

There have been numerous recent publications, since the preliminary observation by Johnson et al. (1971), that showed the presence of protein kinase(s) and phosphatase(s) in isolated brain myelin fractions (Carnegie et al., 1973, 1974; Steck & Appel, 1974; Miyamoto et al., 1974; Miyamoto & Kakiuchi, 1974, 1975; Miyamoto, 1975, 1976; McNamara & Appel, 1977; Yourist et al., 1978; Miyamoto et al., 1978). Among the myelin polypeptides, basic protein(s) were shown to be mainly phosphorylated. In all these studies the endogenous phosphorylation of basic proteins was only minimally increased in the presence of adenosine cyclic 3′,5′-phosphate (cAMP) and that only when a Triton X-100 dispersed myelin preparation was used; divalent cation dependence of the myelin kinase, however, was not examined in detail (Miyamoto, 1975). We recently reexamined the effects of various divalent cations and cAMP on the endogenous kinase-catalyzed phosphorylation of myelin-associated polypeptides and found that calcium, in micromolar amounts, markedly stimulated their phosphorylation either in the central nervous system (Petrali et al., 1980a,b; Sulakhe et al., 1978, 1980) or peripheral nervous system myelin preparations (Petrali & Sulakhe, 1979); cAMP on the other hand was a weak stimulator.

Despite the fact that isolated myelin preparations possess endogenous protein kinase(s), it is not as yet conclusively documented that these enzymes are indeed myelin enzymes and not due to the presence of contaminating membrane fragments in the commonly isolated myelin preparations (Norton, 1977). In the present study we have therefore examined endogenous phosphorylation of basic proteins in subfractions of myelin prepared by continuous (Waehneldt, 1977, 1978) and discontinuous density gradients (Zimmerman et al., 1975; Fujimoto et al., 1976) as well as in isolated myelin-like membrane fraction, SN_4 (Waehneldt, 1977; Waehneldt et al., 1977). Our results indicated that the so-called basal and cAMP-stimulated kinases are enriched in the myelin-like membrane fraction (SN_4), whereas the Ca^{2+}-stimulated kinase is enriched in a typical multilamellar myelin. Among the gradient subfractions of myelin Ca^{2+}-stimulated phosphorylation was higher in the lighter fractions and lowest in the heavy fraction, and this pattern was similar to the content of basic proteins in the gradient subfractions. cAMP-stimulated kinase appeared higher in the medium-heavy subfractions of myelin, and a reliable detection of its activity invariably required the presence of Triton in the assay irrespective of the gradient fractions tested. Our results thus strongly support the view that the calcium-stimulated protein kinase is a myelin enzyme, whereas the cAMP-stimulated protein kinase appears to be located mainly in the myelin-like membrane isolated from rat brain.

Experimental Procedures

Materials

[γ-^{32}P]ATP (10–40 Ci/mmol) was purchased from New England Nuclear, Montreal, Canada. cAMP, Triton X-100, and electrophoretic reference proteins were obtained from Sigma Chemical Co., St. Louis, MO. Low and high molecular weight oligomers of albumin were obtained from BDH Chemicals, Toronto, Canada. All other reagents were of highest purity commercially available from Bio-Rad Chemicals, Richmond, CA, Anachemia Ltd., Montreal, Canada, and Fisher Scientific Co., NJ. Solutions were prepared and all glassware and plasticware thoroughly rinsed with deionized glass-distilled water. Partially purified calmodulin-deficient cAMP phosphodiesterase, purified calmodulin (both from bovine heart; Teo & Wang, 1973; Teo et al., 1973) and purified modulator binding proteins [from bovine brain, see Wang & Desai (1977) and Sharma & Wang (1979)] were kindly supplied by Drs. R. K. Sharma and J. H. Wang of the University of Manitoba, Winnipeg, Canada.

Methods

Isolation of Myelin, SN_4, and SN_1 from White Matter Homogenate. All tissue fractionations were carried out at 4

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°C. Male Wistar rats (190–240 g) were killed by decapitation, brains were removed, and the white matter was carefully separated from the gray matter on an ice-chilled petri dish. White matter (250 mg wet weight per rat) was obtained mostly from the brain stem and homogenized in 8.5 volumes (v/w) of 10% (w/v) sucrose, containing 10 mM Tris-HCl (pH 7.8) and 0.2 mM dithiothreitol (sucrose T-D buffer) with 3–4 strokes (750 rpm) with a loosely fitting Teflon pestle in a glass homogenizer. Myelin, SN_4, and SN_1 prepared from the homogenate as described by Waehneldt et al. (1977) were rehomogenized in double-distilled water and centrifuged at 40000*g* for 20 min; this step was repeated 3 times to obtain final fractions. The final pellets obtained were resuspended in sucrose T-D buffer and assayed immediately following isolation.

Isolation of Myelin Subfractions. Myelin subfractions were prepared from myelin isolated as described above (with SN_4 removed) by discontinuous and continuous sucrose density gradient centrifugation. The continuous density gradient (Waehneldt, 1978) consisted of a linear sucrose gradient, 0.32–1.0 M sucrose (ISCO Model 570 gradient former), overlayered with myelin preparation suspended in water. Centrifugation was carried out for 4 h at 65000*g* (IEC B-60 ultracentrifuge). Thereafter, 2-mL samples were collected with a gradient fractionator (ISCO Model 183 with a cushion of 1.2 M sucrose) and protein density profiles of the myelin distribution on the gradient were determined by a UV-coupled flow-through absorbance spectrophotometer. The fractions pooled (see vertical lines, Figure 6) from various gradient tubes were diluted with T-D buffer, and the pellets were obtained by centrifuging at 40000*g* for 20 min. Discontinuous gradients were prepared [modification of Zimmerman et al. (1975); Fujimoto et al. (1976); McIntyre et al. (1978)] by layering successive 6-mL aliquots of 0.8, 0.7, 0.62, 0.53, and 0.40 M sucrose. Myelin as described above was overlayered on the sucrose gradient and centrifuged for 16 h at 65000*g* (Fujimoto et al., 1976). Various fractions were collected at the sucrose interfaces with a Pasteur pipet and diluted with T-D buffer, and pellets were obtained by centrifugation at 40000*g* for 20 min. Fractions obtained were suspended in sucrose T-D buffer as described above. Protein estimation was done by the method of Lowry et al. (1951).

Determination of Endogenous Protein Kinase Catalyzed Phosphorylation. Phosphorylation was carried out at 30 °C in a reaction mixture (0.15 mL) which consisted of 30 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride (Tris-HCl) (pH 7.4), 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA), 1 mM $MgCl_2$, 50 μM [γ-^{32}P]ATP [sp act. 300 counts/(min pmol)], and 100 μg of membrane protein, and, when present, $CaCl_2$ was 1.05 mM (50 μM in excess over EGTA) and cAMP 5 μM. At the concentration used, Ca^{2+} and cAMP gave maximal stimulatory effects on phosphorylation [also see Petrali et al. (1980a,b); Sulakhe et al. (1980)]. The assays were carried out in the presence of Triton X-100 (0.05% v/v) or in the absence of detergent. The reaction was started by addition of labeled substrate to the assay tubes following preincubation of the rest of the assay components for 4 min (30 °C) and was terminated after 2 or 15 min by addition of 30 μL of "solubilizing buffer" (Dunkley et al., 1976) or 2 mL of ice-cold trichloroacetic acid (10%) containing 2 mM P_i (see below). Changes made in the assay protocol are described wherever appropriate.

Determination of ^{32}P Radioactivity Incorporated into Total Protein. Incorporation of ^{32}P into total protein was determined by a modification of the method of Kuo & Greengard (1970). For this, a portion (50 μL) of the solubilized protein from the phosphorylation assay was mixed with 1 μmol of ATP, and 2 mL of 10% trichloroacetic acid containing 2 mM P_i was added. Following this, 0.1 mL of 0.5% bovine serum albumin was added as a carrier protein. After standing at 0 °C for 10 min, the mixture was centrifuged and the supernatant was removed by aspiration. The precipitate was dissolved in 0.1 mL of 1 M NaOH and reprecipitated with 2 mL of 10% trichloroacetic acid containing 2 mM P_i. This step was repeated once more. The final pellet was solubilized in 0.3 mL of 1 M NaOH, and the radioactivity was measured in a liquid scintillation counter. In some experiments the phosphorylation reaction was terminated with 10% trichloroacetic acid containing 2 mM P_i and was then processed in a manner identical with that described above; the results obtained with either procedure were essentially similar.

NaDodSO$_4$–Polyacrylamide Gel Electrophoresis. The solubilized membrane proteins were fractionated by sodium dodecyl sulfate ($NaDodSO_4$) (0.1%)–polyacrylamide slab-gel electrophoresis as described by Laemmli (1970). The lower (resolving) gel (19 × 0.1 cm) contained 10% (w/v) acrylamide, 0.27 (w/v) bis(acrylamide), 0.375 M Tris-HCl (pH 8.8), and 0.1% (w/v) $NaDodSO_4$. The upper (stacking) gel containing 3% (w/v) acrylamide, 0.08% (w/v) bis(acrylamide), 0.125 M Tris-HCl (pH 6.8), and 0.1% $NaDodSO_4$ was overlaid on the lower gel to a length of 1 or 2 cm. Both gels were chemically polymerized by addition of 0.025% (w/v) ammonium persulfate and 0.025% (w/v) *N*,*N*,*N*′,*N*′-tetramethylethylenediamine. The electrode buffer contained 0.025 M Tris, 0.166 M glycine, and 0.1% $NaDodSO_4$, pH 8.3. Ninety microliters of each sample, containing equal amounts of protein, was applied to the gel. The gels were run at 20 mA (constant current, 80 min) until the tracking dye had migrated 1.5 cm into the resolving gel and then run at 30 mA for about 2 h until the tracking dye had migrated 15.5 cm into the resolving gel. The resolving gel was placed in 25% isopropyl alcohol containing 10% acetic acid with several changes to remove $NaDodSO_4$, and proteins were then stained for 2 h with 0.025% Coomassie Blue in 25% isopropyl alcohol containing 10% acetic acid and then diffusion-destained with 10% acetic acid with several changes. The gel was then treated with methanol (70%)–glycerol (3%) solution and dried on Whatman No. 1 filter paper under vacuum.

Estimation of Protein Content of Separated Polypeptide Bands following Electrophoresis. With the assumption that the intensity of staining (by Coomassie Blue) is proportional to the amount of protein in a given band, the relative proportion of separated polypeptide bands was estimated as follows. The stained gel (prior to drying) was photographed and the (glossy) print was scanned on a Joyce-Loebl densitometer (reflectance spectrophotometry). The area under a given (major) peak was estimated by planimetry or by cutting out the peak and weighing it; both procedures gave essentially similar estimations. In addition, gels were loaded with three different concentrations (10, 20, and 30 μg) of protein so that the estimated relative proportions of polypeptide bands in the membrane fraction studied represented a fairly reliable assessment of the polypeptide content of major peaks, which is expressed as the percent of total protein.

Autoradiography and Quantitation of ^{32}P Incorporation into Individual Proteins. Initially, ^{32}P incorporation into polypeptide bands was determined by two procedures. (A) The gel was sliced (2-mm slice), and the slices were counted by Cerenkov radiation, and (B) the gel was dried (see above) and was placed in contact with Kodak X-Omat R film for 1–3

Table I: Major Polypeptides of Myelin, SN_4, and SN_1[a]

fraction	yield (mg of protein/g of tissue)	content of major polypeptide (% of total) MGP (110K)	WP (52–55K)	PLP (24K)	LBP (19K)	SBP (16K)	high M_r range (>45K)	low M_r range (<45K)
myelin	11.37	3.1	3.7	35.4	19.9	22.4	3.1	11.8
SN_4	1.99	8.5	10.6	17.0	13.3	19.1	14.3	17.0
SN_1	1.32	11.4	10.9	4.2	2.8	4.2	49.5	16.6

[a] Highly purified myelin, SN_4, and SN_1 were isolated from rat brain white matter as described under Methods. MGP, myelin-associated glycoprotein; WP, Wolfgram peptide (triplet); PLP, proteolipid protein; LBP, large basic protein; SBP, small basic protein. 20 μg of protein was layered in each well, and the values are means of three preparations (variations did not exceed 10%) and are estimated from the densitometric scans of the Coomassie Blue stained gels. Sum of high (>45K) and low (<45K) molecular weight proteins excludes major proteins already listed.

days. The resulting autoradiogram revealed those bands into which ^{32}P had been incorporated. The optical density (at 595 nm) of the bands on the film was measured on a Joyce-Loebl scanning densitometer, and ^{32}P incorporation into individual protein bands was expressed in arbitrary units based on the calculation of the area under the peak or by cutting out the peak and weighing it. Results obtained by either procedure were in excellent agreement. As a routine, procedure B was adopted since it was a simple and convenient method, and additionally, compared to procedure A, it allowed a more precise identification of the phosphorylated bands with the Coomassie Blue stained bands on the dried gel.

Estimation of the Molecular Weight of Proteins. The apparent molecular weights of the protein bands on $NaDodSO_4$ gels were estimated by two well-established methods: (1) comparing the mobility of the bands with the mobilities of proteins of known molecular weight and (2) confirming the apparent molecular weights by plotting the mobilities of major proteins obtained at three different resolving gel concentrations (7.5, 10, and 12.5%) on a Ferguson plot (Ferguson, 1964). The following proteins were used as molecular weight standards: myosin (200 000), phosphorylase *a* (94 000), bovine serum albumin (68 000), ovalbumin (45 000), and carbonic anhydrase (32 000). In addition, low and high molecular weight oligomers of albumin were used. A standard curve was generated by plotting the logarithm of the molecular weight vs. the relative migration of the standard proteins (midpoint of the tracking dye was taken as 1).

Assay for Calmodulin-like Proteins in Myelin Extract. Calmodulin-deficient cAMP phosphodiesterase was assayed ($\pm Ca^{2+}$) according to the method of Sharma & Wang (1979) in the absence and presence of varying amounts of purified calmodulin or myelin extract. A standard curve was constructed for purified calmodulin on the basis of the Ca^{2+}-dependent stimulation of the phosphodiesterase. Myelin extract was prepared by initially incubating (at 0 °C for 30 min) myelin (5 mg of protein) suspended in sucrose T-D buffer (0.5 mL) containing 2 mM EGTA and then centrifuging it at 40000*g* for 1 h [also see Sulakhe et al. (1980)]. The supernatant fluid was assayed for calmodulin-like activity. In some experiments the supernatant fluid was dialyzed overnight against 100 volumes of buffer (10 mM Tris-HCl, pH 7.8, 0.2 mM dithiothreitol, 1 mM magnesium acetate, and 1 mM imidazole) prior to assay.

Results

Polypeptide Composition and Endogenous Phosphorylation of Myelin, SN_4, and SN_1. Figure 1 illustrates the Coomassie Blue stained polypeptide patterns of myelin, SN_4, and SN_1 isolated essentially by the method of Waehneldt et al. (1977). Briefly, proteolipid protein (PLP) and large and small basic

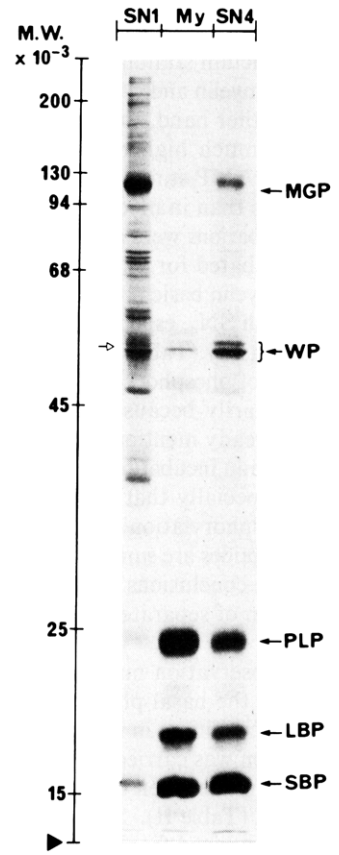

FIGURE 1: Polypeptide composition of myelin, SN_4 and SN_1. Fractions were isolated by the method of Waehneldt et al. (1977) and were electrophoresed by the method of Laemmli (1970). MGP, myelin associated glycoprotein; WP, Wolfgram polypeptides; PLP, proteolipid protein; LBP, large basic protein; SBP, small basic protein. Open arrow indicates regulatory subunit of cAMP-dependent protein kinase. Twenty micrograms of protein was layered for each fraction.

protein (LPB and SBP, respectively) were present in much greater amounts of myelin compared to SN_4 and SN_1. Wolfgram polypeptides (WP) and myelin-associated glycoprotein (MGP) were, on the other hand, enriched in SN_4 and SN_1 compared to myelin, with the Wolfgram peptides being present in higher amount in SN_4 and the glycoprotein in SN_1 (see Table I). In general, high molecular weight polypeptides (M_r > 50 000) were prominent in the SN_1 fraction and, to some extent, in SN_4. These observations are in good agreement with those by Waehneldt and his co-workers (Waehneldt et al., 1977; Matthieu & Waehneldt, 1978). It may be worth pointing out here that the less heavily stained peptide (middle peptide, indicated by the open arrow) in the Wolfgram region was identified to contain the regulatory subunit of the membrane-associated cAMP-stimulated kinase (also see below),

and its content is indeed greater in SN_4 than in myelin (see Table I).

Endogenous phosphorylation of these fractions and the effects of cAMP (5 μM) and Ca^{2+} (50 μM) on phosphorylation are shown in Figure 2A (Triton absent in the assay) and in Figure 2B (Triton present in the assay).[1] In myelin, cAMP failed to stimulate basal phosphorylation of LBP and SBP when the detergent was absent and modestly stimulated it with the detergent present. Also note that autophosphorylation of the regulatory subunit (see Figure 2B) of the endogenous kinase was readily evident when Triton was included in the phosphorylation assay, and cAMP (but not Ca^{2+}) showed a stimulatory effect on its phosphorylation.[2] Also, it is evident from Figure 2B that phosphorylation of R is considerably more in SN_4 than in myelin. The general pattern of phosphorylation of basic proteins was similar in all fractions with the exception that the degree of calcium stimulation over basal phosphorylation was highest in myelin and cAMP stimulation was absent in myelin. On the other hand, the basal phosphorylation of LBP and SBP was much higher in SN_4 and SN_1 than in myelin, and further cAMP stimulation was invariably higher in these two fractions than in myelin, especially when Triton was present and incubations were carried out for short periods (2 min). When incubated for a longer period, the extent of phosphorylation of myelin basic proteins was somewhat higher with myelin than with SN_4, especially when Mg^{2+} and Ca^{2+} were included in the assay (Table II).[3] It was also noted that while the initial rate of phosphorylation of SBP and LBP was higher in SN_4, primarily because of the higher content of kinases in SN_4 as already mentioned above, the steady-state phosphorylation (15-min incubation, see Table II) was slightly higher in myelin, especially that due to Ca^{2+}. The results obtained on the phosphorylation of total membrane protein and individual polypeptides are summarized in Table II. These data also support the conclusions derived from the autoradiographic examination of separated phosphoproteins (Figure 2 and Table II).

One interesting observation made with SN_1 deals with a marked reduction in the basal phosphorylation of LBP and SBP under the assay conditions in which Ca^{2+} and Triton were present and incubation was carried out for 15 min. A similar observation was also made when total membrane phosphorylation was determined (Table II). Since the fraction SN_1 likely contains membranes derived from neuronal tissue, it was not clear whether or not the Ca^{2+}-dependent dephosphorylation observed is due to myelin-specific phosphoprotein phosphatase. Further support to this view also comes from the work of Waehneldt et al. (1977) that indicated considerable activities of acetylcholinesterase in SN_1, an observation that has been

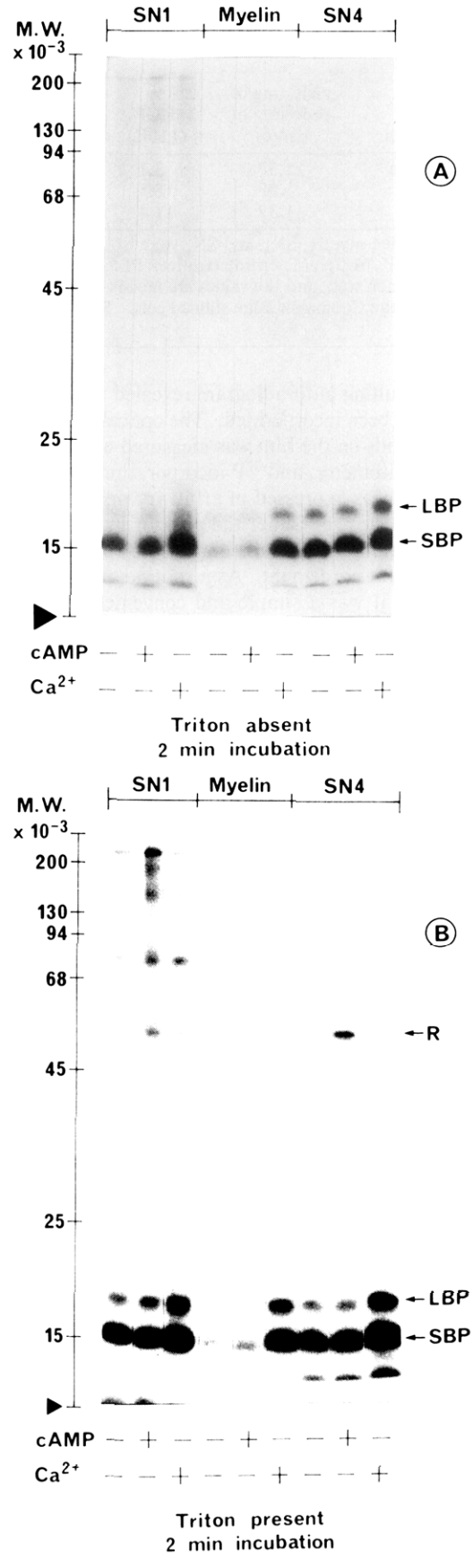

FIGURE 2: Autoradiographic localization of phosphorylated proteins in SN_1, myelin, and SN_4. Incubations were carried out for 2 min under standard phosphorylation assay conditions with (B) or without (A) Triton X-100. When present, cAMP was 5 μM and Ca^{2+} was 50 μM. LBP, large basic protein; SBP, small basic protein. Exposure time for autoradiograms (A) and (B) was 68 h.

[1] At the concentrations used, cAMP and Ca^{2+} gave optimal (stimulatory) effect on endogenous phosphorylation of these fractions [also see Petrali et al. (1980b); Sulakhe et al. (1980)].

[2] That the R band likely represented the regulatory subunit of membrane-associated cAMP-dependent protein kinase was supported by the following observations: (A) it migrated identically with the phosphorylated regulatory unit of exogenous (bovine heart) cAMP-dependent protein kinase and (B) effects of cAMP and Ca^{2+} (stimulatory and inhibitory, respectively) were similar on the autophosphorylation of the R band and the regulatory subunit of exogenous kinase.

[3] Ca^{2+}-stimulated phosphorylation was found to be exclusively particulate, and myelin, among the subcellular fractions isolated, contained about 85–90% of the total Ca^{2+}-stimulated phosphorylation in particulate fractions. Relative to homogenate, Ca^{2+}-stimulated phosphorylation (as *nanomoles of ^{32}P incorporated per milligram of protein*) was enriched three- to fourfold in myelin. Also, recovery of the Ca^{2+}-stimulated phosphorylation in myelin was seven- to ninefold greater compared to SN_4.

Table II: Endogenous Phosphorylation of Basic Proteins in Myelin, SN_4, and SN_1[a]

fraction	Triton in assay	membrane ^{32}P incorporation (pmol/mg of protein)			basic protein phosphorylation (arbitrary units)					
					small basic protein			large basic protein		
		basal	+cAMP	$+Ca^{2+}$	basal	+cAMP	$+Ca^{2+}$	basal	+cAMP	$+Ca^{2+}$
Experiment A										
SN_1	−	185	180 (−5)	256 (71)	22	25 (3)	38 (16)	9	12 (3)	16 (7)
SN_4	−	188	195 (7)	306 (118)	30	34 (4)	47 (17)	13	16 (3)	22 (9)
myelin	−	55	55 (0)	132 (77)	12	10 (−2)	25 (13)	5	5 (0)	13 (8)
SN_1	+	435	665 (230)	682 (247)	35	37 (2)	65 (30)	15	14 (−1)	27 (12)
SN_4	+	271	348 (77)	641 (370)	31	37 (5)	73 (42)	13	18 (5)	28 (15)
myelin	+	86	95 (9)	324 (238)	11	12 (1)	41 (30)	4	6 (2)	18 (14)
Experiment B										
SN_1	+	480	616 (136)	139 (−341)	26	30 (4)	11 (−15)	12	16 (4)	4 (−8)
SN_4	+	375	517 (142)	630 (255)	31	36 (5)	60 (29)	13	17 (4)	25 (12)
myelin	+	251	295 (44)	670 (419)	21	23 (2)	65 (44)	10	12 (2)	27 (17)

[a] Myelin, SN_4, and SN_1 were isolated (see Methods) and incubated under standard assay for 2 min (experiment A) or 15 min (experiment B). ^{32}P incorporation into the membrane fractions and, following electrophoresis, into the basic proteins (expressed as arbitrary units) was determined. Results shown are taken from a representative experiment typical of three separate experiments. Numbers in parentheses represent the difference between cAMP or Ca^{2+} and basal phosphorylation.

Table III: Major Polypeptides of Myelin Subfractions Obtained on a Discontinuous Sucrose Density Gradient[a]

fraction	yield (mg of protein/g of tissue)	content of major polypeptide (% of total)						
		MGP (110K)	WP (52–55K)	PLP (24K)	LBP (19K)	SBP (16K)	high M_r range (>45K)	low M_r range (<45K)
light	1.5	0	2	22	19	33	11.8	12.0
light medium	2.6	1.3	4.4	20.9	17.9	27	11.8	16.1
medium	5.8	3.2	9.0	18.6	16.5	24.5	10.5	17.5
medium heavy	1.0	4.3	11.5	17.0	13.0	18.0	12.9	23.0
heavy	0.4	7.2	11.9	5.8	1.1	3.9	38.7	31.1

[a] Myelin subfractions were obtained on a discontinuous sucrose density gradient following isolation of highly purified myelin from rat brain white matter as described under Methods. MGP, myelin-associated glycoprotein; WP, Wolfgram peptide (triplet); PLP, proteolipid protein; LBP, large basic protein; SBP, small basic protein. 25 μg of protein was layered in each well, and the values are means of three preparations (variations did not exceed 10%) and are estimated from densitometric scans of the Coomassie Blue stained gels. Sum of minor high (>45K) and low (<45K) molecular weight proteins excludes major proteins already listed.

interpreted by Waehneldt and co-workers to indicate neuronal membrane contaminants in SN_1. That the observed phosphatase in SN_1 is not likely a myelin enzyme is further supported by other observations from our laboratory (Petrali et al., 1980b) in which, although phosphatases were detected in myelin, the stimulatory effect of Ca^{2+} on such phosphatases was not evident.

There are, however, some unexpected findings noted from the data presented in Figure 2 and Table II. For example, despite their considerably reduced contents in SN_1 and SN_4 (see Figure 1 and Table I), the degree of basal phosphorylation of LBP and SBP was relatively much higher than anticipated compared to that in myelin. Whether or not this is due to phosphorylation of different serines and/or threonines by the (different) kinases present in these three fractions remains to be investigated. Our previous work has, however, shown that in a typical myelin fraction under all assay conditions (basal, with cAMP and with Ca^{2+}), phosphorylation of serine accounted for nearly 80–90% of phosphorylation with 10–20% being that of threonine residues (Petrali et al., 1980b; Sulakhe et al., 1980); in this work Ca^{2+} was found to promote phosphorylation primarily of serine residues.

Polypeptide Compositions and Endogenous Phosphorylation of Myelin Subfractions Obtained by Discontinuous Sucrose Density Gradients. When myelin was further fractionated on a discontinuous sucrose density gradient (see Methods), at least four distinct bands were obtained, and these were arbitrarily designated as light (0.4–0.53 M sucrose interface), light medium (0.53–0.62), medium (0.62–0.70), and medium heavy (0.70–0.80). In addition, a pellet, designated as heavy, was also obtained. Such myelin subfractions differed from each other in the percentage distribution of the polypeptides as shown in Figure 3 and Table III. In general, the lighter fractions contained greater amounts of proteolipid protein as well as small and large basic proteins than the heavier fractions, whereas the reverse was true in the case of Wolfgram polypeptides as well as myelin-associated glycoprotein. Similar findings have been reported previously by various other laboratories (Zimmerman et al., 1975; Waehneldt, 1978; Waehneldt et al., 1977; Matthieu & Waehneldt, 1978; McIntyre et al., 1978; Shults et al., 1978), although the gradient used in the present study is somewhat different from those previously reported in order to achieve better resolution of myelin subfractions.

Endogenous phosphorylation of LBP and SBP in the absence and presence of calcium is presented in Table IV. Note that the medium–heavy (density) fraction showed a higher initial rate ($\pm Ca^{2+}$) of phosphorylation (2-min incubation), whereas the maximum steady-state phosphorylation (15-min incubation) with Ca^{2+} present was higher in the light and light–medium fractions. The latter two fractions also showed much better calcium stimulation of LBP and SBP over their basal phosphorylation; this was true especially when the steady-state phosphorylation was examined (see experiment B, Table IV). Not shown in Table IV is the effect of cAMP which was maximal in the medium–heavy fraction (also see below for phosphorylation of continuous-gradient myelin subfractions).

Polypeptide Compositions and Phosphorylation of Myelin Subfractions Obtained by Continuous Sucrose Density Gradient Centrifugation. Results obtained in this series of ex-

Table IV: Endogenous Phosphorylation of Basic Protein in Myelin Subfractions Obtained by Discontinuous Sucrose Density Gradient[a]

fraction	^{32}P incorporation (pmol/mg of protein)		phosphorylation (arbitrary units)			
			small basic protein		large basic protein	
	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$
			Experiment A			
light	38	125 (87)	9	22 (13)	4	7 (3)
light medium	76	126 (50)	9	23 (14)	5	8 (3)
medium	73	159 (86)	10	23 (13)	6	10 (4)
medium heavy	105	174 (69)	13	21 (8)	7	11 (4)
heavy	114	114 (0)	2	3 (1)	3	2 (−1)
			Experiment B			
light	344	796 (452)	33	80 (47)	9	27 (18)
light medium	342	747 (405)	41	83 (42)	14	40 (26)
medium	327	612 (285)	33	63 (30)	16	34 (18)
medium heavy	374	610 (236)	27	61 (34)	16	30 (14)
heavy	182	174 (−8)	16	14 (−2)	7	7 (0)

[a] Myelin was fractionated by discontinuous sucrose gradient centrifugation, and the gradient subfractions (see Methods) were phosphorylated under standard assay conditions (Triton X-100 present) for 2 min (experiment A) and 15 min (experiment B). For other details, see Methods. Results are taken from representative experiments and are means of duplicate determinations. Numbers in parentheses represent the difference between cAMP or Ca^{2+} and basal phosphorylation.

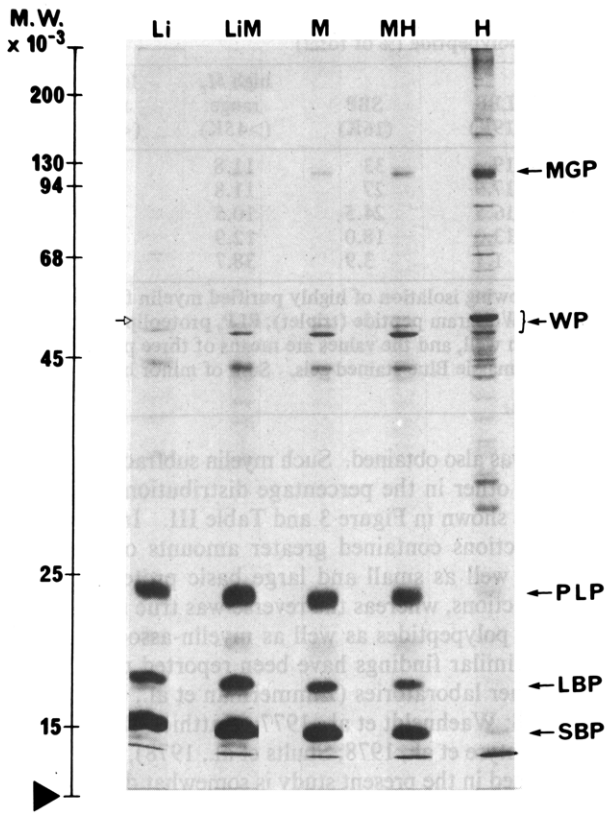

FIGURE 3: Polypeptide composition of myelin subfractions obtained by discontinuous sucrose density gradient centrifugation of myelin. For details see Methods and for nomenclature of proteins see Figure 1. Thirty micrograms of protein was layered for each fraction.

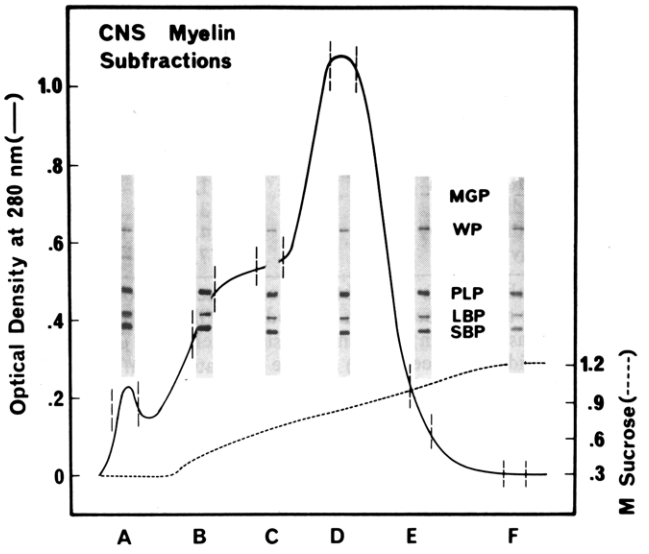

FIGURE 4: Fractionation of myelin by continuous sucrose density gardient centrifugations. Distribution of protein and polypeptide compositions of selected gradient fractions are shown. Twenty micrograms of protein was layered for each fraction (A–F) whose polypeptide patterns are shown.

periments have been summarized in Figure 4 (polypeptide profiles) and Table V (phosphorylation of total protein and small and large basic protein). The continuous sucrose density gradient employed was that of Waehneldt (1978) and the pattern of distribution of myelin subfractions following centrifugation was generally similar to that reported by Waehneldt except that in our hands there was a bimodal distribution of the applied material. As shown in Figure 4, fraction A represented some unfractionated material, fractions B, C, and D represented light, light–medium, and medium density myelin fractions, and fractions E and F represented medium–heavy and heavy fractions, respectively. The polypeptide compositions of the gradient subfractions were similar to those described earlier (Table III) with the discontinuous sucrose density gradient fractions. A noteworthy exception, however, was that the content of proteolipid protein did not differ significantly within fractions B–E (i.e., between light and medium–heavy subfractions). This observation has been reported by Waehneldt (1978) in the continuous-gradient myelin fractions (isolated from rat forebrain). His work and our study nevertheless do show a much reduced amount of proteolipid protein in the heavy fraction (F). At least from the polypeptide profiles it appeared that the continuous gradient subfractions displayed relatively fewer differences compared to those obtained by the discontinuous gradient in our hands. Nevertheless, there are noticeable differences in the phosphorylation of LBP and SBP within the continuous gradient subfractions (Table V). It was found that cAMP-stimulated phosphorylation of LBP and SBP including that of the R protein tended to be higher in fractions D and E, which are medium and medium–heavy subfractions (not shown). Calcium-stimulated phosphorylation, on the other hand, was quite marked in all gradient subfractions and generally was highest in fraction D. The latter observation was indeed expected since all these

Table V: Endogenous Phosphorylation of Basic Proteins in Myelin Subfractions Isolated by Continuous Sucrose Density Gradient Centrifugation of Myelin[a]

fraction	^{32}P incorporation [pmol/(mg of protein 15 min)]			phosphorylation (arbitrary units)					
				small basic protein			large basic protein		
	basal	+cAMP	+Ca^{2+}	basal	+cAMP	+Ca^{2+}	basal	+cAMP	+Ca^{2+}
A	102	128 (26)	242 (140)	31	32 (1)	52 (21)	9	11 (2)	12 (3)
B	128	148 (20)	356 (228)	23	25 (2)	43 (20)	8	9 (1)	16 (8)
C	145	168 (23)	368 (223)	32	35 (3)	58 (26)	15	17 (2)	29 (14)
D	142	171 (29)	410 (268)	24	29 (5)	59 (35)	15	18 (3)	31 (16)
E	97	119 (22)	271 (174)	17	22 (5)	49 (32)	14	18 (4)	25 (11)
F	49	47 (−2)	92 (43)	15	15 (0)	21 (6)	4	5 (1)	21 (6)

[a] Myelin subfractions were isolated by the method of Waehneldt and co-workers (Waehneldt, 1978) and were incubated for 15 min under standard assay conditions (Triton X-100 present). For other details, see Methods. Fraction A represented unfractionated material (also see Figure 6).

subfractions contained both basic proteins (see Figure 4). In other words, there appeared again a parallel relation between the content of basic protein and their Ca^{2+}-stimulated phosphorylation. One could suggest that the continuous-gradient subfractions (despite the fact that the centrifugation was carried out for 4 h to attain isopycnic equilibrium) do not reveal as clear-cut differences within the myelin subfractions as has been the case with the discontinuous-gradient subfractions. This appears to be true both when polypeptide compositions as well as phosphorylation of the gradient subfractions were investigated.

Presence of Calmodulin-like Proteins in Myelin. Numerous recent studies indicate that a protein factor, now called calmodulin (Cheung et al., 1978), is required to mediate the stimulatory effect of Ca^{2+} on a number of enzymes [see reviews by Wang & Waisman (1979) and Cheung (1980)]. It was thus of interest to know (1) whether calmodulin is needed for the stimulatory effect of Ca^{2+} on myelin phosphorylation and (2) whether calmodulin-like protein is present in myelin. Elsewhere we reported that EGTA treatment of myelin decreased the Ca^{2+}-stimulated phosphorylation of basic proteins. Further, purified calmodulin (bovine heart) or EGTA extract of myelin restored it to the control value, an observation that suggested a likely role of calmodulin in the Ca^{2+} stimulatory effect on myelin phosphorylation (Sulakhe et al., 1980). Figure 5 shows that EGTA extract restores the activity of calmodulin-deficient cAMP phosphodiesterase to the same extent as calmodulin. From these data, we have estimated that the "EGTA-removable" amount of calmodulin-like protein in myelin represents 0.4 ± 0.02 μg/mg of myelin protein, whereas the total (EGTA-removable plut tightly bound) calmodulin content is ~1 ± 0.1 μg/mg of myelin protein. Because of its low content, it was difficult to visualize calmodulin and to estimate reliably its content in myelin from the Coomassie Blue stained polypeptide profile of myelin, which was routinely obtained by loading 20–30 μg of membrane protein. An additional problem is that the molecular weight of calmodulin (M_r 16 500–18 000) is very close to other myelin major proteins, e.g., LBP (M_r 18 000 ± 1000) and DM-20 (M_r 20 000 ± 1000), so that its presence is obscured by the latter polypeptides.

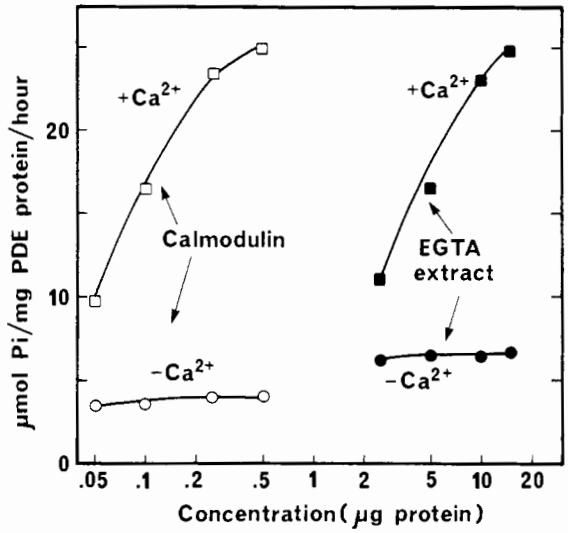

FIGURE 5: Presence of calmodulin-like protein in myelin extract. Calmodulin-deficient cAMP phosphodiesterase (bovine heart) was incubated for 30 min at 30 °C in the absence and presence of varying concentrations of calmodulin (bovine heart) or EGTA extract prepared from myelin. Assays were carried out in the absence and presence of Ca^{2+} according to the method of Sharma & Wang (1979).

Discussion

The main purpose of the study was to test whether protein kinases, which reportedly are present in commonly isolated myelin preparations, represent myelin-specific enzymes or are due to the presence of contaminating membrane fragments (Carnegie et al., 1973; Steck & Appel, 1974; Miyamoto, 1975). From the previously published reports this conclusion could not be reached because of the purity of myelin preparations used (Norton, 1977). Our study for the first time provides strong evidence that at least the Ca^{2+}-stimulated protein kinase is a myelin-specific enzyme since it was reproducibly present in the highly purified myelin from rat brain white matter. Previous work from our laboratory (Sulakhe et al., 1980) indicated a likely role of calmodulin-like protein in the stimulatory effect of Ca^{2+} on myelin phosphorylation.[4] The results presented in Figure 5 support the view of the presence of calmodulin-like protein in myelin; SN_4 also contained calmodulin (results not shown). cAMP-stimulated kinase appeared to be considerably more enriched in SN_4 as well as in medium and medium–heavy discontinuous-gradient subfractions. In fact, if one were to closely examine the polypeptide compositions of SN_4 and medium–heavy discontinuous-gradient subfractions, these are indeed very similar, if not identical. Electron microscopic observations indicate that SN_4 and medium–heavy fractions generally consist of small membrane vesicles with the occasional presence of triple-layered structures. This is in sharp contrast to the light myelin ultrastructure, which mainly consists of multilamellar compact

[4] Recently, we found that a heat-stable calmodulin binding protein (0.6 μg) (M_r of 70 000, isolated from beef brain) selectively inhibited (70%) the Ca^{2+}-stimulated phosphorylation of myelin basic proteins without any effect on basal and cAMP-stimulated phosphorylation.

membrane vesicles (Waehneldt, 1977). It is thus possible that cAMP-stimulated kinase is primarily that of an "early" myelin (Norton, 1977), which in many ways resembles the fraction SN_4 (Waehneldt et al., 1977). An implication of this postulate is that cAMP-stimulated protein kinase tends to be deeply buried within the membrane matrix in the typically isolated adult myelin preparations. The evidence that this enzyme is readily detected only in the presence of Triton further supports this view as already stated in earlier studies by Miyamoto (1975). It has been recognized that, while cAMP-stimulated kinase is present in early myelin or SN_4, the basic proteins are inserted into the compact myelin in the subsequent stages of myelin assembly (Zimmerman et al., 1975; Waehneldt et al., 1977). Thus, while calcium-stimulated kinase is present in both SN_4 and myelin, its maximal activity in adult myelin may in part be the result of incorporation into the membrane of basic proteins and calcium-regulated proteins such as calmodulin. However, virtually nothing is known at the moment about the content of calmodulin during the formation of compact myelin from its precursor membrane (early myelin), and hence further work is undoubtedly required to ascertain such a possibility. Nevertheless, a rather remarkable correlation between the content of basic proteins and their calcium-sensitive phosphorylation within the discontinuous-gradient subfractions argues strongly in favor of myelin basic proteins as highly specific substrates of a myelin calcium-dependent kinase. The observations that the affinities toward free Ca^{2+} (i) of the phosphorylating system (Petrali et al., 1980b; Sulakhe et al., 1980), (ii) of high-affinity Ca^{2+} binding site in isolated myelin (Hemminki, 1974), and (iii) of the isolated calmodulin protein (Teo & Wang, 1973; Wang & Waisman, 1979) are similar implicate calmodulin-like proteins in Ca^{2+}-dependent myelin basic protein phosphorylation. To what extent cAMP-dependent kinase, either in vitro or in vivo, regulates phosphorylation of these proteins requires further careful studies.

Even though in some earlier studies myelin protein kinases and/or phosphatases have been investigated, the substrate proteins utilized were not the myelin basic proteins, and hence the likely significance of those studies is not very clear. For example, in Miyamoto's work either histones and protamines were used in the study of solubilized myelin kinase(s) (Miyamoto, 1975; Miyamoto et al., 1978; Miyamoto & Kakiuchi, 1974) or, when myelin basic protein was used as a substrate in the phosphatase study, it was prephosphorylated with exogenous (brain cytosolic) cAMP-dependent protein kinase (Miyamoto & Kakiuchi, 1975). It is clear that exogenous protein kinase catalyzes phosphorylation of serine residues of basic protein which are different from those phosphorylated by the endogenous system (Carnegie et al., 1974). Also, McNamara & Appel (1977) clearly document that the membrane-bound basic protein is a preferred substrate over the soluble basic protein of the myelin-associated phosphatases. In view of the findings presented in our earlier studies (Sulakhe et al., 1978; Petrali et al., 1980a,b; Sulakhe et al., 1980), including the present investigation, and the above-mentioned difficulties in characterizing the myelin-specific kinases and/or phosphatases, it is important that future work must utilize what appears to be the specific protein substrates of myelin enzymes, namely the basic proteins.

Despite the fact that myelin isolated from the central [this study; Petrali et al. (1980a,b); Sulakhe et al. (1980)] and peripheral (Petrali & Sulakhe, 1979, 1980) nervous system contains highly active protein kinases, it is difficult at present to indicate a role for these in myelin function. It could very well be that phosphorylation of basic proteins may determine or modulate compaction of myelin and/or interactions between the basic proteins and other myelin proteins, in particular the proteolipid protein (Carnegie & Dunkley, 1975; Norton, 1977; Rumsby & Crang, 1977; Benjamins & Morell, 1978; Boggs & Moscarello, 1978). Thus, the present observation of the calcium-stimulated phosphorylation of myelin is likely to be of potential significance.

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Conformational Transitions in the Subfragment-2 Region of Myosin†

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ABSTRACT: A differential scanning calorimeter was used to observe thermally induced conformational transitions in subfragment 2 (S-2) of myosin. In addition to an endotherm for the major transition which had been observed by several other methods earlier, a small broad endotherm was noted with a T_m of 41 °C. By analysis of the heat capacity profiles of long and short S-2, this endotherm was assigned to the hinge region. Comparison of the amino acid compositions of S-2 and tropomyosin showed them to be remarkably similar, and in view of their similar behavior in calorimetric studies, it is apparent that interactions stabilizing the coiled-coil structure of S-2 are a hydrophobic interface supported by charged interaction spanning the groove as was suggested for tropomyosin by McLachlan and Stewart [McLachlan, A. D., & Stewart, M. (1975) *J. Mol. Biol. 98*, 293–304].

The multifunctional role of myosin in the molecular mechanism of muscle contraction requires that it contain structural domains (Kirschner & Bisswanger, 1976) suited to each function. Two structural features are obvious: (1) the globular head (subfragment 1), which contains the sites for ATP hydrolysis and actin binding, and (2) the coiled-coil rod, which contains the sites of the molecular interactions which direct the proper self-assembly of myosin molecules into the thick filament and support tension. Association of the various functions with a specific part of the structure has been achieved by enzymatic cleavage. Myosin can be cleaved into subfragment 1 (S-1)[1] and rod by several enzymes (Bâlint et al., 1975; Weeds & Taylor, 1975; Weeds & Pope, 1977). The rod portion can be enzymatically cleaved to yield subfragment 2 (S-2) and light meromyosin (LMM). The susceptibility of these two regions of myosin to enzymatic cleavage has led to the hypothesis that they are functional hinges in the context of the sliding filament model of muscle contraction (Huxley & Hanson, 1954). Current theories are considering changes in conformation of these regions (Harrington, 1971) and changes in relative orientation (Huxley, 1969; Huxley & Simmons, 1971) between the domains as events which provide a molecular basis for the generation of tension in skeletal muscle. The nature of these processes and their coupling to the hydrolysis of ATP is poorly, if at all, understood.

Considerable attention has been focused recently on S-2 (Harrington, 1979a,b). In current models for contraction, it has variously been suggested as (a) a rigid connecting link between LMM and S-1 which because of the hinge regions allows S-1 to engage actin to form a cross bridge (Huxley, 1969), (b) a series-elastic element which is stretched upon tension generation by rotation of S-1 at the actin-S-1 interface and then transmits this force to the myosin thick filament (Huxley & Simmons, 1971), and (c) a source of tension generation via a helix–coil transition in a portion of the coiled-coil structure (Harrington, 1971, 1979a,b). The recent work of Harrington and collaborators provides support for a helix–coil transition in S-2 as the origin of the contractile force. These workers have carefully characterized a less degraded form of S-2 and found that, in contrast to previous studies with shorter S-2, this long S-2 self-associates at physiological ionic strengths (Sutoh et al., 1978). In earlier experiments, Tsong et al. (1979) have shown that the rate constants for opening and closing the coiled-coil strucuture of S-2 are in the same range observed for the quick-recovery process in muscle fibers (Huxley & Simmons, 1971). From these and other observations, these authors have suggested a model for contraction in which S-2 is in close contact with the thick filament in the relaxed state. Following S-1 binding to actin, S-2 is released from the thick filament and contracts via a helix–coil transition to generate tension (Harrington, 1979b). In this paper we report on the properties of the helix–coil conformational transitions of S-2 as measured by a differential scanning calorimeter. Evidence is presented for a second helix–coil transition which is assigned to the hinge region of S-2.

Materials and Methods

Myosin was prepared from rabbit skeletal muscle and characterized as described earlier (Goodno & Swenson, 1975a,b). It was stored at 4 °C as a pellet which was the

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[1] Abbreviations used: S-1, subfragment 1 of myosin; S-2, subfragment 2 of myosin; LMM, light meromyosin; HMM, heavy meromyosin; NaDodSO$_4$, sodium dodecyl sulfate.

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