Adenosine 3':5'-Monophosphate-regulated Phosphoprotein System of Neuronal Membranes

I. SOLUBILIZATION, PURIFICATION, AND SOME PROPERTIES OF AN ENDOGENOUS PHOSPHOPROTEIN* 

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An endogenous substrate for adenosine 3':5'-monophosphate-dependent protein kinase has been solubilized, and purified about 5,000-fold to apparent homogeneity, from a particulate fraction of bovine cerebral cortex enriched in synaptic membranes. This endogenous substrate, referred to as Protein I, is apparently specific to nervous tissue, and is composed of two types of polypeptides, present in a proportion of 1 (Protein Ia, 86,000 daltons) to 2 (Protein Ib, 80,000 daltons). In the presence of cAMP-dependent Protein I kinase purified from the same membrane fractions, Proteins Ia and Ib contain some sequences similar to those observed in collagen. The sedimentation coefficient of Protein Ia and Protein Ib was determined to be 2.9 S. The data suggest that both Protein Ia and Protein Ib have an elongated shape.

A variety of evidence indicates that adenosine 3':5'-monophosphate plays an important role in several types of neuronal function, including synaptic transmission (for reviews, see Refs. 1 to 7). Other evidence suggests that many of the effects of cAMP in various tissues, including nervous tissue, may be mediated through regulation of the phosphorylation of specific proteins (for reviews, see Refs. 6 and 8 to 10). In view of the possible importance of protein phosphorylation in synaptic function, we have undertaken a systematic study of the phosphorylation system present in synaptic membranes.

When subcellular fractions of nervous tissue rich in synaptic membranes are incubated in the presence of $[\gamma-^32P]ATP$, there is a marked cAMP-dependent phosphorylation of two endogenous substrate proteins, catalyzed by endogenous protein kinase (11). One of these natural substrate proteins (Protein I) appears to be specific to neuronal membranes (11), whereas the other substrate protein (Protein II) appears to have a widespread tissue distribution (12) and, in fact, appears to be the regulatory subunit of a cAMP-dependent protein kinase. This paper describes the solubilization, purification, and some properties of Protein I. The following paper is concerned with the purification and properties of Protein I kinase (13).

EXPERIMENTAL PROCEDURES

Materials – Fresh calf brains were obtained from a local slaughterhouse, transported on ice to the laboratory, and processed immediately. cAMP, ammonium sulfate (enzyme grade), bovine serum albumin, myoglobin, and aldolase were obtained from Schwarz/Mann; catalase and ferritin from Rehlinger Mannheim; phosphorylase $c$ (twice crystallized), pronase, Tris base, Hepes, Triton X-100, and 2-mercaptoethanol from Sigma; collagenase (Form III) from Advanced BioResearch Corp.; soluble skin collagen and carboxypeptidase $A$ from Worthington Biochemical; sucrose, glycine, and ammonium persulfate from Fisher; dithiothreitol from Aldrich; protocol from New England Nuclear; hydroxylapatite and Bio-Gel P-150 and P-300 from Bio-Rad; Ampholine (pH 3 to 10 and 9 to 11) from LKB; acrylamide and $N,N',N''$-tetramethylethylenediamine and Kodak Royal X-Omat film (RP R-54) from Eastman Kodak. $[\gamma-^32P]ATP$ was prepared by the method of Post and Sen (14).

Preparation of Neuronal Membrane Fractions – Neuronal membrane fractions were prepared by the following modification of the procedure of De Robertis et al. (15). All operations were carried out at $4\degree C$. Centrifugations were performed in a Sorvall RC 2 B centrifuge, using a GSA rotor. Cerebral cortices (weighing approximately 1.5 kg) obtained from 1 dozen calf brains were rinsed twice with 2 volumes of 0.92 $\mu$ sucrose, and homogenized in 4 volumes of 0.32 $\mu$ sucrose for 40 s in a Waring Blender with a 2-min interval in ice after the first 20 s of homogenization. The homogenate was mixed with an equal volume of 0.32 $\mu$ sucrose, and centrifuged at 10,000 $x$ g for 10 min. The resulting supernatant fluid was further centrifuged at 10,000 $x$ g for 20 min, and the pellet thus obtained was suspended in 0.32 $\mu$ sucrose for a final volume of 600 ml. The suspended material (containing mitochondria, synaptosomes, myelin, etc.) was subjected to osmotic shock by diluting with 9 volumes of cold water, and homogenized in a Teflon-glass homogenizer. The homogenate was centrifuged at 20,000 $x$ g for 30 min; the resulting pellet was resuspended to a final volume of 1 liter using 0.32 $\mu$ sucrose, rehomogenized in the Teflon-glass homogenizer, frozen, and used within 1 week. This

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§ The abbreviations used are: cAMP, adenosine 3':5'-monophosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate.

1 I. Uno, T. Ueda, and P. Greengard, manuscript in preparation.
preparation, referred to as M-l, using the terminology of De Robertis et al. (15), contained 30 to 35% of the Protein I present in the whole homogenate and was used as the starting material (Step 1) for the solubilization and purification of Protein I. In a few experiments, the M-1 membrane fraction was layered onto a sucrose gradient containing 0.8 m, 0.9 m, 1.0 m, and 1.2 m sucrose, and centrifuged in a swing-out rotor (SW 25.1) at 100,000 x g for 2 h. The material which banded in the interface between 0.8 and 0.9 m sucrose was collected, diluted with 2 volumes of ice-cold distilled water, and centrifuged at 150,000 x g for 30 min. The resulting pellet was suspended in 0.32 M sucrose, homogenized, and used as a synaptic membrane preparation designated M-l (0.9), using the terminology of De Robertis et al. (15). Proteins Ia and Ib (the two components of Protein I [see below]) in the M-1 (0.9) fraction exhibited mobilities which banded in the interface between 0.8 and 0.9 M sucrose, and centrifuged at 150,000 x g for 30 min. Treatment of the M-l fraction (12 to 18 mg of protein/ml) with detergents such as Triton X-100 (final concentration, 0.2% or 1%) or deoxycholate (final concentration, 0.2%) for 30 min was found to be the most effective in solubilizing Protein I; using either of these procedures, the amount of Protein I found in the supernatant fraction (as can be seen in the autoradiograph shown in Fig. 3). The M-l fraction was used as the starting material for the routine purification procedure for Protein I described below. The relative peak heights of the optical density tracings in the positions corresponding to Protein Ia and Protein Ib were used to estimate the relative amounts of [32P]phosphate incorporated into Proteins Ia and Ib. A band containing Proteins Ia plus Ib was cut from one slot of each of the dried slab gels, dissolved in protosol, and radioactivity determined in a liquid scintillation spectrometer, thus providing a means for the conversion of the arbitrary units obtained by the densitometric measurement to conventional radioactivity measurements. Protein determination was carried out by the method of Lowry et al. (17) with bovine serum albumin as protein standard.

Solubilization of Protein I—Several procedures were tested for their ability to solubilize Protein I from the M-1 membrane fraction. Pastore et al. (15) showed that Protein I into the supernatant fraction upon centrifugation at 150,000 x g for 30 min. Treatment of the M-1 fraction (12 to 18 mg of protein/ml) with detergents such as Triton X-100 (final concentration, 0.2% or 1%) or deoxycholate (final concentration, 0.2%) for 30 min was found to be the most effective in solubilizing Protein I; using either of these procedures, the amount of Protein I found in the supernatant fraction (as can be seen in the autoradiograph shown in Fig. 3). The M-l fraction was used as the starting material for the routine purification procedure for Protein I described below. The relative peak heights of the optical density tracings in the positions corresponding to Protein Ia and Protein Ib were used to estimate the relative amounts of [32P]phosphate incorporated into Proteins Ia and Ib. A band containing Proteins Ia plus Ib was cut from one slot of each of the dried slab gels, dissolved in protosol, and radioactivity determined in a liquid scintillation spectrometer, thus providing a means for the conversion of the arbitrary units obtained by the densitometric measurement to conventional radioactivity measurements. Protein determination was carried out by the method of Lowry et al. (17) with bovine serum albumin as protein standard.

Extraction and Assay of Protein I from Various Tissues—Various tissues from 150-g male rats were minced and homogenized in 30 volumes of ice-cold distilled water at 1,200 rpm with 12 strokes in a Teflon-glass homogenizer (0.15 to 0.2 mm clearance). Aliquots (4 ml) of each homogenate (containing approximately 3.5 mg of protein/ml) were centrifuged at 150,000 x g for 30 min. The resulting pellet was suspended and homogenized in 4 ml of ice-cold distilled water, and was adjusted to pH 3 with 0.01 M sodium hydroxide. The supernatant solution, obtained upon centrifugation at 26,000 x g for 15 min, was immediately adjusted to pH 6 with 0.01 M sodium hydroxide, and centrifuged at 26,000 x g for 10 min. Aliquots (60 ml) from each supernatant were incubated under standard Protein I assay conditions, and 50 ml of each sample were subjected to SDS-gel electrophoresis andautoradiography in order to determine the amount of [32P]phosphate which had been incorporated into Protein Ia, as described below. A band containing Proteins Ia plus Ib was cut from one slot of each of the dried slab gels, dissolved in protosol, and radioactivity determined in a liquid scintillation spectrometer, thus providing a means for the conversion of the arbitrary units obtained by the densitometric measurement to conventional radioactivity measurements. Protein determination was carried out by the method of Lowry et al. (17) with bovine serum albumin as protein standard.

Amino Acid Analysis—The amino acid composition of Protein Ia and of Protein Ib was determined with a Beckman 121 A amino acid
For this purpose, Protein Ia and Protein Ib were prepared as follows: Protein I (100 µg) was subjected to SDS-polyacrylamide slab gel electrophoresis according to the method of Fairbanks et al. (18). The gel (11 cm x 16 x 4 mm) contained 5.6% acrylamide, 0.15% N,N'-methylenebisacrylamide, 1% SDS, and 2 mM EDTA in 0.04 M Tris-acetate (pH 7.4), and electrophoresis was carried out at 80 mA for 18.5 h. One middle and both end channels of the gel were cut out, stained and destained, as described above, in order to visualize Protein Ia and Protein Ib; the remaining parts of the gels were fixed in the same solution except that Coomassie blue was omitted. With the aid of the stained pieces of gel, the Protein Ia and Protein Ib bands in the unstained gel were localized, cut out, crushed into small pieces, and incubated at 50°C in the presence of 2% SDS for 5 days. The supernatant solution obtained upon centrifugation at 150,000 × g for 30 min contained recovered Protein Ia and Protein Ib.

In a preliminary experiment using [32P]Phosphoproteins Ia and Ib, it was found that this procedure yielded greater than 70% extraction of Protein Ia and Protein Ib. Proteins Ia and Ib thus extracted from the gel were dialyzed against 500 volumes of 0.2%, then 0.02%, and finally 0.003% SDS solution for 16 h each time. The samples of extracted Protein I were then lyophilized and hydrolyzed in 6 N HCl at 110°C for 24 and 72 h.

Carbon determinations of [32P]Phosphoprotein Acid Residues in Phosphorylated Proteins Ia and Ib—Purified Protein I (100 µg) was phosphorylated in a final volume of 1 ml under otherwise standard Protein I assay conditions, and the phosphorylation terminated by the addition of 0.5 ml of SDS-stop solution. The entire sample was applied to two slab gels (1.5 mm thickness) containing stacking gel, and subjected to electrophoresis in the Tris-glycine buffer system, as described above, except that the separation gel contained 8% acrylamide, 0.22% N,N'-methylenebisacrylamide. The electrophoresis time was doubled to increase the separation of the Protein Ia and Ib bands from each other. Following staining, destaining, and drying of the gels, the Coomassie blue-stained Protein Ia and Protein Ib bands were cut out, and the strips of gel were allowed to swell in water, crushed into small pieces, and incubated at 50°C for 5 days in 2% SDS for the extraction of Protein Ia and Protein Ib. The extracted proteins were dialyzed as described in the preceding section, lyophilized, and hydrolyzed in 6 N HCl at 110°C for 5.5 h. The hydrolysates were lyophilized and each dissolved in 200 µl of 5 mM phosphoserine, 5 mM phosphothreonine solution; 100-µl aliquots were subjected to high voltage electrophoresis in 2.1% formic, 8.7% acetic acid buffer (pH 1.9) at 5000 V for 3 h as described by Allerton and Perlmann (19). Under these conditions, authentic phosphoserine and phosphothreonine were hydrolyzed 66% and 25%, respectively. Inorganic phosphate was determined by the method of Rockstein and Herron (20).

Isoelectric Focusing—Analytical isoelectric focusing was performed, using an LKB 2117 Multiphor apparatus, on a 8.4 cm x 5.5 x 0.13 cm gel containing 6% acrylamide, 0.13% N,N'-methylenebisacrylamide, 2% Ampholine (in the pH range of 3 to 10 or 9 to 11) by slight modification of the method of Righetti and Drysdale (21), as described in the LKB Instructions. Samples were directly applied to the gel surface in the center, and electrophoresis was carried out horizontally at a constant current of 25 mA until the applied voltage increased to 500 V (approximately 45 min) and then that voltage was maintained for 5 h. Immediately following electrophoresis, a channel of the gel devoid of samples was cut into 5-mm slices, which were eluted in 0.5 ml of 10 mM NaCl for 2 h, and the pH of the eluates was measured. The remaining portion of the gel was fixed in 10% trichloroacetic acid containing 3.5% sulfosalicylic acid and 30% methanol, the gel washed in a solution containing 8% acetic acid, 25% ethanol, stained with 0.11% Coomassie blue R250 in 8% acetic acid, 25% ethanol, and then destained in 8% acetic acid, 25% ethanol.

Carbohydrate Analysis—Purified Protein I was analyzed for carbohydrate by gas-liquid chromatography as described by Reinhold (22).

RESULTS AND DISCUSSION

Purification of Protein I

Step 1: M-1 Membrane Fraction—M-1 membrane fraction was prepared from bovine cerebral cortex as described under "Experimental Procedures," and frozen. All subsequent steps were carried out at 4°C, all centrifugations were performed in a Sorvall RC 2-B centrifuge with a GSA rotor, and all containers were plastic. Unless stated otherwise, 5 mM sodium phosphate buffer (pH 7.0) was used.

Step 2: pH 3 Extract—The frozen M-1 membrane preparation (1 liter) was thawed, brought to pH 3 by the gradual addition of 0.1 N HCl and, after 5 min, centrifuged at 26,000 × g for 15 min.

Step 3: pH 6 Supernatant—The pH 3 extract was immediately adjusted to pH 6 by the gradual addition of 0.1 N NaOH. (It was important not to exceed this pH.) A fluorescent protein precipitate was observed, and this precipitate was removed by centrifugation at 26,000 × g for 10 min.

Step 4: First Hydroxylapatite Column Eluate—The pH 6 supernatant (860 ml) was divided into two halves, and each half was applied to a column (6.4 cm x 11.5 cm) of hydroxylapatite (42 g each) which had been equilibrated with 5 mM sodium phosphate buffer (pH 7.0). Elution was carried out with 0.1 M NH4Cl. The initial 15 ml was discarded and the following 170 ml, which contained Protein I, was collected.

Step 5: Ammonium Sulfate Precipitate—The hydroxylapatite eluate fractions were combined (340 ml) and brought to 80% saturation by the addition of 191 g of solid ammonium sulfate, with stirring, over 15 min. Stirring was continued for an additional 15 min and the mixture was allowed to stand for 1 h. The precipitate was collected by centrifugation, dissolved in 5 mM sodium phosphate buffer (pH 7.0) containing 0.2 mM dithiothreitol, and dialyzed against 400 volumes of the same buffer for 10 h with one change of buffer after 4 h. It was found necessary to carry out Steps 2 through 5 within 24 h; a significant loss of Protein I activity was observed when the hydroxylapatite eluate was allowed to stand overnight.

Step 6: Second Hydroxylapatite Column Eluate—The dialyzed protein solution (6.3 ml) was applied to a column of hydroxylapatite (1.3 x 5 cm) which had previously been equilibrated with 5 mM sodium phosphate buffer (pH 7.0), and the column was then washed with 4 ml each of 5 mM and 50 mM sodium phosphate at a flow rate of 0.65 ml/min. The Protein I was then eluted with 80 mM sodium phosphate at the same flow rate. Fractions (2 ml) were collected, and aliquots were assayed for Protein I under standard conditions. The fractions enriched in Protein I (usually Fractions 1 to 4) were pooled.

Step 7: Bio-Gel P-150 Column Eluate—The pooled fractions of the hydroxylapatite eluate were subjected to gel filtration on a column of Bio-Gel P-150 (100 to 200 mesh, 1.7 x 78 cm), which had previously been equilibrated with 5 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl. The column was eluted with the same solution; 2-ml fractions were collected, and assayed for Protein I. Fractions showing the highest specific activity of Protein I (usually Fractions 15 to 17) were pooled, and dialyzed for 4 h against 400 volumes of 5 mM sodium phosphate buffer (pH 7.0) with a change of buffer after 2 h. The Protein I thus obtained was essentially homogeneous, as judged by high resolution SDS-gel electrophoresis. When necessary, the purified Protein I was concentrated by vacuum dialysis.

Typical results of the procedure used for the purification of Protein I are shown in Figs. 2 and 3 and in Table I. Protein Ia and Protein Ib were purified approximately 3,000- and 4,700-fold, respectively, with respect to the M-1 membrane fraction. Using either 32P incorporation or protein staining as the criterion, the ratio of Protein Ia to Protein Ib appeared to decrease at the first hydroxylapatite column chromatography step, probably due to the removal of a contaminating protein which had a molecular weight similar to that of Protein Ia, but remained constant at a ratio of approximately 1 to 2 through-
Neuronal Membrane Phosphoprotein

PURIFICATION OF PROTEINS Ia AND Ib
(Monitored by SDS Gel Electrophoresis)

A. PROTEIN STAINING

B. AUTORADIOGRAPH

FIG. 2 (top). Protein-staining pattern of Protein I preparations at each stage of purification. Samples, containing (1) 46.0 µg (M-1 membrane fraction); (2) 50.3 µg (pH 3 extract); (3) 21.0 µg (pH 6 supernatant); (4) 5.7 µg (first hydroxyapatite column eluate); (5) 16.5 µg (ammonium sulfate precipitate); (6) 10.5 µg (second hydroxyapatite column eluate); (7) 2.0 µg (Bio-Gel P-150 eluate) of protein, in a solution of 3% SDS, 2% mercaptoethanol, 0.063 M Tris/HCl (pH 6.7), and 5% glycerol, were subjected to polyacrylamide slab gel electrophoresis in sodium dodecyl sulfate. The volume of each sample was 60 µl. Electrophoresis and protein staining with Coomassie blue were carried out as described under "Experimental Procedures." This photograph was taken from the wet gel.

Fig. 3 (bottom). Comparison of the electrophoretic mobilities of phosphoproteins in the purified Protein I preparation with those in membrane fractions M-1 (0.9) and M-1. The M-1 (0.9) (125 µg of protein) and M-1 (210 µg of protein) membrane fractions were incubated in a final volume of 100 µl for 10 s at 30° in the absence or presence of 10 mM cyclic AMP in 50 mM sodium morpholinoethane sulfonate buffer, pH 6.2, 10 mM MgCl₂, and 5 µM [γ-³²P]ATP. Purified Protein I (Bio-Gel P-150 eluate, 1 µg in the presence, and 4 µg in the absence of exogenous protein kinase, containing a mixture of Proteins Ia and Ib) was incubated for 10 s in the absence or presence of cyclic AMP and protein kinase under otherwise standard assay conditions, as described under "Experimental Procedures." All reaction mixtures were preincubated at 30° for 1 min prior to the addition of [γ-³²P]ATP. Following termination of the reaction with 50 µl of SDS-stop solution, aliquots (50 µl) were subjected to SDS-gel electrophoresis and autoradiography for the analysis of Protein I phosphorylation. A, protein-staining pattern of the dried gel; B, autoradiograph obtained from the dried gel.
TABLE I
Purification of Proteins Ia and Ib from bovine cerebral cortex

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Protein Activity</th>
<th>Specific activity</th>
<th>Purification*</th>
<th>Yield*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>Ia m</td>
<td>Ib m</td>
<td>Ia pmol</td>
<td>Ib pmol</td>
</tr>
<tr>
<td>1.</td>
<td>M-1 membrane fraction</td>
<td>1,000</td>
<td>18,400</td>
<td>2,300</td>
<td>3,160</td>
</tr>
<tr>
<td>2.</td>
<td>pH 3 extract</td>
<td>860</td>
<td>2,860</td>
<td>16,110</td>
<td>22,150</td>
</tr>
<tr>
<td>3.</td>
<td>pH 6 supernatant</td>
<td>860</td>
<td>712</td>
<td>15,760</td>
<td>22,580</td>
</tr>
<tr>
<td>4.</td>
<td>First hydroxylapatite column eluate</td>
<td>340</td>
<td>40</td>
<td>5,850</td>
<td>11,900</td>
</tr>
<tr>
<td>5.</td>
<td>Ammonium sulfate precipitate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Second hydroxylapatite column eluate</td>
<td>6.0</td>
<td>0.58</td>
<td>1,520</td>
<td>3,310</td>
</tr>
<tr>
<td>7.</td>
<td>Bio-Gel P-150 column eluate</td>
<td>6.0</td>
<td>0.58</td>
<td>1,520</td>
<td>3,310</td>
</tr>
</tbody>
</table>

* Calculations based upon the assumption that the M-1 membrane fraction contained Protein Ia and Protein Ib in the same amounts as were present in the pH 3 extract.

The fact that the yield of Protein Ia was apparently lower than that of Protein Ib was probably due to the removal at Step 4 of a contaminating phosphorylated protein with an electrophoretic mobility similar to that of Protein Ia.

Stoichiometry and Site of Phosphorylation

The stoichiometry of phosphorylation of Proteins Ia and Ib is shown in Fig. 4. It was found that 0.75 and 0.74 mol of \(^{32P}\)phosphate were incorporated/mol of Protein Ia and Protein Ib, respectively. In some experiments, Protein I (0.3 µg) was preincubated for 60 min with 0.8 unit of a partially purified Protein I phosphatase in an effort to remove any endogenous phosphate present on the appropriate amino acid residue; the Protein I phosphatase was then inactivated by treatment at pH 3 for 10 min at 30°C, prior to incubation at pH 7.4 with \(\gamma^{32P}\)ATP and Protein I kinase. When this was done, the maximal amounts of \(^{32P}\)incorporated into Proteins Ia and Ib increased to 0.83 and 0.81 mol, respectively.

In order to determine the nature of the amino acid residues into which radioactive phosphate had been incorporated, \(^{32P}\)Phosphoproteins Ia and Ib were eluted from the gel, subjected to acid hydrolysis, and the hydrolysates analyzed for \(^{32P}\)phosphoserine and \(^{32P}\)phosphothreonine by high voltage paper electrophoresis, as described under "Experimental Procedures." Of the total phosphate incorporated into Proteins Ia and Ib, respectively, 86% and 90% were found to be associated with phosphoserine, and 1% and 0.8% with phosphothreonine, after correction for hydrolysis of authentic phosphoserine and phosphothreonine; 7.4% and 5.1% of the radioactive phosphate was associated with other amino acids or small peptides, and the remainder was recovered as inorganic phosphate.

Fig. 4 (left). Stoichiometry of phosphorylation of Proteins Ia and Ib in purified preparation of Protein I. Bio-Gel P-150 eluate (3.2 Kz of a mixture of Protein Ia and Protein Ib) was incubated for the indicated periods of time under standard assay conditions, and analyzed for the incorporation of \(^{32P}\)phosphate into Proteins Ia (○—○) and Ib (■—■).

Fig. 5 (right). Isoelectric focusing of Protein I. Purified Protein I (6 µg of Bio-Gel P-150 eluate) was subjected to electrophoresis at 4°C in a 5% acrylamide slab gel containing 2% Ampholine (pH 9 to 11) as described under "Experimental Procedures."
Amino Acid and Carbohydrate Analyses

The results of the amino acid analyses of Protein Ia and Protein Ib are shown in Table II. The two proteins have a low content of hydrophobic amino acids. Both of them have a high proportion of proline and glycine. These features of the amino acid composition are reminiscent of collagen which contains even higher proportions of these two amino acids (25).

In one experiment, purified Protein I (160 μg/ml) was subjected to enzymatic hydrolysis for 16 h at 30°C using carboxypeptidase A (3 μg/ml) and pronase (3 μg/ml), and the amounts of glutamine and asparagine in Protein I were estimated by subtracting the amounts of glutamate and aspartate recovered in the enzymatic hydrolysate from the respective amounts of glutamate and aspartate recovered in the acid hydrolysate. Using this procedure, substantial amounts of glutamine and asparagine were found in Protein I: the glutamine/glutamate ratio was calculated to be 5.1:1, and the asparagine/aspartate ratio to be 1.2:1. These high proportions of glutamine and asparagine indicate that the net negative charge of the protein is much less than would be expected from the amino acid composition shown in Table II.

Partial specific volumes of 0.725 and 0.726 ml/g were estimated for Protein Ia and Protein Ib, respectively, from the amino acid composition, using the method of calculation of Cohn and Edsall (26).

In other experiments, when [32P]Phosphoprotein I (the unseparated mixture of [32P]Phosphoproteins Ia and Ib) was subjected to limited hydrolysis by thermolysin, and analyzed for [32P]Phosphopeptides by high voltage paper electrophoresis (pH 1.9 or 6.5) in one dimension, followed by descending chromatography (1-butanol/acetic acid/H2O (3:l:l)) in the second dimension, a single major radioactive peptide was observed. This observation suggests that Protein Ia and Protein Ib may have an identical primary structure in the region of the phosphorylated serine residue.

<table>
<thead>
<tr>
<th>Table II</th>
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<tbody>
<tr>
<td><strong>Amino acid composition</strong></td>
</tr>
<tr>
<td>These values are averages of duplicate determinations.</td>
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<tr>
<td></td>
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<tr>
<td>--------------------------</td>
</tr>
<tr>
<td>Aspartate*</td>
</tr>
<tr>
<td>Threonine*</td>
</tr>
<tr>
<td>Serine*</td>
</tr>
<tr>
<td>Glutamate*</td>
</tr>
<tr>
<td>Proline</td>
</tr>
<tr>
<td>Glycine</td>
</tr>
<tr>
<td>Alanine</td>
</tr>
<tr>
<td>Half-cystine*d</td>
</tr>
<tr>
<td>Valine</td>
</tr>
<tr>
<td>Methionine</td>
</tr>
<tr>
<td>Isoleucine*</td>
</tr>
<tr>
<td>Leucine*</td>
</tr>
<tr>
<td>Tyrosine</td>
</tr>
<tr>
<td>Phenylalanine</td>
</tr>
<tr>
<td>Lysoleic acid</td>
</tr>
<tr>
<td>Histidine</td>
</tr>
<tr>
<td>Arginine</td>
</tr>
<tr>
<td>Trpophan*</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

*Values include any asparagine present prior to hydrolysis.
*Values include any glutamine present prior to hydrolysis.
*Values include any cysteic acid after performic acid oxidation (23).
*Values include any asparagine present prior to hydrolysis.
*Values determined spectrophotometrically (24).

Collagenase Treatment

Collagen contains high proportions of proline and glycine, is composed of two types of subunit in a ratio of 1 to 2, and exists in a triple-stranded helical form (27). The high content of proline and glycine in Proteins Ia and Ib, and the 1:2 ratio of these two peptides, raised the possibility that Proteins Ia and Ib might contain collagen-like sequences. For this reason, we examined the effect of collagenase on Protein I.

When [32P]Phosphoprotein I was incubated with a commercial preparation of highly purified bacterial collagenase, it was degraded to what appeared to be a single, stable radioactive 48,000-dalton peptide through a series of radioactive intermediate peptides with apparent molecular weights of 68,000, 62,000, and 53,000 (Fig. 6).

Results qualitatively similar to those shown in Fig. 6 were obtained when 60 μg of Protein I and 1320 units of commercial collagenase were incubated in a volume of 1.2 ml under the conditions described in the legend to Fig. 6 and the degrada-
tion products visualized by Coomassie blue staining of the gel. The effect of this collagenase preparation on several other proteins was similarly examined by gel electrophoresis using this staining procedure. When 60 µg of soluble skin collagen was incubated under the same conditions, virtually all peptides disappeared within 15 min. In contrast, no proteolysis of 60 µg of ferritin, phosphorylase a, bovine serum albumin, ovalbumin, or chymotrypsinogen was observed when these proteins were incubated under the same conditions for 2 h. The data suggest that both Protein Ia and Protein Ib are composed of a collagenase-resistant peptide fragment (48,000 daltons) and a collagenase-sensitive peptide fragment. It is noteworthy that the phosphorylated serine residues occur in the collagenase-resistant peptide fragments. Consistent with this observation, collagen did not serve as a substrate for cAMP-dependent protein kinase. Certain other proteins have been reported to possess collagen-like properties, including acetylcholinesterase (28) and the Clq component of human complement (29).

**Molecular Weight Determinations**

The molecular weights of Proteins Ia and Ib were determined to be 86,000 and 80,000, respectively, by the standard SDS-polyacrylamide slab gel electrophoresis procedure, as described under "Experimental Procedures," using myoglobin (17,200), chymotrypsinogen (25,700), ovalbumin (45,000), bovine serum albumin (68,000), and phosphorylase a (94,000) as standard marker proteins. However, somewhat higher molecular weight values (90,000 for Protein Ia and 86,000 for Protein Ib) were obtained when other SDS-polyacrylamide gel electrophoresis systems without stacking gel (18, 30) were used. Using the sucrose density gradient centrifugation procedure of Martin and Ames (31) the sedimentation coefficient of Protein Ia and Protein Ib was estimated to be 2.9 S (Fig. 7), corresponding to a molecular weight of 37,000 for a globular protein. This molecular weight value, obtained upon the assumption that Proteins Ia and Ib are globular proteins, is far smaller than the values of 86,000 and 80,000 estimated from SDS-polyacrylamide gel electrophoresis. This discrepancy suggests that Proteins Ia and Ib are highly elongated proteins.

When purified Protein I was subjected to gel filtration chromatography, Protein Ia and Protein Ib were eluted in the same position. The peak position corresponded to a Stokes radius of 59 Å (Fig. 8). This value is far greater than would be expected for a globular protein with a molecular weight of 86,000 or 80,000, again indicating that Proteins Ia and Ib are highly elongated peptides. Using values of 2.9 S for the sedimentation coefficient, of 59 Å for the Stokes radius, and of 0.726 ml/g for the partial specific volume, an approximate molecular weight of 71,000 is calculated for Proteins Ia and Ib, according to the equation

\[
M = \frac{6\eta N a}{1 - \frac{d}{p}}
\]

where \(N\) = Avogadro's number, \(\eta\) = viscosity of water at 20°, \(a\) = Stokes radius, \(s\) = sedimentation coefficient at 20° (\(s_{20w}\)), \(d\) = partial specific volume, and \(p\) = density of water at 20° (32). This molecular weight value is much closer to the molecular weight of monomeric Protein Ia and Protein Ib, determined by SDS-polyacrylamide gel electrophoresis, than to that of complexed Protein Ia and Protein Ib. This indicates that, under the conditions used for sucrose density gradient centrifugation and gel filtration, Protein I is a dispersed mixture of monomeric Protein Ia and Protein Ib, rather than a complex of Protein Ia and Protein Ib. Moreover, the values obtained for \(a\), \(s\), and \(d\) have also been used to calculate the frictional coefficients and axial ratios (32, 33) for Proteins Ia and Ib and the calculations suggest that they are highly elongated molecules.

Sedimentation velocity experiments were carried out at 44,000 rpm at 5°, using an 0.053% solution of purified Protein I (a higher concentration than used in the sucrose density gradient centrifugation and gel filtration studies), in a Beckman model E analytical centrifuge equipped with ultraviolet absorption scanner. The centrifugal patterns indicated the pres-

**Fig. 7.** Sucrose density gradient centrifugation of Protein I. Sucrose density gradient centrifugation of purified Protein I was carried out by the procedure of Martin and Ames (31), in a 4.6 ml volume, using a 5 to 20% linear gradient in 5 mM sodium phosphate buffer (pH 7.0), with an SW 20 rotor at 37,500 rpm for 18.5 h at 4°. A mixture of Protein I (15 µg), bovine serum albumin (25 µg), and myoglobin (25 µg) in a volume of 100 µl of 5 mM sodium phosphate buffer (pH 7.0) was layered onto the gradient. After centrifugation, fractions of 7 drops were collected. One aliquot (60 µl) of each fraction was assayed for Protein I as described under "Experimental Procedures," and a second aliquot (60 µl) was analyzed for marker proteins (bovine serum albumin, 4.3 S; myoglobin, 2.1 S) by SDS-gel electrophoresis and protein staining.

**Fig. 8.** Estimation of Stokes radius of Protein I by gel filtration. A solution (0.48 ml) containing 250 µg each of several standard marker proteins or 120 µg of purified Protein I (Bio-Gel P-150 eluate) in 5 mM sodium phosphate buffer, 0.1 M NaCl, was applied to a column (0.9 x 51.2 cm) of Bio-Gel P-300 which had been equilibrated with 5 mM sodium phosphate, 0.1 M NaCl. The marker proteins were: (1) myoglobin (Stokes radius, 14 Å); (2) bovine serum albumin (Stokes radius, 35 Å); (3) aldolase (Stokes radius, 47 Å); (4) catalase (Stokes radius, 52 Å); and (5) ferritin (Stokes radius, 79 Å). Fractions (0.55 ml) were collected and 0.1 ml aliquots were analyzed for the individual marker proteins and Protein I by SDS-polyacrylamide gel electrophoresis and protein staining, as described under "Experimental Procedures." Proteins Ia and Ib showed identical elution profiles. The data were treated as described by Siegel and Monty (32).
ence of many aggregated species with sedimentation coefficient values ranging widely from approximately 4 to 100 S. When the recovered sample was subjected to gel filtration under the conditions described in the legend to Fig. 8, except for the use of 200 µg rather than 120 µg of Protein I, Protein Ia and Protein Ib were eluted in the void volume. These observations suggest that Protein Ia and Protein Ib tend to aggregate at higher protein concentrations. The tendency to aggregate is not uncommon among membrane-derived proteins.

Absorption Spectrum

Protein I had an absorption maximum at 277 nm and an absorption minimum at 253 nm, with an $A_{277}/A_{253}$ ratio of 1.57. The extinction coefficient of Protein I at 277 nm, $E_{1%}^{	ext{277}}$, was calculated to be 6.74. There was no absorption in the visible wavelength region.

Thermal Stability

Preincubation of Protein I at elevated temperatures reduced its ability to serve as substrate for Protein I kinase. As shown in Fig. 9, Protein Ia and Protein Ib were inactivated to a similar extent by increasing the temperature; partial inactivation was observed at 45° and complete inactivation at 80° under the conditions tested.

Tissue Specificity

In an earlier study (11), the phosphorylation of endogenous Protein I by endogenous Protein I kinase was observed in brain tissue, but not in nonneuronal tissues. It could not be determined from that study whether the absence of demonstrable Protein I phosphorylation in nonneuronal tissues was due to an absence of Protein I itself, or to some other cause, such as, for example, an absence of endogenous Protein I.
In the present study, we have re-examined the tissue specificity of Protein I, by using the extraction and assay methods described under "Experimental Procedures," in which a purified Protein I kinase preparation is used to phosphorylate extracted Protein I. Protein I was present in both cerebrum and cerebellum, but was not detected in spleen, lung, kidney, liver, or heart (Fig. 10), nor in human erythrocytes (data not shown). In other experiments, Protein I was not detected in cells of neural origin devoid of synaptic structure, including nerve axons (bovine lingual nerve) and neuroblastoma (Cl300 or NIE 115) and glioma (C6) cells in culture.

Further evidence for the synaptic localization of Protein I comes from studies of the time course of the appearance of this membrane protein during maturation of the brain. Thus, studies by Lohmann et al.\(^3\) have indicated that there is a correlation between the time course of synaptogenesis and the appearance of Protein I in the brain of the developing rat.

Finally, we have prepared antibodies to purified Protein I for several purposes, including determining its cytochemical localization in nervous tissue. Preliminary experiments\(^4\) using these antibodies verify the localization of Protein I in the synaptic region.

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