Isolation and Characterization of the Nervous System-specific Protein 14-3-2 from Rat Brain

PURIFICATION, SUBUNIT COMPOSITION, AND COMPARISON TO THE BEEF BRAIN PROTEIN

(Received for publication, July 30, 1974)

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SUMMARY

A procedure is described for the isolation of the nervous system-specific protein designated 14-3-2 from rat brain. The methods utilized were salt precipitation, DEAE-cellulose ion exchange chromatography, Sephadex G-150 gel filtration, and column isoelectric focusing. The native 14-3-2 protein has an isolectric point of 4.7 in the absence of denaturing agents and 5.0 in the presence of 2.0 M urea. The protein, as isolated, appears homogeneous since it migrates as a single band on Tris-glycine (pH 8.9), sodium dodecyl sulfate as isolated, appears homogeneous since it migrates as a single band on Tris-glycine (pH 8.9), sodium dodecyl sulfate

polyacrylamide gel electrophoresis in 8 M urea exhibited a molecular weight of 78,000. Sedimentation velocity and equilibrium data indicate a homogeneous component of molecular weight 78,000. Sedimentation of 14-3-2 in 6 M guanidine HCl containing 0.02% glutathione yielded a molecular weight of 39,000, indicating the dimeric nature of the protein as isolated. The rat brain protein seems to be composed of one subunit type, since polyacrylamide gel electrophoresis in 8 M urea yields a single protein component. Sodium dodecyl sulfate gel electrophoresis of rat brain 14-3-2 produced one sharp band with a relative mobility corresponding to a molecular weight of 48,000. Specific anti-14-3-2 serum has been prepared from both New Zealand white rabbits and goats.

Rat 14-3-2 is very similar in amino acid composition to the beef brain protein and to antigen α. The antigenic properties of rat and beef 14-3-2 are also similar, since beef 14-3-2 antiserum reacts well with rat 14-3-2 and vice versa. Electrophoretic mobilities of denatured rat and beef 14-3-2 (0.1% sodium dodecyl sulfate and 8 M urea) are identical. Despite these similarities the two proteins are completely resolved on Tris-glycine gels. The sedimentation behavior of the beef and rat proteins are also different, indicating a difference in the association state and conformation of the two preparations.

The existence of tissue-specific proteins is a predictable consequence of cell differentiation, since it can be expected that cell types performing specific physiological functions would have to possess unique proteins. Recently a number of investigators have reported on the existence and isolation of several nervous system-specific proteins (1-4). Several reviews discussing the localization and properties of these proteins have appeared recently (5, 6). The functions of the nervous system proteins isolated to date remain largely unknown, with the exception of the enzymes required for the biosynthesis and degradation of neurotransmitters and structural proteins such as myelin-specific proteins.

Two especially intriguing proteins that have been isolated from brain are the S-100 and 14-3-2 proteins (1-4). Both are soluble acidic proteins. Several lines of evidence indicate that the S-100 protein is probably localized in glial cells and the 14-3-2 in neurons (7). Both proteins have been implicated as playing a key role in nervous tissue function, since their levels increase dramatically coincident with the functional maturation of the nervous system (8). The S-100 protein from beef brain has been well characterized physically and chemically. The protein is highly acidic, has a molecular weight of 21,000 and is composed of three nonidentical subunits (9, 10). It is affected specifically by calcium, which causes it to undergo a dramatic conformational change giving rise to several stable forms of the protein (11). Functionally the S-100 protein has been implicated in the learning process by virtue of the fact that it increases in quantity during learning and the injection of anti-S-100 serum interferes with the learning process (12). The specific function of S-100 is not as yet known.

The 14-3-2 protein has been isolated from beef brain and a molecular weight of approximately 50,000 reported (13, 14). This protein is also highly acidic, but few data on its physical and chemical properties are available. Bennett and Edelman have isolated a protein from rat brain which they called antigen α (1). This protein is probably similar, if not identical with 14-3-2, since it reacts with beef 14-3-2 antiserum and has a similar electrophoretic mobility (15). The purification procedure described for antigen α yields a mixture of what appears to be aggregation states of the protein, making it difficult to characterize the product physically and chemically.

Upon attempting to prepare 14-3-2 from rat brain we found it necessary to develop a purification scheme that would consistently yield a homogeneous sample of 14-3-2. It was also necessary to obtain the product in reasonable yields, since the amount of tissue available from rats is limited. The following report describes a purification procedure for rat brain 14-3-2 that con-
sistently yields a homogeneous product as determined by elec-
rophoretic and sedimentation results. Physical and chemical
properties of the isolated 14-3-2 are also reported. It is antici-
pated that knowledge of the structure of this protein will facil-
tate the elucidation of its function in the nervous system.

MATERIALS AND METHODS

**Protein Determination**—The concentration of pure 14-3-2 frac-
tions was determined spectrophotometrically after determining
that the E\textsubscript{280} is equal to 0.5. Preliminary identification
of 14-3-2 fractions was accomplished by reaction against beef anti-
14-3-2 serum on Ouchterlony plates. The antiserum and beef
protein were a kind gift from Dr. B. Moore (Washington Uni-
versity School of Medicine, St. Louis, Mo).

**DEAE-cellulose Columns**—The column routinely used was 2.6
X 70 cm. DEAE-52 (Whatman preswollen) was prepared by-
washing in 0.5 N HCl and 0.5 N NaOH according to the procedure
recommended by the manufacturer. The final equilibration
buffer was 10 mM Tris-phosphate, pH 7.5. About 2 to 4 g of pro-
tein were usually applied to each column in a volume of 50 ml or
less. All columns were run at 4°C with a flow rate of 30 ml per
hour. The sample was applied and 3 column volumes of 0.15
M NaCl in 10 mM Tris-phosphate, pH 7.5, with 900 ml of each solution in the re-
spective gradient reservoirs. The column eluent was monitored
at 280 and 260 nm with an LKB Uvicord monitor.

**Sephadex Columns**—Sephadex G-100, superfine (Pharmacia),
was used in columns (1.5 X 100 and 1.5 X 20 cm). The buffer
used was 100 mM Tris-phosphate, pH 7.5.

**Preparation of Antiserum**—Antisera were prepared from both
New Zealand white rabbits and goats. In each case 1 ml of a 1
mg per ml solution of rat brain 14-3-2 in isotonic (NaCl) Tris-
phosphate (10 mM, pH 7.5) was mixed with 1 ml of Freund’s com-
plete adjuvant (Difco) and emulsified. This emulsion was in-
jected intradermally in quantities of 0.5 ml per injection site.

**Extraction**—Rats (Charles River) were injected with
1 ml of 0.5 N NaCl in an evacuated and sealed tube at 110°C for
22 hours. The hydrolysate was analyzed according to the method
of Spackman et al. (22) with a Jeol model 6AH automatic amino
acid analyzer. For the determination of tryptophan, samples were
hydrolyzed with 0.2 ml of 4 M methanesulonic acid containing
0.2% tryphtamine (25).

**RESULTS**

**Rat Brain 14-3-2 Purification**

**Extraction**—Rats (Charles River) were injected with 1 ml of
Nembutal (10 mg per ml) and killed by exsanguination via the ab-
dominor aorta. The whole brain was removed and a 25% homoge-
nate prepared in 10 mM Tris-phosphate, pH 7.5, using a
Teflon-glass homogenizer. One hundred brains were usually
prepared each day (150 g of tissue). After centrifugation of the
homogenate at 12,000 rpm for 10 min at 4°C in a Sorvall RC 2B
centrifuge, the resulting post-mitochondrial supernatant was
centrifuged at 100,000 x g for 1 hour at 4°C. The high speed
supernatant obtained was used for the isolation of the 14-3-2
protein and is referred to as the crude soluble fraction.

**Salt Precipitation**—The crude soluble fraction was brought to
40% (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} (Mann, ultrapure) saturation by slow addition
of the solid. This solution was stirred for 45 min after the
(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} dissolved. The resulting suspension was centrifuged
at 15,000 rpm for 20 min at 4°C, and the supernatant decanted.
The 40% (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} supernatant fraction was brought to 60%
(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} and stirred for 45 min. This solution was centri-
fuged at 15,000 rpm for 20 min. The supernatant was discarded
and the 60% (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} pellets were resuspended in 10 mM
Tris-phosphate, pH 7.5. This solution was then dialyzed against
100 volumes of buffer with one change. The resulting solution
was centrifuged at 15,000 rpm for 20 min and the supernatant
decanted. This solution is referred to as the P-60 fraction.

**DEAE-cellulose Chromatography**—The P-60 fractions from 400
rats (about 600 g of tissue) were pooled and concentrated to 50
ml or less with an Amicon ultrafiltration apparatus using a
PM-10 membrane. This solution was centrifuged at 15,000 rpm
for 15 min. The supernatant usually contained 3 to 4 g of pro-
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Fig. 1. DEAE-cellulose chromatography of rat brain P-60 fraction. Approximately 3 g of the P-60 fraction in 50 ml of Tris-phosphate buffer were applied on a column (2.6 X 70 cm). The flow rate was adjusted to 30 ml per hour. Fraction volume was 8 ml. The 14-3-2-containing fraction was identified using antiserum after each peak was pooled and dialyzed.

Fig. 2. Sephadex G-150 chromatography of the 14-3-2-containing DEAE-fraction. The sample (120 mg) was applied to a column (1.5 X 100 cm) in a volume of 2 ml. The flow rate was adjusted to 2 ml per hour.

Fig. 3. Isoelectric focusing of the 14-3-2-containing Sephadex fraction. Isoelectric focusing was performed in the pH 4 to 6 range at a 2% Ampholine concentration in the 440-ml column. An LKB linear gradient maker was used to prepare the sucrose gradient (5 to 47%). The sample contained 80 A$_{280}$ units of protein in 15 ml of Tris-phosphate buffer (5 mM). The column was drained at a rate of 50 ml per hour into 2-ml fractions.

protein. The above solution was applied on a DEAE-52 column (2.6 X 70 cm) and eluted as described under "Materials and Methods." A flow rate of 30 ml per hour was found to give the best resolution. Fig. 1 illustrates the A$_{280}$ tracing consistently obtained. The peak labeled 14-3-2 was identified initially by its reaction with beef 14-3-2 antiserum. The fractions corresponding to this peak were isolated, pooled, and dialyzed against 50 volumes of double distilled water with three changes. This solution was then lyophilized. This fraction usually contained 100 to 150 mg of protein.

Gel Filtration—The lyophilized DEAE-fraction was resuspended in 2 ml of 10 m Tris-phosphate, pH 7.5, and applied to a column (1.5 X 100 cm) packed with Sephadex G-150 (superfine). The column eluate was passed through the Uvicord monitor (280 nm) with a typical tracing shown in Fig. 2. The second larger peak contained the 14-3-2. The fractions corresponding to this peak were isolated, pooled, and usually contained about 80 mg of protein.

Isoelectric Focusing—One-half of the sample obtained from Sephadex G-150 chromatography was added slowly to the light and heavy Ampholine solutions. Each Ampholine solution contained 2% pH 4 to 6 ampholytes, 2 m urea, and 0.25% β-mercaptoethanol. The heavy solution contained 47% sucrose and the light solution 5% sucrose. Slow addition of the sample to the Ampholine solutions (room temperature) avoided any precipitation. During the first 12 hours of the run a precipitation ring appeared at the top of the gradient. Initially this precipitate was withdrawn (with current off) with a capillary tube connected to a syringe, after 18 hours of running. Additional cathode solution was then layered over the upper electrode to compensate for the lost volume. However, withdrawal of the precipitate was found to be unnecessary, as it remained at the top of the column and did not affect band resolution. The column was run at room temperature with cold (8°) water flowing. After 72 hours the final stabilized potential was 600 to 700 volts at 4 to 5 ma. Fractions collected from the column were monitored by an LKB Uvicord, consistently producing the A$_{280}$ trace which is illustrated in Fig. 3. The isoelectric point of the rat brain 14-3-2 in 2 M urea is 5.1 ± 0.1. The isoelectric point in the absence of urea was lower, with the 14-3-2 peak usually appearing at pH 4.7. Focusing in the absence of urea produced very low yields (1 to 3 mg of 14-3-2) whereas incorporation of 2 M urea apparently increases the solubility of 14-3-2 and raised the yield to 15 to 20 mg per run. The omission of β-mercaptoethanol also decreased the yield to about 5 mg per run.

The fractions corresponding to 14-3-2 were pooled and dialyzed against 100 volumes (three changes) of 10 m Tris-phosphate, pH 7.5. All of the ampholytes were not removed after dialysis, since a very rapidly migrating band was observed on polyacrylamide electrophoresis of the dialyzed focusing fraction. This band was not observed in the sample applied on the electofocusing column. Complete removal of the nondialyzable ampholyte was effected by lyophilizing the above fraction, resuspending it in 1 ml of 10 m Tris-phosphate, pH 7.5, and applying it to a Sephadex G-150 (superfine) column (1.5 X 20 cm). The sharp symmetrical peak obtained at the expected elution volume was pooled and divided into 250-μl aliquots and stored at −20°. Routine yields of pure 14-3-2 ranged from 15 to 20 mg. The preparation was stable for at least 3 months when stored at −20°. Table I summarizes the purification procedure. The value of 0.14% for the amount of 14-3-2 obtained represents the quantity obtained in the purification and
TABLE I

Summary of rat brain 14-3-2 purification scheme

The protein content of each fraction was estimated by assuming that the \( E_{\text{143}}^{\text{cm}} \) was equal to 1.0 for each crude fraction. The \( E_{\text{143}}^{\text{cm}} \) was determined to be 0.5 for the pure 14-3-2 fraction.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume ml</th>
<th>Protein mg</th>
<th>Per Cent Soluble Prot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble Fraction</td>
<td>1200</td>
<td>13,200</td>
<td>100</td>
</tr>
<tr>
<td>40 to 60% (NH(_4))(_2)SO(_4) Fraction</td>
<td>250</td>
<td>3750</td>
<td>28</td>
</tr>
<tr>
<td>DEAE Cellulose Fraction</td>
<td>120</td>
<td>125</td>
<td>0.90</td>
</tr>
<tr>
<td>Sephadex G-150 Fraction</td>
<td>5</td>
<td>100</td>
<td>0.75</td>
</tr>
<tr>
<td>Isoelectric Focusing Fraction, Passed thru G-150</td>
<td>10</td>
<td>20</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Fig. 4. Polyacrylamide gel electrophoresis patterns of various fractions in the 14-3-2 purification procedure. The gels were 7.5% polyacrylamide Tris-glycine at pH 8.9 and were run at 3 mA per tube for 3 hours. The samples from left to right contain: 0.1 mg of crude soluble fraction, 0.1 mg of P-60 fraction, 0.1 mg of DEAE fraction, 10 and 50 \( \mu \)g of the product. The gel containing 50 \( \mu \)g of 14-3-2 was obtained from a separate experiment.

Fig. 5. Sedimentation equilibrium of native rat brain 14-3-2 (\( \ln A \) versus \( r^2 \) plot). Rat brain 14-3-2 (0.3 mg per ml) was centrifuged at 10,000 rpm for 24 hours at 20°. The protein solution contained 10 mM Tris-phosphate, pH 7.5, and 0.5 M NaCl. The term \( \ln A \) refers to the natural log of the absorbance at 280 nm. The molecular weight values ranged from 45,000 at the top of the cell to 80,000 at the bottom. These data indicate that sulfhydryl group-dependent aggregation is occurring, with the monomer and dimer existing in equilibrium.

Molecular Weight—The molecular weight of the native 14-3-2 was determined by sedimentation equilibrium. A molecular weight of 78,000 was observed in Tris-NaCl, pH 7.5. The plot of \( \ln A \) versus \( r^2 \) was a straight line, indicating a homogeneous population of molecules (Fig. 5). Some samples of rat 14-3-2 which were allowed to stand for prolonged periods at room temperature displayed a small degree of polydispersity due to aggregation. The beef brain 14-3-2 was also run under these conditions and yielded a curve plot of \( \ln A \) versus \( r^2 \), indicating a heterogeneous system. Molecular weight values ranged from 45,000 at the top of the cell to 80,000 at the bottom of the cell. Sedimentation velocity experiments indicated a sharp boundary for rat brain 14-3-2 and a broad boundary for the beef protein. The beef protein displayed an S value of 4.8 and the rat protein a value of 5.6.

Subunit Analysis—The rat brain protein was kept in 6 M guanidine hydrochloride containing 0.02% glutathione for 1 hour at 50°. The protein concentration was 0.3 mg per ml. Sedimentation equilibrium was then performed on this sample. The \( \ln A \) versus \( r^2 \) is shown in Fig. 6. A homogeneous component of molecular weight 39,000 was observed indicating that the native protein was probably a dimer. If glutathione was omitted from the sample a heterogeneous population of molecules was observed with molecular weight values ranging from 40,000 at the top of the cell to 80,000 at the bottom. These data indicate that sulphydryl group-dependent aggregation is occurring, with the monomer and dimer existing in equilibrium.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis of rat brain 14-3-2 using lysozyme, chymotrypsin, ovalbumin, and serum albumin as markers yielded a molecular weight of 48,000, as can be seen in Fig. 7. The rat brain and beef brain 14-3-2 were not resolvable on sodium dodecyl sulfate gels, indicating that they had very similar molecular weights in this system.
Fig. 6. Sedimentation equilibrium of denatured rat brain 14-3-2 (ln A vs. r^2 plot). The sample containing 0.3 mg per ml of rat brain 14-3-2, 6 M guanidine hydrochloride, and 0.02% glutathione was centrifuged at 18,000 rpm for 70 hours.

Fig. 7. Molecular weight determination using 0.1% sodium dodecyl sulfate polyacrylamide gels. Five micrograms of rat 14-3-2 and each marker protein were treated electrophoretically for 4 hours at 9 ma per tube. Relative mobilities were determined from gels containing markers and 14-3-2 as well as from gels where each protein was run individually.

One sharp band was always obtained for the rat brain 14-3-2 in this gel system, further attesting to the homogeneity of the preparation. The sodium dodecyl sulfate gel results were not reconcilable with the sedimentation data, which indicated a molecular weight of 39,000. Recent reports have indicated that the TEMED^1 present in sodium dodecyl sulfate gels affects the molecular weight determination of some brain proteins (24). Sodium dodecyl sulfate gel electrophoresis was performed using one-tenth the usual amount of TEMED but the 14-3-2 molecular weight remained at 46,000 to 48,000. The molecular weights obtained are in good agreement with those obtained by Bennett and Edelman (15) for antigen a and beef brain 14-3-2. This apparent molecular weight 10,000 discrepancy is consistent and appears to be due to anomalous behavior of 14-3-2 on sodium dodecyl sulfate gels. This type of behavior has also been observed with other proteins (25-27).

In an effort to determine whether each of the 14-3-2 subunits had the same net charge, the protein was electrophoretically treated in 8 M urea gels. Fig. 8 shows the pattern obtained for both sodium dodecyl sulfate and 8 M urea gel electrophoresis of 14-3-2. Electrophoresis in 8 M urea at three different pH values (pH 3.2, 3.5, and 4.0) all produced one protein band, indicating that the constituent subunits of the dimer had a similar or identical net charge.

Amino Acid Composition

The amino acid composition of both the rat and beef brain 14-3-2 was determined, with the results shown in Table II. The beef and rat proteins are very similar, both containing a high percentage of acidic amino acids. The amino acid mole percent for the rat and beef proteins are also similar to those obtained by Bennett and Edelman for antigen a (1). Since sulphhydril groups appeared to be very important for maintenance of the proper quaternary structure of 14-3-2, it was of interest to determine the total number of cysteine residues present in the protein.

The abbreviation used is: TEMED, N,N',N''-tetramethyl-ethylenediamine; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).
TABLE II

Amino acid composition of rat and beef 14-3-2

Fifty micrograms each of beef and rat 14-3-2 were used for the analysis of tryptophan as well as for the analysis of the remaining amino acids. Data are presented as mole per cent with cysteine being omitted from the calculations.

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Beef</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>6.13</td>
<td>6.70</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.58</td>
<td>1.62</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.90</td>
<td>4.47</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>12.08</td>
<td>11.90</td>
</tr>
<tr>
<td>Threonine</td>
<td>3.85</td>
<td>3.66</td>
</tr>
<tr>
<td>Serine</td>
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<td>4.67</td>
</tr>
<tr>
<td>Glutamine</td>
<td>11.38</td>
<td>11.38</td>
</tr>
<tr>
<td>Proline</td>
<td>4.03</td>
<td>3.86</td>
</tr>
<tr>
<td>Glycine</td>
<td>9.11</td>
<td>9.35</td>
</tr>
<tr>
<td>Alanine</td>
<td>11.38</td>
<td>11.79</td>
</tr>
<tr>
<td>Valine</td>
<td>6.83</td>
<td>6.91</td>
</tr>
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<td>Methionine</td>
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<td>1.02</td>
</tr>
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<td>Isoleucine</td>
<td>5.60</td>
<td>5.60</td>
</tr>
<tr>
<td>Leucine</td>
<td>9.63</td>
<td>9.96</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.45</td>
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</tr>
<tr>
<td>Phenylalanine</td>
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</tr>
<tr>
<td>Tryptophan</td>
<td>1.75</td>
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</tr>
</tbody>
</table>

molecule. This was done using a spectrophotometric assay employing DTNB. The results indicated that there are 6 cysteine residues per 78,000 molecular weight or 3 residues per subunit. The cysteine determination was also done under non-denaturing conditions to determine the number of exposed sulfhydryl groups in the native conformation. DTNB did not react with native 14-3-2 in the first 60 min of the assay, indicating that little or none of the component sulfhydryl groups were exposed under these conditions.

Rat and beef brain 14-3-2 were similar in several of their properties, such as amino acid composition, antibody cross-reactivity, and mobility in sodium dodecyl sulfate polyacrylamide gels. In order to obtain further information on the degree of similarity between the beef and rat proteins their mobilities were compared on 7.5% polyacrylamide Tris-glycine gels at pH 9.0. Fig. 9 shows the results obtained from such an experiment. It can be seen that the beef protein has a greater electrophoretic mobility than that of the rat. Furthermore, when coelectrophoretically treated both proteins are completely resolved from one another, indicating that they differ significantly in net charge.

Antiserum Preparation

Antiserum to rat brain 14-3-2 were prepared from both rabbits and goats. Two injections of 1 mg of protein each were sufficient to elicit antibody formation. The ability of goats to produce a precipitating antibody for 14-3-2 is fortunate, as large volumes of serum and hence large quantities of antibody can be obtained. Fig. 10 shows an Ouchterlony double diffusion plate obtained with the rabbit antisera in the center well. It can be seen that only one precipitation arc is seen with both pure 14-3-2 (Wells 1 and 2) and the P-60 fraction (Wells 3 to 6). These patterns indicate that the antisera are specific in their reaction with 14-3-2.

Other preliminary data were obtained concerning the physical and chemical properties of rat brain 14-3-2. The CD spectrum indicates that the protein contains 29% helix. Although Ca²⁺
has been shown to exert profound effects on the conformation of the S-100 protein (1) no effect was seen on the electrophoretic mobility or the CD spectrum of the 14-3-2 protein. The cation was also without significant effect on the number of exposed sulfhydryl groups as determined by the DTNB method. Collectively these findings suggest that Ca²⁺ does not significantly affect the conformation of rat brain 14-3-2. The periodic acid-Schiff staining method (28) (gel electrophoresis) was also performed with negative results, indicating that 14-3-2 contained no carbohydrate moieties. A phosphoprotein-staining procedure was also employed (29), which also gave negative results indicating the absence of phosphorus in 14-3-2.

**DISCUSSION**

A purification procedure has been devised for rat brain 14-3-2 utilizing column isoelectric focusing as the final step. The procedure consistently yields a homogeneous product as judged by electrophoresis on three different gel systems (Figs. 4, 6, and 16) as well as its behavior in sedimentation experiments (Figs. 5 and 16). The procedure employed by us is different than that used by Bennett and Edelman to purify antigen α. In their procedure a pH 5.0 precipitation step was used to precipitate protein other than 14-3-2. In our system treatment of any 14-3-2 fraction in that manner led to a great loss of the protein. All washed pH residues were not accessible to DTNB. It is possible that disulfide bridges contribute to the subunit interaction and are thus buried in the center of the molecule. This idea is supported by the fact that dimers only appeared in the 6 M guanidine HCl sedimentation equilibrium experiments when a sulfhydryl-reducing agent was omitted, indicating that dimer formation was probably to some degree disulfide-dependent.

The brain 14-3-2 was homogeneous during sedimentation in a Tris-NaCl system, displaying a molecular weight of 78,000 while the antigen preparation contained two components of molecular weight 45,000 and 72,000. The antigen α preparation also exists as a single component of molecular weight 60,500 in 6.3 M guanidine, while we observed a heterogeneous mixture of species under these conditions. The species observed probably represent a mixture of monomers and dimers. Both preparations yield a single species of 39,000 molecular weight when sedimented in 6 M guanidine under sulfhydryl-reducing conditions, indicating that both are probably composed of the same basic unit.

The 14-3-2 protein as isolated behaves as a stable dimer with little tendency to aggregate into high molecular weight species. Sephadex G-150 chromatography of the pure preparation always produced a single symmetrical peak, further indicating the lack of any significant aggregation. The sedimentation data strongly support the idea that the protein is a dimer because the denatured 14-3-2 molecular weight is a multiple of the native molecular weight. However, the molecular weight of 48,000 obtained on sodium dodecyl sulfate polyacrylamide gels is difficult to explain as it does not fit the sedimentation data. It does not appear that the TEMED present in the gels is affecting the molecular weight determination, as the same results are obtained when one-tenth the usual amount of TEMED is used. Similar molecular weight results were obtained by Bennett and Edelman with the rat antigen α and beef 14-3-2 (15). The possibility exists that 14-3-2 behaves differently in sodium dodecyl sulfate than other proteins, which would account for the decreased mobility of the protein on sodium dodecyl sulfate gels (25-27).

The results obtained in the 8 M urea polyacrylamide gel experiments support the idea that rat brain 14-3-2 is composed of one monomer type. This gel system discriminates denatured proteins according to both size and charge, contrary to sodium dodecyl sulfate electrophoresis which imparts a negative charge to all proteins and discriminates solely on the basis of size. Taken in conjunction with the sedimentation data, it appears that rat brain 14-3-2 is a dimer consisting of identical subunits.

14-3-2 is an excellent antigen in both rabbits and goats. A quantity of 2 mg given in 1-mg injections one month apart is sufficient to produce high antibody titres in both the above animals. The results were identical both with and without the incorporation of methylated serum albumin in the samples used for injection. A precipitation arc was observed on Ouchterlony plates with a 0.50 dilution of the rabbit antiserum and a 1:10 dilution of the goat serum. Higher titres were obtained with subsequent booster injections in the goat. Since 14-3-2 antibodies can be produced in goats, large quantities of blood (500 ml) can be obtained, which makes it feasible to prepare the purified 14-3-2 antibody. Immunological evidence (Fig. 10) supports the contention that rat brain 14-3-2 was isolated in pure form because only one precipitin arc was seen on Ouchterlony plates when 14-3-2 antiserum was reacted with a crude P-60 fraction.

Sulfhydryl group analysis indicated that the protein contained 6 cysteine residues per dimer or 3 residues per monomer unit. The native protein had no exposed sulfhydryl groups, suggesting that the conformation of the molecule was such that the cysteine residues were not accessible to DTNB. It is possible that disulfide bridges contribute to the subunit interaction and are thus buried in the center of the molecule. This idea is supported by the fact that dimers only appeared in the 6 M guanidine HCl sedimentation equilibrium experiments when a sulfhydryl-reducing agent was omitted, indicating that dimer formation was probably to some degree disulfide-dependent.

The rat brain and beef 14-3-2 are very similar, but apparently not identical. The antiserum prepared against beef 14-3-2 cross-reacts very well with rat 14-3-2 and vice versa, indicating that the antigenic determinant for the two proteins is probably very similar. The amino acid compositions of beef and rat 14-3-2 are also remarkably similar (Table II). Moreover, the mobility of 14-3-2 from these two sources on sodium dodecyl sulfate gels and 8 M urea gels was identical. Cœlectrophoresis of the two denatured proteins on these gel systems produced one band. These results support the idea that the monomer unit of both beef and rat 14-3-2 is structurally very similar. However, when the undenatured beef and rat protein are compared on Tris-glycine gels (Fig. 6) complete resolution of the proteins is achieved. This clearly indicates that beef and rat 14-3-2 have significantly different net charges under these conditions. The probable explanation is that one or several amino acids differ between the two proteins. This difference is probably reflected in a conformational alteration which leads to a difference in the exposed charged groups on each molecule. The hypothesis of a conformational difference is also supported by sedimentation data which showed that the beef brain protein had an S value of 4.8 while the rat brain protein had an S value of 5.6. This large difference in S value between rat and beef 14-3-2 may be misleading, since the beef protein yielded a broad interface during sedimentation velocity runs. It should be mentioned that direct comparisons between beef and rat 14-3-2 should be made with caution because it appears that the 14-3-2 protein from beef has a greater tendency toward nonspecific aggregation than that obtained from the rat. The difference observed in sedimentation and electrophoretic patterns may be caused in part by these differences.

The remarkable similarity between beef and rat 14-3-2 that has been demonstrated indicates that evolution has conserved this protein to a high degree, since these two species are phylo-
genetically removed from one another. This suggests that the specific function of this protein is ubiquitous and is essential to the survival of the organism. In attempting to determine the function of the brain specific protein designated 14-3-2 we have adopted the tactic of initially studying its chemical and physical properties in the anticipation that this would yield information contributing to this goal. Other ongoing studies in our laboratory attempting to characterize brain specific proteins functionally include investigation of factors regulating the in vitro synthesis of 14-3-2 and S-100 (30), as well as the axoplasmic transport of 14-3-2. It is our hope that such a broad based attack on the problem of brain specific protein function will be successful and lead to a better understanding of brain function at the molecular level.

Acknowledgments—We express sincere appreciation to Dr. Chunyen Lai and Dr. Miloslav Boublik of The Roche Institute of Molecular Biology for performing the amino acid analysis and CD spectra, respectively.

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