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DARPP-32, A DOPAMINE- AND ADENOSINE 3′:5′-MONOPHOSPHATE-REGULATED PHOSPHOPROTEIN ENRICHED IN DOPAMINE-INNERVATED BRAIN REGIONS

II. Purification and Characterization of the Phosphoprotein from Bovine Caudate Nucleus[1]

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Abstract

DARPP-32 is a neuronal phosphoprotein of M_r = 32,000, originally identified in rat brain (Walaas, S. I., D. W. Aswad, and P. Greengard (1983) Nature *301:* 69–72). This protein has now been identified in bovine caudate nucleus cytosol and purified 435-fold to apparent homogeneity as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purification procedure involved acid extraction at pH 2, CM-cellulose chromatography, DEAE-cellulose chromatography, hydroxylapatite chromatography, and gel filtration on Ultrogel AcA 44. The purified catalytic subunit of cAMP-dependent protein kinase catalyzed the incorporation of 0.96 mol of phosphate/mol of purified DARPP-32. Phosphorylation occurred exclusively on threonine. The isoelectric point of dephospho-DARPP-32 was 4.7, and that of phospho-DARPP-32 was 4.6. The amino acid composition showed a high content of glutamate/glutamine and proline, and a low content of hydrophobic amino acids. DARPP-32 was found to have a Stokes radius of 34 Å and a sedimentation coefficient of 2.05 S, indicating that it exists as an elongated monomer.

Experimental evidence has accumulated implicating adenosine 3′:5′-monophosphate (cAMP) as a second messenger in mediating the physiological actions of several neurotransmitters, including dopamine (for reviews, see Iversen, 1975; Greengard, 1978a, 1981). Dopamine acting at D-1 receptors (dopamine receptors coupled to adenylate cyclase (Kebabian et al., 1972; Kebabian and Calne, 1979)) activates adenylate cyclase and increases intracellular cAMP levels, thereby stimulating cAMP-dependent protein kinase and increasing the state of phosphorylation of specific endogenous substrate proteins (Greengard, 1978b). The preceding paper of this series (Walaas and Greengard, 1984) reported the presence of a cytosolic phosphoprotein of M_r = 32,000 (DARPP-32, *d*opamine- and c*A*MP-*r*egulated *p*hospho*p*rotein of M_r = *32,*000) in rat brain. Dopamine or 8-bromo-cAMP was found to stimulate the phosphorylation of DARPP-32 in slices of rat caudatoputamen (Walaas et al., 1983a; Walaas and Greengard, 1984), demonstrating a sequential link between dopamine, cAMP, and the phosphorylation of a specific substrate protein. In addition, the regional distribution of DARPP-32 in rat brain was found to be very similar to the distribution of dopamine-containing nerve terminals (Walaas and Greengard, 1984; see also Ouimet et al., 1984), for instance, being highest in the neostriatum. The results of those studies suggest that DARPP-32 may be involved in mediating certain of the actions of dopamine acting at D-1 receptors on dopaminoceptive neurons. In this paper we report the presence of DARPP-32 in bovine caudate nucleus, its purification to apparent homogeneity, and the biochemical characterization of this purified DARPP-32.

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Materials and Methods

Materials. The catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart as described (Kaczmarek et al., 1980) and was stored at 4°C in 150 mM potassium phosphate (pH 7.0). [γ-^{32}P]ATP (5 to 10 × 10^7 cpm/nmol) was prepared by the method of Glynn and Chappell (1964) from dipotassium ATP (Sigma Chemical Co.) and carrier-free [^{32}P]orthophosphate (New England Nuclear). L-*O*-Phosphotyrosine was a gift from Dr. Efraim Racker. All chemicals were of reagent grade. As starting material for the purification of DARPP-32, fresh brains from 1- to 3-week-old calves were obtained from a local slaughterhouse and transported on ice to the laboratory. The caudate nuclei were removed within 1 hr after death and were stored at −70°C until use (up to 6 months).

Buffers. All buffer pH values refer to measurements made at 4°C. Buffer A consisted of 10 mM potassium phosphate (pH 7.4), 2 mM EDTA, 15 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma), and 0.1% (v/v) isopropanol (necessary as a solvent for PMSF). Buffer B consisted of 12.5 mM sodium acetate (pH 4.0), 2 mM EDTA, 15 mM 2-mercaptoethanol, 0.1 mM PMSF, 0.1% (v/v) isopropanol, 2 μg/ml pepstatin A (CalMed) and 0.1% (v/v) dimethylsulfoxide (necessary as a solvent for pepstatin A). Buffer C consisted of 10 mM potassium phosphate (pH 7.4), 2 mM EDTA, 200 mM NaCl, 15 mM 2-mercaptoethanol, 0.1 mM PMSF, 0.1% (v/v) isopropanol, and 0.02% sodium azide. Buffer D consisted of 50 mM Tris-HCl (pH 7.6), 0.2 mM EDTA, 50 mM NaCl, and 1.5 mM 2-mercaptoethanol. Buffer E consisted of 10 mM Tris-HCl (pH 7.6), 2 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, and 0.1% (v/v) isopropanol.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and autoradiography. SDS-slab gel electrophoresis was carried out in 10% polyacrylamide/0.27% methylene bisacrylamide in the presence of 2-mercaptoethanol, according to the method of Laemmli (1970). Gels were stained with Coomassie blue R250 (0.1%) in 50% methanol/10% acetic acid and destained in 10% isopropanol/10% acetic acid. Apparent molecular weights were determined with the following standards (all from Sigma): rabbit muscle phosphorylase *b* (94,000), bovine serum albumin (68,000), hen egg ovalbumin (43,000), bovine erythrocyte carbonic anhydrase B (30,000), soybean trypsin inhibitor (20,000), and bovine milk α-lactalbumin (14,400). Gels were dried onto filter paper *in vacuo* and autoradiography was performed with Kodak X-Omat AR film at room temperature or at −70°C with a DuPont Cronex Lightning Plus intensifying screen (Swanstrom and Shank, 1978).

Comparison of endogenous phosphorylation of DARPP-32 in crude cytosol from rat and bovine brain. For studies of endogenous phosphorylation, fresh bovine caudate nuclei were dissected at a local slaughterhouse and transported to the laboratory in ice-cold 0.32 M sucrose. Male Sprague-Dawley rats (150 to 200 gm body weight) were stunned and decapitated, their brains were removed, and the caudatoputamen was dissected and placed in ice-cold 0.32 M sucrose. Samples from each tissue were homogenized in 10 vol (ml/gm) of ice-cold Buffer E with a Teflon-glass homogenizer using 20 up-and-down strokes at 2,100 rpm. The homogenate was centrifuged at 27,000 × *g* for 30 min. The supernatant was decanted and then centrifuged in a Beckman L3-50 Ultracentrifuge using an SW-50.1 rotor at 100,000 × *g* for 1 hr at 4°C. The 100,000 × *g* supernatant was dialyzed against Buffer E for 2 hr to remove endogenous cyclic nucleotides. A 20-μl sample, containing approximately 75 μg of cytosolic protein, was phosphorylated in a reaction mixture (final volume, 0.1 ml) containing 50 mM HEPES (pH 7.4), 1 mM EGTA, 10 mM magnesium acetate, 1 mM 3-isobutyl-1-methylxanthine (IBMX; Aldrich) and 5 μM [γ-^{32}P]ATP, in the absence or presence of 1 μM cAMP. The reaction was carried out at 30°C for the times indicated and was terminated by the addition of "SDS-stop solution" (see below). Following heating at 90 to 100°C for 2 min, the samples were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography.

Protein bands containing DARPP-32 were excised from dried SDS-polyacrylamide gels and subjected to one-dimensional peptide mapping by limited proteolysis (Cleveland et al., 1977) as described (Huttner and Greengard, 1979), using 50 μl of 0.02 mg/ml of *Staphylococcus aureus* V8 protease (Miles Biochemicals). The proteolytic peptides were separated by electrophoresis on an SDS-polyacrylamide slab gel consisting of a 3% stacking gel and a 15% resolving gel and visualized by autoradiography of the dried gel.

Standard phosphorylation assay for DARPP-32. To monitor purification, DARPP-32 was assayed by its ability to accept the γ-phosphate from [γ-^{32}P]ATP in a reaction catalyzed by the purified catalytic subunit of cAMP-dependent protein kinase. The reaction was carried out at 30°C in a reaction mixture (final volume, 0.1 ml) containing 50 mM HEPES (pH 7.4), 1 mM EGTA, 10 mM magnesium acetate, 3 to 6 μg/ml of the catalytic subunit of cAMP-dependent protein kinase, and 50 μM [γ-^{32}P]ATP (200 to 1,000 cpm/pmol). When necessary, samples were adjusted to between pH 7 and pH 8 with Tris base before adding them to the reaction mixture. Following a 2-min preincubation, the reaction was initiated by the addition of the [γ-^{32}P]ATP and was terminated after 60 min by the addition of 25 μl of a five times concentrated "SDS-stop solution" (Laemmli, 1970) to yield the following final concentrations: 50 mM Tris-HCl (pH 7.4), 10% sucrose, 2% (v/v) 2-mercaptoethanol, 1% SDS, with 0.04 mg/ml of pyronin Y as a marker. The samples were heated at 90 to 100°C for 2 min and then subjected to SDS-polyacrylamide gel electrophoresis and autoradiography, as described above. The phosphorylated bands corresponding to the M_r = 32,000 phosphoprotein were cut out, and the amount of ^{32}P incorporated was measured by liquid scintillation spectrometry in 3 ml of scintillation fluid (Liquiscint, National Diagnostics). The "substrate activity" of DARPP-32 is defined as the picomoles of [^{32}P]phosphate incorporated into the M_r = 32,000 band in the above assay.

Radioimmunoassay of DARPP-32. Since the standard phosphorylation assay was judged unreliable for quantitation of substrate activity in crude cytosol preparations due to endogenous protein kinase and phosphoprotein phosphatase activities, a non-equilibrium radioimmunoassay was developed to quantitate the amount of

DARPP-32 at various stages of purification (H. C. Hemmings, Jr., and P. Greengard, manuscript in preparation). For this purpose, rabbit antiserum was prepared against purified bovine DARPP-32, and purified bovine DARPP-32 was radiolabeled with ^{125}I by the chloramine T method (Greenwood et al., 1963). This method gave results in good agreement with those of the standard phosphorylation assay following purification step 2 (pH 2/6 extract).

Time course and stoichiometry of phosphorylation of DARPP-32. Assays were performed essentially as described by Aswad and Greengard (1981b), except that the reaction mixture contained 50 mM HEPES (pH 7.4), 1 mM EGTA, 10 mM magnesium acetate, 2 μM DARPP-32, 6 μg/ml of the catalytic subunit of cAMP-dependent protein kinase, and 200 μM [γ-^{32}P]ATP (200 cpm/pmol).

Identification of the amino acid phosphorylated in DARPP-32. Stained and dried gel slices containing [^{32}P] DARPP-32 were washed with four changes of 50% (v/v) methanol to remove residual SDS, and lyophilized. The dried gel pieces were swollen in 1 ml of 50 mM ammonium bicarbonate containing 150 μg of TPCK-trypsin (Worthington) and incubated at 37°C for 24 hr. The supernatant, which contained greater than 90% of the original radioactivity, was removed, and the gel slice was washed with 0.5 ml of 50 mM ammonium bicarbonate (pH 8.0) for 4 hr at 37°C. The second supernatant was removed, combined with the first, and lyophilized. The lyophilized tryptic digests were resuspended in 200 μl of 50 mM ammonium bicarbonate (pH 8.0), and an aliquot was subjected to partial acid hydrolysis in 250 μl of 6 N HCl at 100°C for 2 hr *in vacuo* (Bylund and Huang, 1976). The hydrolysate was lyophilized to remove HCl, resuspended in 10 μl of 8.7% acetic acid/2.5% formic acid (pH 1.9) buffer, containing 1 mg/ml each of DL-*O*-phosphoserine (Sigma), DL-*O*-phosphothreonine (Sigma), and L-*O*-phosphotyrosine standards and a trace of phenol red, spotted 4 cm from the end of a thin layer cellulose sheet (type 13254, Eastman), and electrophoresed in the same pH 1.9 buffer at 500 V for 4 hr. The cellulose sheet was dried and subjected to electrophoresis at 500 V in a second dimension at pH 3.5 in 10% acetic acid/1% pyridine (Hunter and Sefton, 1980) until the phenol red had migrated 9 cm. The cellulose sheet was dried, stained with 1% ninhydrin in acetone to identify the internal phospho-amino acid markers, and subjected to autoradiography to identify the labeled phospho-amino acids.

Amino acid analysis. Four aliquots, each containing 25 μg of purified DARPP-32 that had been dialyzed against 2.5 mM potassium phosphate (pH 7.4), were lyophilized and then hydrolyzed in 6 N HCl, 0.2% phenol, for 20, 48, 72, and 115 hr at 110°C *in vacuo*. The hydrolysates were analyzed on a Durrum 500 amino acid analyzer by Gary Davis of the Yale Protein Chemistry Facility. Values reported are the average of four time points, except for valine and isoleucine which are the maximum values, and serine and threonine which are extrapolated back to zero time. For determination of cysteine, three aliquots, each containing 1.5 μg of purified DARPP-32 which had been dialyzed extensively against 10 mM potassium phosphate (pH 7.4), were lyophilized and subjected to performic acid oxidation (Moore, 1963) followed by amino acid analysis. A blank, containing DARPP-32 treated with formic acid only, was subtracted. Tryptophan was determined by hydrolysis of two 25-μg aliquots of purified DARPP-32 in mercaptoethanesulfonic acid (Penke et al., 1974), or spectrophotometrically (Goodwin and Morton, 1946) at 280 nm using a 1.17-mg/ml (based on amino acid analysis) solution of purified DARPP-32. The partial specific volume was calculated from the amino acid composition according to the method of Cohn and Edsall (1943).

Isoelectric focusing in polyacrylamide gels. Isoelectric focusing followed the procedure of Righetti and Drysdale (1974) with the following modifications: the concentrations of acrylamide and methylene bisacrylamide were 4% and 0.11%, respectively, and gels contained 5% sucrose and 1.6% ampholytes (final concentration). The ampholyte mixture contained 2 vol of the pH 3.5 to pH 5 solution, 2 vol of the pH 5 to pH 7 solution, and 1 vol of the pH 4 to pH 6 solution (all from LKB). Gels were 4 mm in diameter and 110 mm in length. The anolyte (bottom) was 10 mM H_3PO_4 and the catholyte (top) was 20 mM NaOH (degassed). Samples (50 μl) were applied to the top of gels in 10% sucrose/1% pH 3 to pH 10 ampholytes and overlayed with 50 μl of 1% pH 3 to pH 10 ampholytes to prevent direct contact of the sample with the catholyte. Focusing was carried out at 4°C at constant 500 V for 12 to 16 hr. Gels were fixed overnight in a solution made up to 9 gm of sulfosalicylic acid, 30 gm of trichloroacetic acid, and 180 ml of water. The gels were stained by procedure A of Vesterberg et al. (1977) and then cut into 2-mm slices, and radioactivity was measured by liquid scintillation spectrometry. For pH gradient determination, gels run in parallel were cut into 2-mm slices and incubated with 1 ml of 10 mM NaCl for 1 hr at room temperature, and the pH value was determined at 4°C.

Determination of Stokes radius by gel filtration. Gel filtration was carried out by the method of Whittaker (1963) in a column (2.5 × 93 cm) of Ultrogel AcA 44 (LKB) previously equilibrated with Buffer C. The column was calibrated with the following standards (all from Sigma): bovine serum albumin (35.5 Å), hen egg ovalbumin (27.6 Å), bovine pancreas α-chymotrypsinogen (20.9 Å), and bovine pancreas ribonuclease A (16.4 Å). Determination of the Stokes radius of DARPP-32 was carried out according to the method of Laurent and Killander (1964).

Determination of sedimentation coefficient by linear sucrose density gradient ultracentrifugation. Linear sucrose density gradient ultracentrifugation was carried out at 50,000 rpm for 40 hr at 4°C in a Beckman L3-50 Ultracentrifuge using an SW 50.1 rotor. The 5-ml linear sucrose gradients (5 to 20%) were made in Buffer D. The standard proteins (all from Sigma) were hen egg ovalbumin (3.55 S), bovine erythrocyte carbonic anhydrase B (2.85 S), and bovine heart cytochrome *c* (1.9 S). After centrifugation, fractions were collected from the bottom of the tubes and analyzed by SDS-polyacrylamide gel electrophoresis and protein staining, and the sedimentation coefficient was determined by the method of Martin and Ames (1961).

Protein determinations. Protein was determined by the method of Lowry et al. (1951), following precipitation with cold trichloroacetic acid, using polyadenylic acid

(Sigma) as a carrier. Spectrophotometrically standardized bovine serum albumin (Sigma, fraction V powder) was used as a standard.

Results

DARPP-32 in crude cytosol from bovine caudate nucleus

Identification of DARPP-32 in bovine caudate nucleus. DARPP-32 was originally identified in rat brain, where its phosphorylation was found to be regulated in slices of caudatoputamen by dopamine and by 8-bromo-cAMP (Walaas et al., 1983a). In the present study, bovine caudate nucleus was investigated as a possible source of starting material for the large-scale purification of DARPP-32.

When endogenous phosphorylation was studied in a crude cytosol fraction from rat caudatoputamen (Fig. 1*A*), the addition of cAMP was found to stimulate the phosphorylation of two phosphoprotein bands in the M_r = 32,000 to 35,000 range, the lower of which (Fig. 1*A*, *upper arrow*) was DARPP-32 (cf. Walaas et al., 1983a; Walaas and Greengard, 1984). When endogenous phosphorylation was studied in a crude cytosol fraction from bovine caudate nucleus, two phosphoprotein bands were observed in the same molecular weight range, but with slightly lower molecular weight values than for the corresponding bands from rat caudatoputamen. We conclude that the lower band seen in bovine cytosol (Fig. 1*A*, *lower arrow*) is bovine DARPP-32, since it was found to be homologous to rat DARPP-32 by several criteria, including acid solubility, peptide mapping, and immunoreactivity. Thus, the lower band (DARPP-32), but not the upper band, of each doublet was soluble at pH 2 (data not shown). In addition, DARPP-32 from rat and calf brain yielded similar one-dimensional peptide maps upon limited proteolysis with *S. aureus* V8 protease (Fig. 1*B*). Based upon these experiments demonstrating the similarity of bovine and rat DARPP-32, bovine caudate nucleus was chosen as the starting material for the purification of DARPP-32. Finally, subsequent experiments showed that antibody prepared against DARPP-32 purified from bovine caudate nucleus immunoprecipitated phosphorylated DARPP-32 from rat caudatoputamen, and that rat DARPP-32 cross-reacted with bovine DARPP-32 in a radioimmunoassay (H. C. Hemmings, Jr., and P. Greengard, manuscript in preparation).

Time course of phosphorylation of DARPP-32 in crude cytosol. In crude cytosol from fresh bovine caudate nucleus, DARPP-32 was found to be one of the most rapidly phosphorylated proteins in the presence of 1 μM cAMP/1 mM IBMX. Under the conditions tested, ^{32}P incorporation into DARPP-32 was half-maximal by 10 sec (Fig. 2).

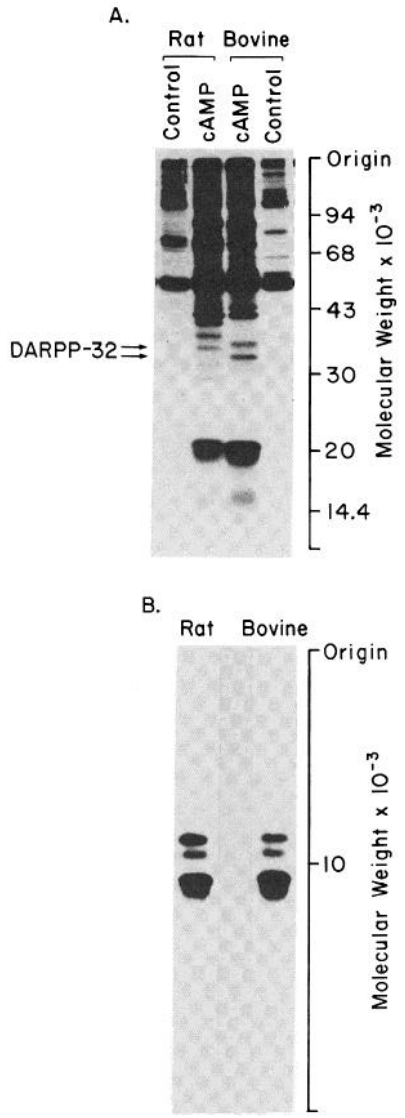

Figure 1. A, Endogenous phosphorylation in crude cytosol from rat caudatoputamen (*lanes 1* and *2*) and bovine caudate nucleus (*lanes 3* and *4*). Rat samples represent 56 μg of protein, and bovine samples represent 62 μg of protein. Phosphorylation was carried out for 60 sec with no cAMP (control) or 1 μM cAMP as indicated, and the samples were analyzed on a 10% SDS-polyacrylamide gel. The autoradiogram shows increased phosphorylation of a doublet in the M_r = 32,000 to 35,000 range with cAMP in both species, with the doublet having slightly higher relative molecular weight values in the rat. The *lower band* of each doublet, corresponding to DARPP-32, is indicated by an *arrow*. *B*, Phosphopeptide pattern obtained upon digestion with 1 μg of *S. aureus* V8 protease of the DARPP-32 bands indicated by the *arrows* in *A*. [^{32}P]Phosphopeptides were separated on a 15% SDS-polyacrylamide gel and visualized by autoradiography.

Purification of bovine DARPP-32

All purification steps were performed at 0 to 4°C. Centrifugations were carried out in a Sorvall RC 2-B centrifuge, using a GSA rotor.

Step 1: Homogenization. Frozen bovine caudate nuclei (510 gm) were thawed in 4 ml/gm of room temperature Buffer A and homogenized for 2 min using a model STD Tissumizer (Tekmar Co.) fitted with a model 182E shaft and generator. The homogenate was centrifuged at 16,000 × *g* for 30 min and the supernatant was decanted. The pellet was then rehomogenized in 2 vol of ice-cold Buffer A for 2 min and centrifuged at 16,000 × *g* for 30

min, and the supernatant was decanted and combined with the first supernatant.

Step 2: Acid extraction. The combined supernatants (final pH 7.0) were brought to pH 2 by the slow addition of 2 N H_2SO_4 with stirring. A flocculent precipitate began to appear at pH 5 which turned brown at pH 3. The brown precipitate was removed by centrifugation at 16,000 × *g* for 20 min. The resulting clear amber pH 2 supernatant was then brought to pH 6 by the slow addition of 2 N NH_4OH with stirring. The white precipitate which formed was removed by centrifugation at 16,000 × *g* for 20 min.

Step 3: CM-cellulose chromatography. The amber pH 6 supernatant was made 12.5 mM in sodium acetate by the addition of 5 M sodium acetate (pH 4.0), and 2 μg/ml in pepstatin A by the addition of a 2-mg/ml stock solution in dimethyl sulfoxide. The pH was then adjusted to 4.0 with 2 N H_2SO_4. At this stage in the purification, DARPP-32 was found to be soluble at pH 4.0, but precipitation occurred at later stages when DARPP-32 was more concentrated. It was therefore important to carry out this step early in the purification when DARPP-32 was in a crude and less concentrated state. CM-cellulose (125 gm) (CM-52, Whatman) previously equilibrated with Buffer B was added and the resulting slurry was stirred for 1 hr. The CM-cellulose was recovered by filtration on hardened filter paper (Whatman 54) and was washed three times with 200 ml of Buffer B. The light brown resin was suspended in an equal volume of Buffer B, poured into a column 5 cm in diameter, and washed with an additional 200 ml of Buffer B. The column (5 × 14 cm) was eluted at a flow rate of 2 ml/min with a 1-liter linear gradient of 0 to 0.5 M NaCl in Buffer B. DARPP-32 eluted as a single broad peak at a conductivity of 7.2 millimho (Fig. 3). The fractions containing DARPP-32 were pooled and dialyzed overnight against two changes of 4 liters of Buffer A.

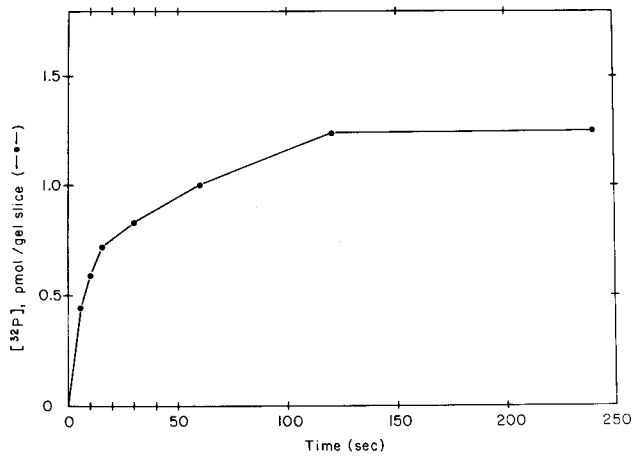

Figure 2. Quantitation of cAMP-dependent phosphorylation of DARPP-32 in crude cytosol from bovine caudate, as a function of incubation time. Crude caudate cytosol was subjected to endogenous phosphorylation in the presence of 1 μM cAMP for various periods of time as described under "Materials and Methods." Following SDS-polyacrylamide gel electrophoresis in a 10% gel, bands containing DARPP-32 were excised. Data are expressed as picomoles per gel slice determined by liquid scintillation spectrometry of bands containing DARPP-32.

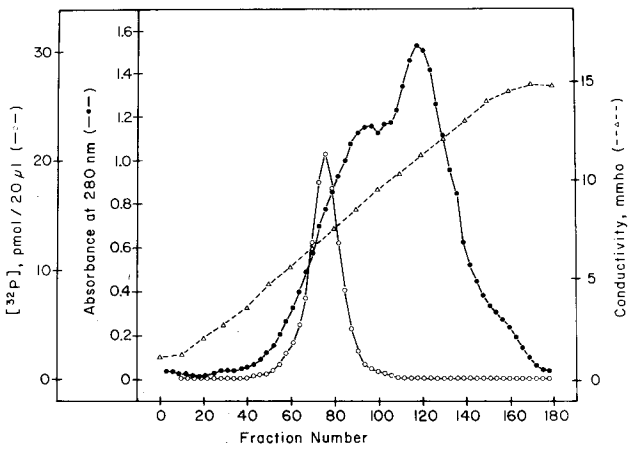

Figure 3. Chromatography of pH 2 soluble proteins on CM-cellulose at pH 4.0 (step 3). The flow rate was 2 ml/min and 5.5-ml fractions were collected. Aliquots (20 μl) of every third fraction were assayed for DARPP-32 (O—O) by the standard phosphorylation assay.

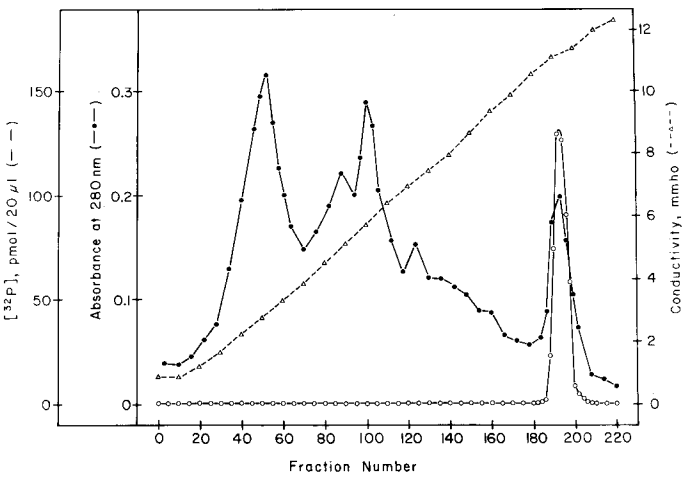

Figure 4. Chromatography of step 3 pool on DEAE-Sephacel at pH 7.4 (step 4). The flow rate was 1 ml/min and 2.5-ml fractions were collected. Aliquots (20 μl) of every fifth fraction were assayed for DARPP-32 (O—O) by the standard phosphorylation assay.

Step 4: DEAE-cellulose chromatography. The pooled and dialyzed fractions from the CM-cellulose column were then centrifuged at 16,000 × *g* for 20 min and the supernatant was loaded onto a column (2.5 × 20 cm) of DEAE-cellulose (DEAE-Sephacel, Pharmacia) previously equilibrated with Buffer A. After washing the column with 100 ml of Buffer A, the protein was eluted at a flow rate of 1 ml/min with a 500-ml linear gradient of 0 to 0.35 M NaCl made up in Buffer A. A single peak of DARPP-32 corresponding to a peak in absorbance at 280 nm eluted at a conductivity of 11 millimho (Fig. 4). The peak fractions were pooled and dialyzed overnight against two changes of 4 liters of Buffer A.

Step 5: Hydroxylapatite chromatography. The pooled and dialyzed fractions from the DEAE-cellulose column

were loaded onto a column (1.5 cm × 6 cm) containing hydroxylapatite (BioGel HTP, Bio-Rad) previously equilibrated in Buffer A. The column was washed with 20 ml of Buffer A, and the protein eluted at a flow rate of 0.5 ml/min with a 100-ml linear gradient from Buffer A to Buffer A containing an additional 0.24 M potassium phosphate (pH 7.4). DARPP-32 eluted as a single peak at a conductivity of 4.3 millimho which corresponded to the major peak of absorbance at 280 nm (Fig. 5).

Step 6: Gel filtration on Ultrogel AcA 44. The peak fractions from the hydroxylapatite column were pooled and concentrated to 5 ml using an Amicon ultrafiltration cell fitted with a PM-10 membrane. This material was applied to a column (2.5 × 93 cm) containing Ultrogel AcA 44 (LKB) gel filtration medium previously equilibrated with Buffer C, and eluted at a flow rate of 20 ml/hr. A single symmetrical peak of DARPP-32 was eluted as shown in Figure 6. The peak fractions were pooled and dialyzed against 4 liters of 10 mM HEPES (pH 7.4), 2 mM EDTA, 15 mM 2-mercaptoethanol, and 0.1 mM PMSF, and were stored at −20°C. Under these conditions the protein was stable, although some breakdown occurred after repeated freezing and thawing.

A summary of the purification procedure for DARPP-32 is presented in Table I. Substrate activity was determined using a radioimmunoassay technique for DARPP-32, since direct assay of the step 1 material (crude cytosol) by the standard phosphorylation assay was unreliable for quantitative purposes. This purification procedure has been carried out five times, with no significant variation in the results. Figure 7*A* shows the protein staining pattern, and Figure 7*B* shows the autoradiogram of phosphorylated samples from each step of the purification procedure. Protein staining of the purified DARPP-32 showed a single band of M_r = 32,000 and autoradiography indicated that all of the ^{32}P co-migrated with this band, suggesting a homogeneous preparation. Purified [^{32}P] DARPP-32 co-migrated on SDS-polyacrylamide gels with the M_r = 32,000 protein phosphorylated by the addition of cAMP to crude bovine cytosol. Moreover, the two proteins gave identical one-dimensional peptide maps (data not shown). This indicates that the purified phosphoprotein of M_r = 32,000 is identical to the phosphoprotein of M_r = 32,000 whose phosphorylation is regulated by cAMP in crude bovine cytosol.

Characterization of purified bovine DARPP-32

Stoichiometry of phosphorylation. The stoichiometry of phosphorylation of purified DARPP-32, catalyzed by

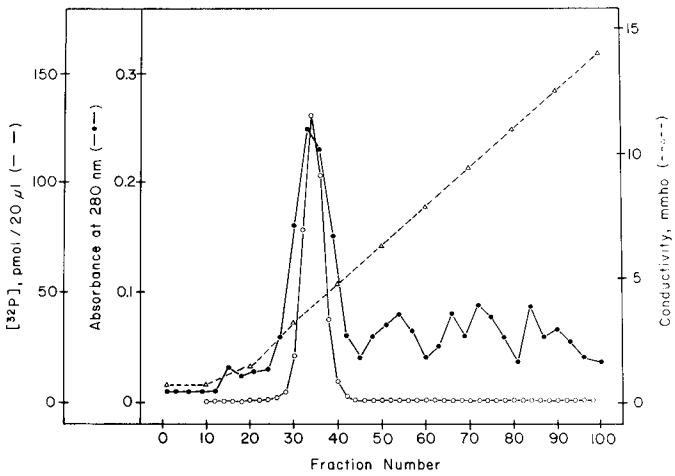

Figure 5. Chromatography of step 4 pool on hydroxylapatite at pH 7.4 (step 5). The flow rate was 0.5 ml/min and 1.0-ml fractions were collected. Aliquots (20 µl) of every second fraction were assayed for DARPP-32 (O—O) by the standard phosphorylation assay.

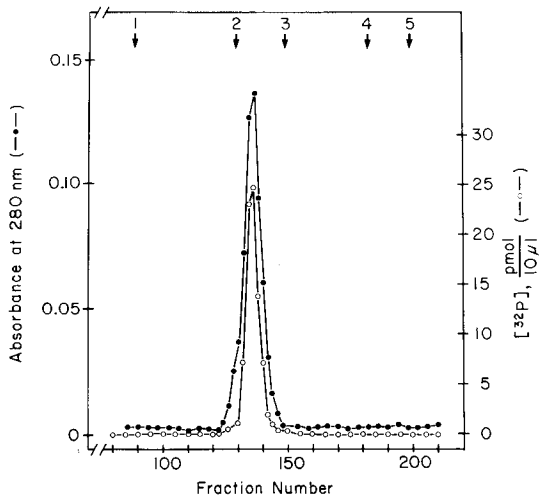

Figure 6. Gel filtration of step 5 pool on Ultrogel AcA 44 at pH 7.4 (step 6). The flow rate was 20 ml/hr and 2.0-ml fractions were collected. Aliquots (10 µl) were assayed for DARPP-32 (O—O) by the standard phosphorylation assay. The *arrows* denote the positions of elution of: *1*, blue dextran (void volume); *2*, bovine serum albumin (35.5 Å); *3*, ovalbumin (27.6 Å); *4*, α-chymotrypsinogen (20.9 Å); and *5*, ribonuclease A (16.4 Å).

TABLE I

Purification of DARPP-32 from 510 gm of bovine caudate nucleus

Step	Volume	Protein	Substrate Activity[a]	Specific Activity	Purification	Yield
	ml	*mg/ml*	*nmol*	*nmol/mg*	*-fold*	%
1. 16,000 × *g* supernatant	2580	3.7	662	0.069	1	100
2. pH 2/6 extract	2570	0.52	622	0.46	6.7	94
3. CM-cellulose	230	0.75	184	1.1	15.9	28
4. DEAE-cellulose	56	0.10	142	25	362	21
5. Hydroxylapatite	9.4	0.37	97	28	406	15
6. Ultrogel AcA 44	29.5	0.057	50	30	435	8

[a] Determined by radioimmunoassay.

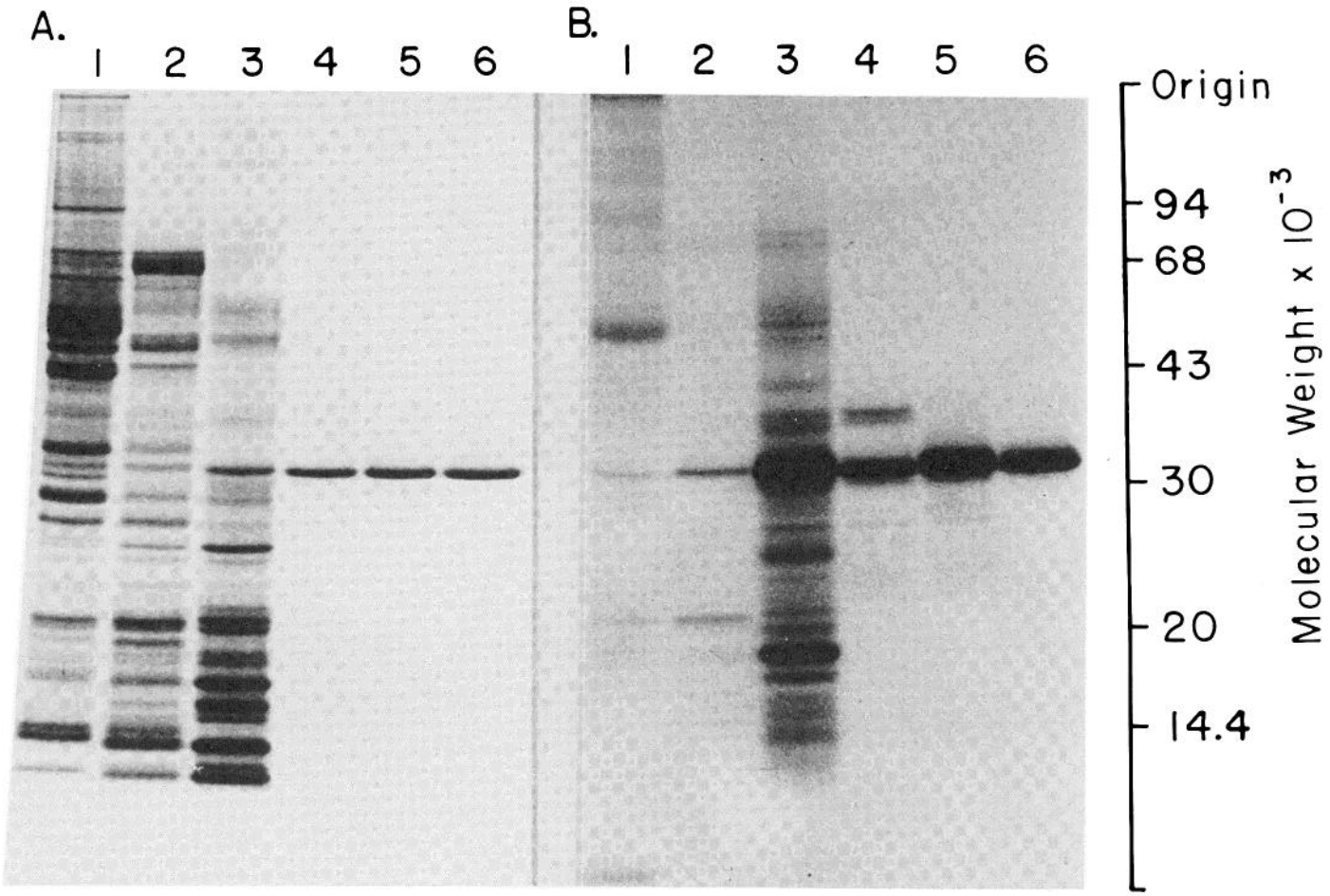

Figure 7. Purification of DARPP-32 monitored by SDS-polyacrylamide gel electrophoresis. Protein staining pattern (*A*) and autoradiogram (*B*) of the dried gel are shown. *Lane 1*, 55 μg of 16,000 × *g* supernatant (step 1); *lane 2*, 31 μg of pH 2/6 extract (step 2); *lane 3*, 45 μg of pool from CM-cellulose column (step 3); *lane 4*, 4.0 μg of pool from DEAE-cellulose column (step 4); *lane 5*, 4.0 μg of pool from hydroxylapatite column (step 5); *lane 6*, 3.4 μg of pool from Ultrogel AcA 44 column (step 6). All samples were assayed with exogenous catalytic subunit of cAMP-dependent protein kinase using the standard phosphorylation assay.

the catalytic subunit of bovine heart cAMP-dependent protein kinase, is shown in Figure 8. It was found that 0.96 mol of [^{32}P]phosphate was incorporated per mol of DARPP-32 after 60 min. Longer incubation times did not result in any significant increase in the amount of [^{32}P]phosphate incorporated.

Site of phosphorylation. In order to determine which amino acid(s) was phosphorylated by the catalytic subunit of cAMP-dependent protein kinase, [^{32}P]DARPP-32 was proteolyzed with trypsin in a gel slice, the phosphopeptides eluted and subjected to acid hydrolysis, and the hydrolysate analyzed for [^{32}P]phosphoamino acids by thin layer electrophoresis. As shown in Figure 9, phosphorylation occurred exclusively on threonine. No significant radioactivity co-migrated with either phosphoserine or phosphotyrosine.

Isoelectric point. Isoelectric focusing in polyacrylamide gels of purified dephospho-DARPP-32 resulted in a single band corresponding to an isoelectric point of 4.7. Phosphorylation of DARPP-32 prior to isoelectric focusing shifted the isoelectric point to 4.6 (Fig. 10).

Amino acid analysis. The amino acid composition of purified DARPP-32 is shown in Table II. The composition is remarkable for its high content of glutamate/glutamine, which accounts for 25.3 mol % of the protein and is consistent with the acidic isoelectric point of DARPP-32. The protein is also rich in proline which

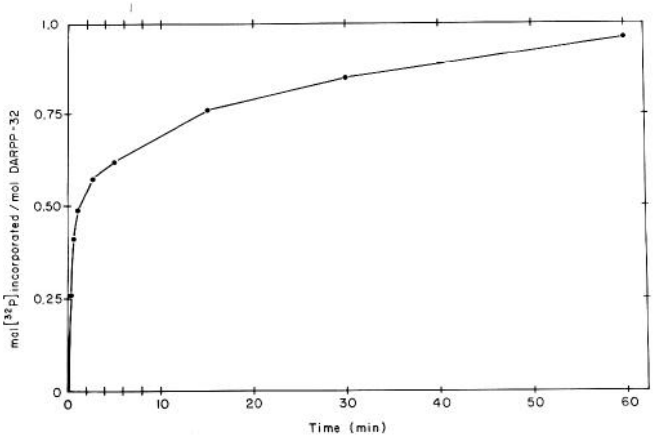

Figure 8. Time course and stoichiometry of phosphorylation of purified DARPP-32 catalyzed by purified catalytic subunit of cAMP-dependent protein kinase. DARPP-32 (2 μM) was incubated for the indicated periods of time and analyzed for the incorporation of [^{32}P]phosphate.

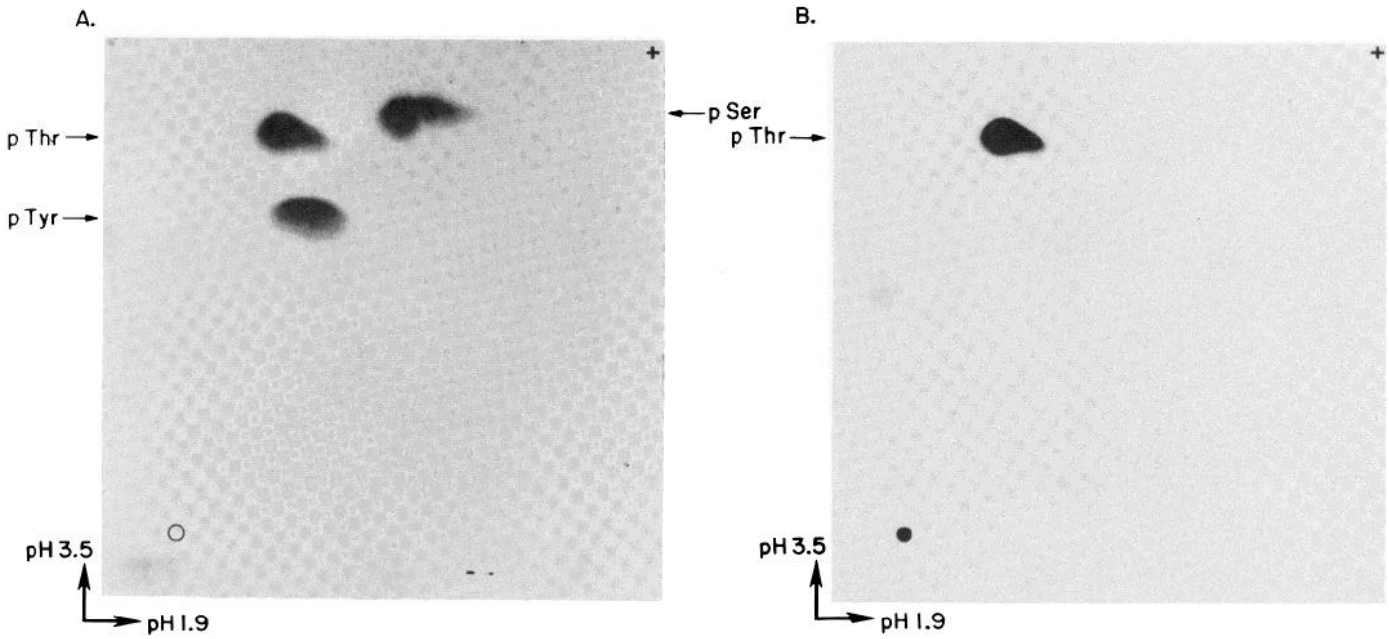

Figure 9. Identification of [^{32}P]phosphothreonine after acid hydrolysis of purified [^{32}P]DARPP-32. *A*, Ninhydrin staining of phosphoamino acid markers; *B*, autoradiogram of [^{32}P]phosphoamino acids. Electrophoresis was carried out first at pH 1.9 in the horizontal dimension, and then at pH 3.5 in the vertical dimension. Origin was at the *lower left corner*. *pSer*, phosphoserine; *pThr*, phosphothreonine; *pTyr*, phosphotyrosine.

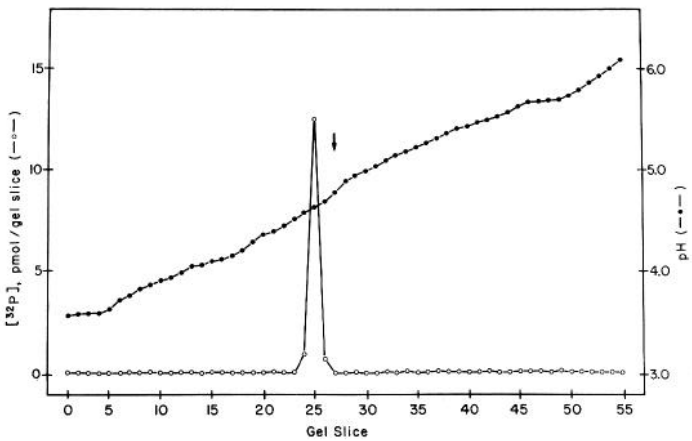

Figure 10. Isoelectric focusing in polyacrylamide gels of purified DARPP-32. Dephospho-DARPP-32 and [^{32}P]phospho-DARPP-32 were analyzed in parallel tube gels. Staining of the gel containing the dephospho-DARPP-32 resulted in a single band at the position indicated by the *arrow*. The gel containing the [^{32}P]phospho-DARPP-32 was cut into 2-mm slices and analyzed by liquid scintillation spectrometry (○—○). A third gel was used to determine the pH gradient (●–●).

TABLE II

Amino acid composition of DARPP-32

Hydrolyses were carried out for 24, 48, 72, and 115 hr at 110°C in 6 N HCl and 0.2% phenol.

Amino Acid	Mol %	Residues/Mole[a]
Asx	7.3	21.3 (21)
Thr[b]	2.0	6.0 (6)
Ser[b]	8.7	25.4 (25)
Glx	25.3	74.0 (74)
Pro	12.5	36.6 (37)
Gly	8.2	24.1 (24)
Ala	5.8	17.1 (17)
Cys[c]	0.5	1.6 (2)
Val	3.0	8.8 (9)
Met	0.8	2.3 (2)
Ile	1.7	5.1 (5)
Leu	7.0	20.4 (20)
Tyr	1.3	3.8 (4)
Phe	0.7	2.0 (2)
His	2.8	8.1 (8)
Lys	3.8	11.2 (11)
Arg	7.8	23.0 (23)
Trp[d]	0.7	2.0 (2)
Total		292.8 (292)

[a] The number of amino acid residues was calculated on the basis of a molecular weight of 32,000.

[b] Obtained by extrapolation to zero time.

[c] Determined as cysteic acid after performic acid oxidation (Moore, 1963).

[d] Determined after hydrolysis in mercaptoethanesulfonic acid (Penke et al., 1974) and spectrophotometrically (Goodwin and Morton, 1946).

makes up 12.5 mol %. The seven hydrophobic amino acids (leucine, isoleucine, valine, tyrosine, phenylalanine, tryptophan, and methionine) together constituted only 15 mol % of the total. The partial specific volume calculated from the amino acid composition was 0.711 ml/gm.

Heat stability. DARPP-32 is heat stable. When a sample of purified DARPP-32 was heated in a boiling water bath for 5 min, no precipitation was observed. Heat-treated DARPP-32 accepted 85% as much ^{32}P as an unheated sample when measured by the standard phosphorylation assay. The ^{32}P was incorporated into the same site in heated samples as in unheated samples, as indicated by the one-dimensional peptide maps obtained

upon limited proteolysis with *S. aureus* V8 protease (data not shown).

Molecular weight determinations. The relative molecular weights (M_r) of rat DARPP-32 (Walaas and Greengard, 1984) and of bovine DARPP-32 (Fig. 1) have been determined by SDS-polyacrylamide gel electrophoresis under reducing conditions using the Laemmli buffer system. The precise value calculated for the molecular weight of DARPP-32 is somewhat arbitrary, since it depends upon the molecular weight value assumed for the standard carbonic anhydrase, which has been reported as either 29,000 (Armstrong et al., 1966) or 30,000 (Reynaud et al., 1971). Using an M_r value of 29,000 for carbonic anhydrase, we have calculated the M_r of rat DARPP-32 to be 32,000, and that of bovine DARPP-32 to be 30,500. Using an M_r value of 30,000 for carbonic anhydrase, we have calculated the M_r of rat DARPP-32 to be 33,500 and that of bovine DARPP-32 to be 32,000.

The Stokes radius of purified DARPP-32, determined by gel filtration on Ultrogel AcA 44, was found to be 34 Å (Fig. 11). For a typical globular protein, this Stokes radius corresponds to M_r = 59,000, suggesting that DARPP-32 is either a dimer or an elongated, asymmetrical molecule. Using the linear sucrose density gradient ultracentrifugation procedure of Martin and Ames (1961), the sedimentation coefficient of DARPP-32 was determined to be 2.05 ± 0.10 S (Fig. 12). For a typical globular protein, this sedimentation coefficient corresponds to M_r = 15,000, also suggesting that DARPP-32 is a highly elongated monomer. The Stokes radius, sedimentation coefficient, and partial specific volume allowed calculation of the frictional ratio and molecular weight of DARPP-32. The frictional ratio (f/f_0) calculated according to equation 2 of Siegel and Monty (1966) was 1.72, indicative of an asymmetric, elongated tertiary structure and corresponding to an axial ratio of 13.5 for a prolate ellipsoid (Shachman, 1959). Using values of 2.05 S for the sedimentation coefficient, 34 Å for the Stokes radius, and 0.711 ml/gm for the partial specific volume, a molecular weight of 27,600 ± 1,500 was calculated. This value is 86% of the M_r = 32,000 estimated by SDS-polyacrylamide gel electrophoresis, suggesting that DARPP-32 may bind less detergent than do typical globular proteins, contain bound carbohydrate or lipid, or fail to assume a random coil conformation in SDS, all of which would lead to slower migration upon SDS-polyacrylamide gel electrophoresis and consequently a higher apparent relative molecular weight. However, DARPP-32 was not stained by the periodic acid-Schiff stain in polyacrylamide gels (Glossmann and Neville, 1971), suggesting that it is not a glycoprotein.

A summary of physical and chemical properties of DARPP-32 is presented in Table III.

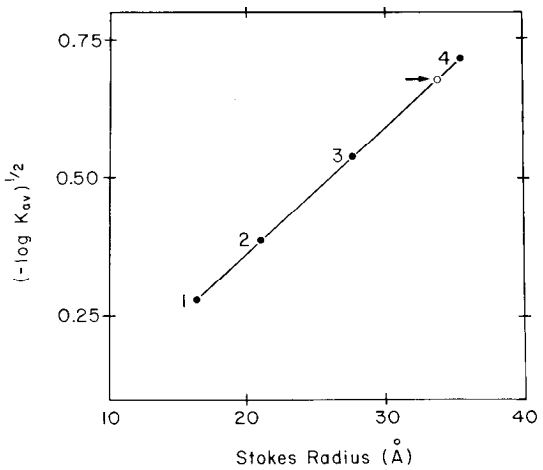

Figure 11. Determination of the Stokes radius of purified DARPP-32 by gel filtration on Ultrogel AcA 44. The column was calibrated with the following marker proteins: *1*, ribonuclease A (16.4 Å); *2*, α-chymotrypsinogen (20.9 Å); *3*, ovalbumin (27.6 Å); and *4*, bovine serum albumin (35.5 Å). The position of DARPP-32 is indicated by the *arrow*. The data were treated as described by Siegel and Monty (1966).

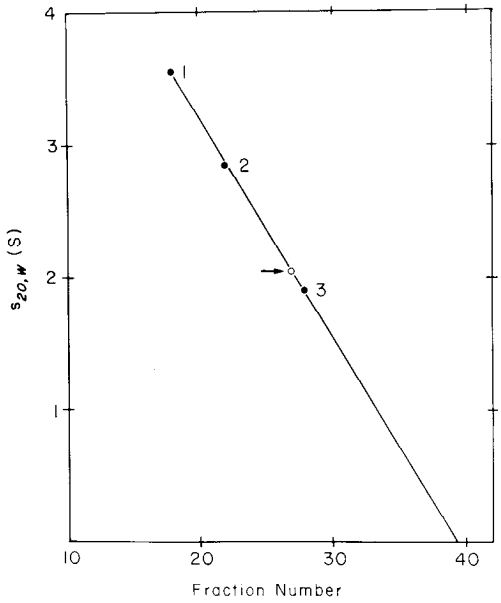

Figure 12. Determination of the sedimentation coefficient ($s_{20,w}$) of purified DARPP-32 by sucrose density gradient ultracentrifugation. The marker proteins used were: *1*, ovalbumin (3.55 S); *2*, carbonic anhydrase (2.85 S); and *3*, cytochrome *c* (1.9 S). The position of DARPP-32 is indicated by the *arrow*.

Discussion

cAMP was found to stimulate the phosphorylation of DARPP-32 in crude cytosol prepared from bovine caudate nucleus. DARPP-32 has been purified 435-fold with an 8% recovery to apparent homogeneity from this source by selective acid precipitation followed by four successive chromatographic steps (Table I). Preparation of an apparently homogeneous protein with only a 435-fold purification indicates that DARPP-32 is highly abundant in the caudate nucleus, comprising roughly 0.2% of the total cytosolic protein. The purification procedure took advantage of the acid solubility of DARPP-32 as the first step. In addition to eliminating endogenous protein kinase and phosphoprotein phosphatase activities, making the assay for DARPP-32 more reliable, inclusion of an acid treatment early in the purification provided a 7-fold purification with excellent yield. DARPP-32 which had been partially purified without subjecting the protein to the denaturing effects of acid pH exhibited gel filtration and ion exchange prop-

TABLE III
Summary of physical and chemical properties of DARPP-32

Property	Method of Determination	Value
Molecular weight (M_r)	SDS-polyacrylamide gel electrophoresis	32,000
	Stokes radius and sedimentation coefficient[a]	27,600
Isoelectric point (pI)	Polyacrylamide gel isoelectric focusing	
	Phospho form	4.6
	Dephospho form	4.7
Stokes radius (a)	Gel filtration	34 Å
Sedimentation coefficient ($s_{20,w}$)	Sucrose density gradient centrifugation	2.05 S
Frictional ratio (f/f_0)[b]	Stokes radius and sedimentation coefficient	1.72
Axial ratio[c]	Stokes radius and sedimentation coefficient	13.5
Amino acid composition		High glutamate/glutamine and proline
		Low hydrophobic amino acids
Extinction coefficient ($E^{1\%}_{280}$)[d]	Spectrophotometry	5.03
Partial specific volume (ν)	Amino acid analysis	0.711 ml/gm

[a] The molecular weight was calculated using the Stokes radius obtained from gel filtration, the sedimentation coefficient derived from sucrose density gradient centrifugation, and the partial specific volume derived from the amino acid analysis according to the following equation (Siegel and Monty, 1966): $M = \frac{6\pi\eta N a s_{20,w}}{(1 - \nu\rho)}$ where η = viscosity of water at 20°C, N = Avogadro's number, a = Stoke's radius, s = sedimentation coefficient, ν = partial specific volume, and ρ = density of water at 20°C.

[b] The frictional ratio was calculated according to the following equation (Siegel and Monty, 1966): $f/f_0 = a/\left(\frac{3\nu M}{4\pi N}\right)^{1/3}$

[c] Value for a prolate ellipsoid (Schachman, 1959).

[d] Determined for a 1.17-mg/ml solution of purified DARPP-32 in 10 mM potassium phosphate (pH 7.4), based on amino acid analysis.

erties identical to those of DARPP-32 purified by the scheme shown in Table I, suggesting that acid treatment does not alter the properties of the protein (data not shown).

Studies of the hydrodynamic properties of DARPP-32 indicated a sedimentation coefficient of 2.05 S, a Stokes radius of 34 Å, and a frictional ratio (f/f_0) of 1.72. Such a high frictional ratio suggests that DARPP-32 exists in solution as a prolate ellipsoid. Similar high frictional ratios have been explained by a nonspherical, elongated molecular shape in the cases of protein phosphatase inhibitor-1 (an inhibitor of protein phosphatase-1 in its phosphorylated form (Huang and Glinsmann, 1976; Nimmo and Cohen, 1978)) and of G-substrate (a neuronal phosphoprotein (Aswad and Greengard, 1981a)). The molecular weight calculated from the hydrodynamic measurements of purified DARPP-32 is ~27,600. Analysis of the purified preparation by SDS-polyacrylamide gel electrophoresis revealed the presence of a single polypeptide with an apparent M_r = 32,000, indicating that DARPP-32 behaves in solution as an asymmetric monomer. The lower molecular weight obtained by hydrodynamic measurements suggests that DARPP-32 may migrate anomalously upon SDS-polyacrylamide gel electrophoresis for the reasons described under "Results." A similar anomaly was observed by Aitken et al. (1982) for phosphatase inhibitor-1, which exhibits an apparent M_r = 26,000 upon SDS-polyacrylamide gel electrophoresis (Nimmo and Cohen, 1978) but has a molecular weight of 19,000 when determined by amino acid sequencing (Aitken et al., 1982) or sedimentation equilibrium centrifugation (Nimmo and Cohen, 1978).

Phosphorylation of purified DARPP-32 catalyzed by the catalytic subunit of cAMP-dependent protein kinase occurred exclusively on threonine with a stoichiometry of 0.96 mol/mol, indicating a single phosphorylatable site. All substrates for cAMP-dependent protein kinase previously studied have been found to be phosphorylated on serine residues, with the exception of phosphatase inhibitor-1, which is phosphorylated on a threonine residue (Cohen et al., 1977). G-substrate is specifically phosphorylated by cGMP-dependent protein kinase, also on a threonine residue (Aswad and Greengard, 1981a).

DARPP-32 shares several properties with phosphatase inhibitor-1 and G-substrate in addition to having similar hydrodynamic properties and being phosphorylated on threonine. Thus, phosphatase inhibitor-1 (Nimmo and Cohen, 1978), G-substrate (Aswad and Greengard, 1982a), and DARPP-32 (present study) all exhibit solubility at acid pH, heat stability, an acidic isoelectric point, a low content of hydrophobic amino acids, a relatively low molecular weight, and unfolded tertiary structure.

Comparison of the amino acid compositions of DARPP-32 (Table II) and phosphatase inhibitor-1 (Nimmo and Cohen, 1978) reveals a striking similarity. Moreover, although G-substrate and phosphatase inhibitor-1 differ in their overall amino acid compositions, the sequences of the two phosphorylation sites on G-substrate are similar to the sequence around the phosphorylation site of phosphatase inhibitor-1 (Aitken et al., 1981) and to the sequence around the phosphorylation site of DARPP-32 (K. Williams, H. C. Hemmings, Jr., W. Konigsberg, and P. Greengard, manuscript in prepa-

ration). Phosphatase inhibitor-1 (Huang and Glinsmann, 1976) and G-substrate (P. F. Simonelli, H. -C. Li, A. C. Nairn, and P. Greengard, unpublished observations) both appear to function as protein phosphatase inhibitors. It will be of interest to determine whether DARPP-32 also functions as a protein phosphatase inhibitor.

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