ARPP-21, A Cyclic AMP-Regulated Phosphoprotein Enriched in Dopamine-Innervated Brain Regions. I. Amino Acid Sequence of ARPP-21B from Bovine Caudate Nucleus

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ARPP-21 (cAMP-regulated phosphoprotein, $M_r = 21,000$ as determined by SDS-PAGE) is a major cytosolic substrate for cAMP-stimulated protein phosphorylation in dopamine-innervated regions of the rat CNS. It has recently been purified to homogeneity from bovine caudate nucleus and characterized (Hemmings and Greengard, 1989). ARPP-21 is isolated as 2 isoforms, ARPP-21A and ARPP-21B. The amino acid sequence of purified bovine ARPP-21B has now been determined by gas-phase sequencing. The S-¹⁴C-carboxymethylated protein was subjected to enzymatic cleavage with trypsin, chymotrypsin, subtilisin, and endoproteinase Lys-C. The resulting peptides were purified by high-performance liquid chromatography, and selected peptides were subjected to amino acid analysis and/or amino acid sequencing by automated Edman degradation. ARPP-21B consists of a single NH₂-terminal blocked polypeptide chain of 88 residues, with a calculated molecular mass of 9561 Da, including an NH₂-terminal acetyl group inferred by deblocking with an acylaminopeptidase. This molecular mass is significantly lower than earlier estimates based on SDS-PAGE or hydrodynamic measurements. The seryl residue phosphorylated by cAMP-dependent protein kinase (Hemmings et al., 1989) is located at position 55. The molecule contains 1 cysteinyl residue, at position 71, and contains no methionyl, tyrosyl, phenylalanyl, tryptophanyl, or histidinyl residues. Determination of the primary structure of ARPP-21, one of several phosphoproteins localized to dopaminergic neurons in the basal ganglia, provides a framework for further investigations into the molecular mechanisms involved in dopaminergic neurotransmission.

The phosphorylation of specific proteins is a prominent molecular mechanism by which a number of neurotransmitter effects are mediated (for review, see Nestler and Greengard, 1984). ARPP-21 (cAMP-regulated phosphoprotein, M_r , 21,000) is a cytosolic neuronal phosphoprotein that was originally identified

in dopamine-innervated regions of mammalian brain as a prominent substrate for cAMP-dependent protein kinase (Walaas et al., 1983). The phosphorylation of ARPP-21 is stimulated in intact cells by 8-bromo-cAMP (Girault et al., 1988; Hemmings et al., 1989), forskolin, or vasoactive intestinal peptide (Girault et al., 1988). ARPP-21 has been purified from bovine caudate nucleus and biochemically characterized (Hemmings and Greengard, 1989). It is an acidic protein (pI 4.6) with an apparent $M_r = 21,000$ as determined by SDS-PAGE. cAMP-dependent protein kinase phosphorylates a single seryl residue in ARPP-21 with favorable kinetic parameters (Hemmings and Greengard, 1989; Hemmings et al., 1989). The regional distribution of ARPP-21 in rat brain, determined by immunocytochemistry using specific monoclonal antibodies (Ouimet et al., 1989), correlates with the distribution of dopamine-innervated neurons that possess D-1 dopamine receptors. These findings have led to the hypothesis that ARPP-21 mediates some of the physiological effects of dopamine and other neurotransmitters acting through cAMP on dopaminergic neurons.

A detailed analysis of the structural and biochemical properties of ARPP-21 and other phosphoproteins concentrated in the basal ganglia has been undertaken as an approach to elucidating the molecular mechanisms involved in synaptic transmission and neurotransmitter interactions in the striatum (Hemmings et al., 1987). ARPP-21 is isolated from bovine brain as 2 isoforms (ARPP-21A and ARPP-21B), which are separable by anion-exchange chromatography. They are present in approximately equal amounts and are indistinguishable by a number of biochemical and immunochemical criteria (Hemmings and Greengard, 1989). The amino acid sequence surrounding the site in ARPP-21B phosphorylated by cAMP-dependent protein kinase was determined recently (Hemmings et al., 1989). In order to further characterize ARPP-21, we have determined its complete amino acid sequence. Below, we report the results of the direct amino acid sequencing of bovine brain ARPP-21B. In the accompanying paper, we report the molecular cloning and nucleotide sequence of the cDNA derived from bovine brain ARPP-21 mRNA, which was selected using 2 synthetic oligonucleotide probes based on the amino acid sequence. Determination of the amino acid and nucleotide sequences of ARPP-21 provides the basis for detailed studies of the function and regulation of ARPP-21.

Materials and Methods

Materials. Unless specified otherwise, materials were as described previously (Williams et al., 1986). ARPP-21A and ARPP-21B were purified from bovine caudate nucleus as described previously (Hemmings and

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Figure 1. The complete amino acid sequence of bovine ARPP-21B. Solid lines indicate peptides that were sequenced; dashed lines indicate that the amino acid composition of the peptide is consistent with the proposed sequence. The single-headed arrow indicates the last residue that could be sequenced in each peptide, which, because some of the peptides were only partially sequenced, is not necessarily the COOH-terminus of the peptide. Peptides were derived from ARPP-21B by cleavage with chymotrypsin (C), endoproteinase Lys-C (L), subtilisin (S), and trypsin (T). The leftward arrow indicates the residue released by carboxypeptidase Y. The box indicates the single difference between the amino acid sequence determined directly and the sequence deduced from the cDNA nucleotide sequence. P indicates the phosphorylatable seryl residue at position 55.

Greengard, 1989); the purified proteins were reduced and S-¹⁴C-carboxymethylated as described (Williams et al., 1986), dialysed extensively against 5 mM NH₄HCO₃, and lyophilized. Prior to enzymatic digestion, ARPP-21A and ARPP-21B thus obtained were further purified by reverse-phase high performance liquid chromatography (HPLC) using a Vydac C-18 column as described below for the purification of ARPP-21B peptides.

Enzymatic digestion of ARPP-21. Purified, reduced, and S-¹⁴C-carboxymethylated ARPP-21A and ARPP-21B (0.5 nmol each) were subjected to proteolytic digestion by endoproteinase Lys-C for 24 hr at 37°C in 0.2 ml of 25 mM Tris-HCl (pH 7.6) and 2 M urea with a substrate/enzyme ratio of 400:1 (wt/wt). Proteolytic digestion of 5 nmol S-¹⁴C-carboxymethylated ARPP-21B by endoproteinase Lys-C was carried out for 24 hr at 37°C in 0.2 ml of 25 mM Tris-HCl (pH 7.6) and 2 M urea with a substrate/enzyme ratio of 400:1 (wt/wt). Proteolytic digestion of 49 nmol S-¹⁴C-carboxymethylated ARPP-21B by TPC-treated trypsin was carried out for 24 hr at 37°C in 25 mM NH₄HCO₃ and 2 M urea with a substrate/enzyme ratio of 25:1 (wt/wt). Proteolytic digestion of 22 nmol S-¹⁴C-carboxymethylated ARPP-21B by subtilisin (Sigma, type XXVII) was carried out for 2 hr at 37°C in 25 mM NH₄HCO₃ and 2 M urea with a substrate/enzyme ratio of 25:1 (wt/wt). The NH₂-terminal blocked tryptic peptide T-1 (10 nmol) was digested by subtilisin for 2 hr at 37°C in 25 mM NH₄HCO₃ with a substrate/enzyme ratio of 25:1 (wt/wt). The above reactions were terminated by injection of the reaction mixture directly onto an HPLC column. Carboxypeptidase Y digests of 2 nmol aliquots of intact ARPP-21B or purified peptides were carried out for 1 hr at 37°C in 0.2 M sodium acetate with a substrate/enzyme ratio of 100:1 (wt/wt). The reactions were terminated by acidification and injection of the mixture directly onto a Beckman 121 M ion-exchange amino acid analyzer. The NH₂-terminal blocked subtilisin peptide S-2 (0.5 nmol) was cleaved with acylaminopeptidase at pH 7.5 for 72 hr at 25°C as described (Jones and Manning, 1985; Jones et al., 1986).

Purification and amino acid sequencing of ARPP-21B peptides. Peptides resulting from the enzymatic cleavage of ARPP-21B were separated by reverse-phase HPLC using a Waters Associates HPLC system employing a Vydac C-4 or C-18 column (0.46 × 25 cm, 300 Å pore size, 5 μm particle size). The column was equilibrated with 0.05% (vol/vol) trifluoroacetic acid (buffer A) at a flow rate of 0.7 ml/min, and the peptides were separated by elution with increasing concentrations of

buffer B [0.05% (vol/vol) trifluoroacetic acid and 80% acetonitrile] as follows: 0–90 min (0–37.5% B), 90–135 min (37.5–75% B). Fractions were collected automatically by peaks as detected by absorbance at 210 nm using an ISCO model 2150 peak detector. Amino acid analysis was performed following acid hydrolysis and precolumn derivatization with phenylisothiocyanate using a Waters Associates Pico-Tag system (Stone and Williams, 1986). Peptides selected for sequence analysis were subjected to automated gas-phase amino acid sequencing using an Applied Biosystems 470 A amino acid sequencer as described (Williams et al., 1986).

Results

General strategy

Three chymotryptic peptides comprised 95% of the total amino acid residues of ARPP-21B (Fig. 1). Additional peptides and amino acids needed to complete the sequence were obtained from cleavages made with endoproteinase Lys-C, subtilisin, trypsin, and carboxypeptidase Y. The sequence of the blocked NH₂-terminal residue was determined using an acylaminopeptidase. Approximately 2 mg purified ARPP-21B were used in the sequence determination.

Analysis of purified ARPP-21 by HPLC

Purified, reduced, and S-carboxymethylated ARPP-21A and ARPP-21B were analyzed by HPLC as shown in Figure 2. Both proteins were homogeneous as analyzed by SDS-PAGE (Fig. 7 in Hemmings and Greengard, 1989). ARPP-21B was also essentially homogeneous as analyzed by reverse-phase HPLC. However, ARPP-21A was resolved into 2 major peaks by reverse-phase HPLC. A comparison of the later-eluting ARPP-21A peak and ARPP-21B purified by HPLC by analytical reverse-phase HPLC peptide mapping following digestion by endoproteinase Lys-C is shown in Figure 3. A major triplet of peptides eluting near 62 min is present in both digests; however,

Figure 2. Reverse-phase HPLC chromatograms of purified S-carboxymethylated ARPP-21A (upper) and ARPP-21B (lower) from bovine caudate nucleus. 0.5 nmol of each protein was applied to a C-18 column equilibrated in 0.05% (vol/vol) trifluoroacetic acid, which was then eluted with increasing concentrations of acetonitrile as described in Materials and Methods.

the later-eluting peptides of each ARPP-21 isoform appear distinct, suggesting structural heterogeneity between ARPP-21A and ARPP-21B. Heterogeneity between the 2 major peaks of ARPP-21A separated by reverse-phase HPLC (Fig. 2) was also detected by HPLC peptide mapping (data not shown). Given this heterogeneity of purified ARPP-21A, ARPP-21B was chosen for sequence analysis.

Proteolytic cleavage of ARPP-21B with chymotrypsin or endoproteinase Lys-C

The separation HPLC of chymotryptic peptides derived from 24 nmol [32 P]phospho-ARPP-21B is shown in Figure 5 of Hem-

Figure 3. Reverse-phase HPLC chromatograms of peptides from endoproteinase Lys-C digests of the 2 ARPP-21 isoforms. About 0.5 nmol each of ARPP-21A (reverse-phase HPLC purified; the second major peak obtained in Fig. 2) or ARPP-21B (reverse-phase HPLC purified, see Fig. 2) was digested and the resulting peptides separated by HPLC on a C-18 column equilibrated in 0.05% (vol/vol) trifluoroacetic acid and eluted with increasing concentrations of acetonitrile as described in Materials and Methods.

mings et al. (1989). The amino acid sequence of residues 46–64 of ARPP-21B (peptide C-18; Table 1, Fig. 1) was determined in the course of analyzing the phosphorylation site sequence of ARPP-21B (Hemmings et al., 1989). Two other chymotryptic peptides resulting from this digest were useful in establishing the complete sequence of ARPP-21B. Peptides C-21 and C-22 (eluting at 64 and 86 min, respectively, in figure 5 of Hemmings et al., 1989), together with peptide C-18, comprised 84 of the 88 total amino acids of ARPP-21B (Fig. 1). Both intact ARPP-21B and peptide C-22 failed to sequence (data not shown), indicating that ARPP-21B has a blocked NH_2 -terminus and that peptide C-22 represents the NH_2 -terminus of ARPP-21B. Although peptide C-22 did not yield to direct amino acid sequencing, its amino acid composition was useful in confirming the

Table 1. Amino acid compositions of chymotryptic and tryptic peptides from ARPP-21B^a

Amino acid	C-22	C-18	C-21	T-1	T-2	T-3	T-4	T-5	T-6	T-7	T-8
Cys			ND (1)						0.4 (1)	ND (1)	
Asp	3.1 (3)	1.0 (1)	1.1 (1)	2.1 (2)	1.0 (1)	1.1 (1)	1.0 (1)			1.0 (1)	1.0 (1)
Thr	2.9 (3)		1.9 (2)	3.0 (3)				0.9 (1)		1.0 (1)	0.8 (1)
Ser	3.7 (4)	1.6 (2)	3.7 (4)	2.0 (2)	1.9 (2)				2.8 (3)	3.6 (4)	1.1 (1)
Glu	13.8 (13)	3.2 (3)	5.3 (5)	8.4 (8)	6.0 (6)	3.2 (3)	3.0 (3)		2.3 (2)	5.1 (5)	3.0 (3)
Pro	3.0 (3)		0.9 (1)	3.1 (3)						1.0 (1)	1.0 (1)
Gly	4.3 (4)	2.9 (3)	2.2 (2)	4.3 (4)						2.1 (2)	2.1 (2)
Ala	1.1 (1)	1.9 (2)	2.0 (2)	1.1 (1)		1.0 (1)	1.0 (1)		2.2 (2)	2.0 (2)	
Val	2.0 (2)	0.9 (1)	1.1 (1)	1.5 (2)		1.0 (1)	1.0 (1)		1.0 (1)	1.0 (1)	
Met											
Ile	1.4 (2)			1.4 (2)							
Leu	3.9 (4)	1.2 (1)	3.0 (3)	1.0 (1)	3.0 (3)	1.0 (1)	1.0 (1)	1.0 (1)	1.1 (1)	3.0 (3)	1.8 (2)
Tyr											
Phe											
His											
Lys	1.7 (2)	3.8 (4)		1.0 (1)	1.0 (1)						
Arg		2.4 (2)	2.0 (2)		1.0 (1)	2.0 (2)	1.1 (1)	1.0 (1)	1.0 (1)	1.0 (1)	
Trp											
Yield (%)	32	30	17	57	28	8	47	57	6	40	6
Residues	1–41	46–64	65–88	1–29	30–43	45–53	45–52	64–66	67–77	67–88	78–88

^a Determined by ion-exchange amino acid analysis as described under Materials and Methods. Values are expressed as residues per molecule. Values in parentheses are residues per molecule as determined from the sequence. ND, not determined.

Figure 4. Reverse-phase HPLC chromatogram of peptides from endoproteinase Lys-C digest of 5 nmol of S-carboxymethylated ARPP-21B. The C-18 column was equilibrated with 0.05% (vol/vol) trifluoroacetic acid and eluted with increasing concentrations of acetonitrile as described in Materials and Methods.

sequence of the first 41 amino acids of ARPP-21B. Peptide C-22 also provided an "overlap" for peptides S-4 and L-1 (Fig. 1), which had to be ordered based on its amino acid composition. In the absence of an overlapping sequence, the amino acid composition of peptide C-22 confirmed the proposed ordering of peptides S-4 and L-1 and strongly suggested that there were no interposed peptides.

Peptide C-18 contains the single phosphorylation site of ARPP-21B (serine-55, Hemmings et al., 1989), while peptide C-21 contains the only cysteinyl residue in ARPP-21B (Fig. 1). The identification of residue 71 as cysteine was based on the appearance of the characteristic phenylthiohydantoin derivatives of ^{14}C -carboxymethylcysteine at the seventh cycle in the sequence of peptide C-21 and was confirmed by a peak of radioactivity at the same cycle (radioactivity at cycles 69, 70, 71, and 72 was 59, 53, 128, and 47 cpm, respectively). Although peptide C-21 could not be completely sequenced, its amino acid composition is consistent with the proposed COOH-terminal sequence of ARPP-21B.

The preparative separation by HPLC of the peptides resulting from the digestion of 5 nmol ARPP-21B by endoproteinase Lys-C is shown in Figure 4. The differences between the peptide elution pattern shown in Figure 4 and that shown in Figure 3 are probably due to the 10-fold difference in reactant concentrations. Two peptides from this digest were useful in overlapping the 3 chymotryptic peptides described above; peptide L-1 overlapped peptides C-22 and C-18, while peptide L-2 overlapped peptides C-18 and C-21 (Fig. 1). Peptide L-1 was sequenced completely, while peptide L-2 sequenced to threonine-87, the penultimate amino acid of this peptide and of intact ARPP-21B.

Proteolytic cleavage of ARPP-21B with trypsin and analysis of the COOH-terminus

The separation by HPLC of the peptides resulting from the digestion of 49 nmol ARPP-21B by trypsin is shown in Figure 5. Eight of the tryptic peptides isolated appeared to be essentially pure as judged by their amino acid compositions (Table 1). In

Figure 5. Reverse-phase HPLC chromatograms of peptides from tryptic digest of 49 nmol S-carboxymethylated ARPP-21B (upper) and subtilisin digest of 10 nmol of peptide T-1 (lower). For each separation, a C-4 column was equilibrated with 0.05% (vol/vol) trifluoroacetic acid and eluted with increasing concentrations of acetonitrile as described in Materials and Methods.

all cases, their compositions agreed with the proposed sequence of ARPP-21B (Fig. 1). In particular, the composition of peptide T-8 confirmed the presence of leucine as the COOH-terminal residue of ARPP-21B. This conclusion was verified by carboxypeptidase Y digestion of both intact ARPP-21B and peptide T-8. Under the conditions used, leucine release corresponded to an overall yield approaching 100% for ARPP-21B and 75% for peptide T-8. In addition, carboxypeptidase Y digestion of peptide T-8 released 2 or more of the following amino acids which are incompletely resolved under the normal conditions used for ion-exchange chromatography: threonine, serine, glutamine, and/or asparagine.

Proteolytic cleavage of ARPP-21B with subtilisin and analysis of the NH₂-terminus

The amino acid composition of peptide T-1 was consistent with its assignment as the NH₂-terminal tryptic peptide of ARPP-21B (Table 1). This was supported by its failure to sequence, indicating the presence of a blocked NH₂-terminal residue. Several attempts were made to further cleave the blocked peptide T-1. Despite the presence of potential cleavage sites, thermolysin, *S. aureus* V8 protease, and proteinase A each failed to cleave peptide T-1. In contrast, subtilisin proved to be extremely effective. The separation by HPLC of the peptides resulting from the digestion of peptide T-1 by subtilisin is shown in Figure 5. Three of the resulting peptides (S-3, S-4, and S-5) were sequenced (Fig. 1), and the amino acid compositions of the 2 remaining peptides (S-1 and S-2; Table 2) were in agreement with the proposed sequence of ARPP-21B (Fig. 1). Peptides S-1 and S-2 both failed to sequence, confirming their placement at the NH₂-terminus of ARPP-21B.

The NH₂-terminal sequence of ARPP-21B was investigated further by analyzing a subtilisin digest of intact ARPP-21B. The separation by HPLC of the peptides resulting from this digest is shown in Figure 6. Additional peptide S-2 was obtained, and 0.5 nmol of this peptide was treated with an acylaminopeptidase (Jones et al., 1986) prior to sequencing. Based on the first cycle

Table 2. Amino acid compositions of subtilisin peptides derived from T-1^a

Amino acid	S-1	S-2	S-3	S-4	S-5
Asp	0.9 (1)	0.9 (1)		1.2 (1)	1.1 (1)
Thr	0.9 (1)			2.6 (3)	2.0 (2)
Ser	1.8 (2)	1.0 (1)			
Glu	1.9 (2)	1.0 (1)	2.5 (2)	5.4 (5)	1.3 (1)
Pro	1.1 (1)	1.2 (1)		2.9 (3)	1.4 (1)
Gly	1.3 (1)	1.2 (1)		4.2 (4)	1.3 (1)
Ala				1.2 (1)	0.9 (1)
Val			0.7 (1)	0.7 (1)	0.5 (1)
Met					
Ile			0.8 (1)	0.7 (1)	0.5 (1)
Leu	0.7 (1)	0.8 (1)			
Tyr					
Phe					
His					
Lys				1.1 (1)	0.9 (1)
Arg					
Trp					
Yield (%)	5	14	5	9	9
Residues	1-9	1-6	10-13	14-29	20-29

^a Determined by analysis of phenylthiocarbamyl derivatives as described under Materials and Methods. Values are expressed as residues per molecule. Values in parentheses are residues per molecule as determined from the sequence.

yield of the phenylthiohydantoin derivative of glutamic acid, this enzyme hydrolyzed approximately 5% of peptide S-2. As a result, the sequence of residues 2-6 could be determined directly. Based on the amino acid composition of peptide S-2 and the known specificity of the acylaminopeptidase, the first residue was inferred to be *N*-acetylserine. The ordering of peptides S-2 and S-6 and the lack of any intervening residues were confirmed by the amino acid composition of peptide S-1 (Table 2, Fig. 1).

The peptide peak labeled S-5, S-6 in Figure 6 was also subjected to amino acid sequencing. This peak contained 2 peptides in approximately equimolar amounts corresponding to residues 7-18 (peptide S-6) and 20-29 (peptide S-5). Peptide S-6 provided an overlap for peptides S-3 and S-4. The sequences of peptides S-4 and S-5, along with that of peptide S-7, provided 3 independent determinations of residue 25 as aspartic acid, the only residue for which there is disagreement between the directly determined amino acid sequence of ARPP-21B and the sequence derived from analysis of the ARPP-21 cDNA (Kurihara et al., 1989). Based on sequence analysis of the ARPP-21 cDNA, residue 25 was identified as asparagine.

As shown in Table 3, the amino acid composition of ARPP-21B predicted by the proposed sequence (Fig. 1) is in excellent agreement with the composition determined by ion-exchange amino acid analysis.

Discussion

ARPP-21 consists of 2 very similar isoforms, ARPP-21A and ARPP-21B; the complete amino acid sequence of bovine ARPP-21B has now been determined by direct sequencing techniques. ARPP-21B is composed of a single polypeptide chain of 88 residues with a blocked NH₂-terminus. The NH₂-terminal blocking group is inferred to be acetyl based on the substrate specificity of the acylaminopeptidase used for deblocking (Jones and Manning, 1985); *N*^a-acetylserine is one of the most fre-

Figure 6. Reverse-phase HPLC chromatogram of peptides from subtilisin digest of 22 nmol S-carboxymethylated ARPP-21B. The C-18 column was equilibrated with 0.05% (vol/vol) trifluoroacetic acid and eluted with increasing concentrations of acetonitrile as described in Materials and Methods.

quently observed NH₂-terminal residues of blocked proteins (Boissel et al., 1985). The molecular mass of ARPP-21B calculated from the amino acid sequence, assuming the NH₂-terminus is acetylated, is 9561 Da. This value is considerably less than the *M_r* of 21,000 determined by SDS/PAGE (Hemmings and Greengard, 1989). Anomalous slow migration in SDS-PAGE appears to correlate with a relative decrease in SDS binding and an extended conformation (Tung and Knight, 1972) and has been observed for other low-molecular-weight, acidic phos-

Table 3. Experimentally determined amino acid composition of ARPP-21B compared with that predicted by the proposed amino acid sequence^a

Amino acid	Determined	Predicted
Cys	0.54 ^b	(1)
Asx	5.2	(5)
Thr	4.8	(5)
Ser	8.8	(10)
Glx	22.6	(22)
Pro	4.3	(4)
Gly	9.2	(9)
Ala	5.6	(5)
Val	3.7	(4)
Met	0	(0)
Ile	1.6	(2)
Leu	9.1	(9)
Tyr	0	(0)
Phe	0	(0)
His	0	(0)
Lys	6.1	(6)
Arg	6.7	(6)
Trp	0	(0)
Total	88.24	88

^a Determined by ion-exchange amino acid analysis as described under Materials and Methods. Values are expressed as residues per molecule based on a molecular weight of 9,561.

^b Determined as cysteic acid after performic acid oxidation.

Figure 7. Schematic diagram of the predicted secondary structure of ARPP-21. The prediction was generated by a computer program based on the method of Garnier et al. (1978). Residues are depicted in α -helical (loop pattern), β -sheet (sawtooth pattern), and random coil (straight line) conformations. β -Turns are indicated by chain reversals. Charged residues are indicated by a + or -, and conformational boundary residues are numbered. The site of phosphorylation is indicated by P.

phoproteins including inhibitor-1 (Aitken et al., 1982) and DARPP-32 (Williams et al., 1986).

The amino acid compositions obtained from acid hydrolyses of intact ARPP-21B (Table 3, and Hemmings and Greengard, 1989) are in excellent agreement with that predicted from the amino acid sequence. As noted previously (Hemmings and Greengard, 1989) and confirmed herein, ARPP-21B is devoid of tyrosinyl, phenylalanyl, tryptophanyl, histidinyl, and methionyl residues; there is a single cysteinyl residue at position 71. ARPP-21B consists of 25 mol% glutamyl residues, a finding consistent with its low pI. The single seryl residue phosphorylated by cAMP-dependent protein kinase *in vitro* and by a cAMP analog in intact cells (Hemmings et al., 1989) is located at position 55; the amino acid sequence around serine-55 conforms to the general specificity requirements for phosphorylation by cAMP-dependent protein kinase (-Arg-Arg-X-Ser-). Based on the amino acid sequence of ARPP-21B and the substrate specificity requirements of casein kinase II (Kuenzel et al., 1987), serine-32 is a potential phosphorylation site for casein kinase II; however, significant phosphorylation of ARPP-21B by this protein kinase does not occur (J.-A. Girault, H. C. Hemmings, Jr., and P. Greengard, unpublished observations).

The amino acid sequence of bovine ARPP-21B determined directly agrees with that deduced from the nucleotide sequence of a cDNA clone derived from bovine ARPP-21 mRNA except for the NH_2 -terminal residue and position-25 (Kurihara et al., 1989). The differences detected between the amino acid and nucleotide sequences of ARPP-21 demonstrate the need for sequencing at both the polypeptide and polynucleotide levels. Both differences could result from posttranslational modification of ARPP-21B, i.e., by aminopeptidase cleavage of the NH_2 -terminal initiator methionyl residue in the nascent protein (Yoshida and Lin, 1972) or removal of an N^α -acetylmethionyl residue by an acetylaminocylhyrolase (Wold, 1984) and by chemical deamidation of an asparaginyl residue at position 25, either *in vivo* or during purification and sequencing. In fact, an NH_2 -terminal initiator methionyl residue appears to be preferentially cleaved when followed by a seryl (as in ARPP-21B), alanyl, glycyl, or valinyl residue (Boissel et al., 1985), and retained when followed by a charged residue [*cf.* DARPP-32, in which the preserved initiator methionyl residue is followed by an aspartyl residue (Williams et al., 1986; Kurihara et al., 1988)].

The differences observed between the amino acid and nucleotide sequences of ARPP-21 may also be related to the biochemical heterogeneity of ARPP-21 (Hemmings and Greengard, 1989). Thus, separate mRNA species derived from distinct genes could code for the 2 isoforms (ARPP-21A and ARPP-21B) identified in bovine brain (Hemmings and Greengard,

1989). If so, the ARPP-21 cDNA sequence determined by Kurihara et al. (1989), which contains an asparagine at position 25, might be derived from ARPP-21A mRNA, while the amino acid sequence was determined using purified ARPP-21B. Such a difference between ARPP-21A and ARPP-21B would be consistent with their elution from anion-exchange resins in which ARPP-21A elutes earlier and is therefore less acidic. However, it is unlikely that the 2 mRNA species identified for ARPP-21 represent separate messages for ARPP-21A and ARPP-21B as discussed in the accompanying paper (Kurihara et al., 1989). Determination of the structural difference between ARPP-21A and ARPP-21B will require direct amino acid sequencing studies of ARPP-21A.

Comparison of the amino acid sequence of ARPP-21B with the sequences in the Protein Sequence Database (5407 sequences, Release 17.0) and the New Sequences in Preparation Database (3181 sequences, Release 35.0) of the Protein Identification Resource using a computer-based survey revealed no significant similarities with other sequences (no initial sequence similarity score >52). In particular, there was no similarity between the amino acid sequences of ARPP-21B and DARPP-32 (Williams et al., 1986), another cAMP-regulated phosphoprotein which is enriched in dopamine-innervated brain regions (Hemmings et al., 1984; Ouimet et al., 1984; Walaas and Greengard, 1984; Hemmings and Greengard, 1986).

The predicted secondary structure of ARPP-21 is shown in Figure 7. Consistent with hydrodynamic studies (Hemmings and Greengard, 1989), ARPP-21 is predicted to have an extended conformation. The phosphorylation site (Ser-55) is located within a β -turn, a structural feature of a number of substrates for cAMP-dependent protein kinase (Small et al., 1977; Matsuo et al., 1978), including DARPP-32 (Williams et al., 1986). An important role for secondary structural features in the phosphorylation of ARPP-21 by cAMP-dependent protein kinase is suggested by a study of the phosphorylation of a synthetic peptide containing residues 49–61 of ARPP-21. This peptide, which does not include the predicted α -helix and β -sheet structures which flank the phosphorylation site, has a 50-fold higher apparent K_m value than intact ARPP-21B, possibly due to the absence of a β -turn.

The function of ARPP-21 is unknown. Its interesting regional distribution in the rat CNS (Ouimet et al., 1989) and the regulation of its state of phosphorylation in intact neurons by a cAMP analog, forskolin, or vasoactive intestinal peptide (Girault et al., 1988; Hemmings et al., 1989) suggest a role as an intracellular messenger involved in signal transduction in a specific subset of neurons related to the dopamine system. The determination of the primary structure of ARPP-21 and its

cDNA (Kurihara et al., 1989) will allow further studies of the role of this phosphoprotein in signal transduction by a number of powerful techniques, including the biochemical analysis of synthetic peptides based on the complete amino acid sequence, the production of phosphorylation-state-specific antibodies to synthetic phosphopeptides, site-directed mutagenesis, and *in situ* hybridization.

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