Purification of Proline-rich Proteins from Parotid Glands of Isoproterenol-treated Rats

Joseph Muenzer, Carolyn Bildstein, Michael Gleason, and Don M. Carlson

From the Department of Biochemistry, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106, and Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907


The Journal of Biological Chemistry

Prolonged isoproterenol treatment of rats is known to cause hypertrophy and hyperplasia of the parotid glands. Our results show that a dramatic increase in the synthesis or accumulation in the parotid glands of a series of proteins rich in proline also occurs with isoproterenol treatment. After 10 days of treatment (5 mg of isoproterenol/day) these proline-rich proteins (PRPs) comprise more than 50% of the total soluble proteins in parotid gland homogenates. The PRPs are rapidly labeled in vivo by a single intraperitoneal injection of [3H]proline with maximum incorporation occurring at about 3 h. More than 90% of the [3H]proline found in parotid gland homogenates is incorporated into PRPs with less than 1% of the radioactivity in a-amylase. Tritium incorporated into PRPs was isolated as [3H]proline after acid hydrolysis. One acidic and six basic [3H]-labeled PRPs were isolated from the 100,000 x g supernatant fraction of parotid gland homogenates by Sephadex G-100 and ion exchange chromatography. The six basic proteins accounted for about 90% of the total PRPs isolated.

Proteins rich in proline have been isolated from human parotid gland saliva (1-9), and from rat (10) and monkey (11) parotid glands. These proteins characterize contain not only high amounts of proline (25 to 40%), but also high quantities of glycine, glutamic acid or glutamine, and aspartic acid or asparagine. The contents of aromatic and sulfur-containing amino acids are very low or not detected. The isolation and partial characterization of the proline-rich protein (PRP) from rat parotid glands resulted from observations following isoproterenol treatment (10), which is known to cause hypertrophy and hyperplasia of the parotid glands (12-15). The amount of this protein, undetected in normal gland extracts, increased dramatically.

Upon stopping the isoproterenol treatment, both the size of the parotid glands and the amounts of proline-rich protein returned to normal within about 10 days. The amino acid composition of this proline-rich protein was strikingly similar to the analysis reported by Amsterdam et al. (15) for a zymogen granule membrane fraction from normal rat parotid glands. There have been recent reports on the detection of different types of proline-rich proteins associated with the secretory granule membrane and in the contents of the secretory granule (16-18). The proline-rich protein fraction detected by Keller et al. (16) in the contents of the secretory granule appears to be similar to the proline-rich protein isolated earlier (10) and to some human salivary proline-rich proteins (2, 3). In addition, proline-rich proteins apparently are loosely associated with secretory granule membranes (17, 18) and these differ from the proline-rich proteins previously described. The basic proline-rich proteins isolated and characterized in these and subsequent studies (19) are similar to the membrane-associated proline-rich proteins. The presence of the proline-rich proteins in saliva and their association with secretory granule membranes suggest that these proteins may be involved in the exocrine secretory reaction and possibly as structural components of the secretory granule membrane.

In this study, an in vivo labeling procedure was developed using [3H]proline which facilitated the identification and isolation of the proline-rich proteins since these proteins exhibit little or no absorbance at 280 nm and are not fixed upon staining of polyacrylamide gels. This paper reports on the isolation of several [3H]proline-rich proteins from parotid glands of isoproterenol-treated rats and a subsequent paper (19) describes the physical and chemical properties of these unusual proteins. Proline-rich proteins in parotid gland homogenates of isoproterenol-treated rats accounted for greater than 50% of the total soluble protein fraction.

EXPERIMENTAL PROCEDURES

Materials and Methods—Isoproterenol-HCl and phenylmethanesulfonfluoride were from Sigma. [3H]Proline was from New England Nuclear (specific activity, 30 to 50 Ci/mmol). NCS reagent was from Research Products International Corp. Blue dextran was from Pharmacia. 2,5-Diphenyloxazole (PPO) and 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) were from Packard. Unless otherwise indicated all chemicals and materials were obtained from commercial sources.

Liquid scintillation counting was used to detect radioactivity. Tissue homogenates and membrane fractions were solubilized with NCS reagent prior to counting. The tissue sample (0.1 ml) and NCS reagent (0.9 ml) which was previously diluted 10% with distilled water were incubated at 37°C for 4 to 8 h prior to adding 10 ml of toluene scintillation fluid (7 g of 2,5-diphenyloxazole and 0.5 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene/liter). Radioactivity in soluble samples was detected by adding 1 part of sample plus water to 8 parts of toluene-Triton X-100 scintillation fluid (2 parts of toluene and 1 part of Triton X-100, 8.0 g of 2,5-diphenyloxazole/liter, and 0.25 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene/liter). Radioactivity in membrane fractions was determined by counting 0.1 ml of sample directly. The tissue sample (0.1 ml) and NCS reagent (0.9 ml) were added to 10 ml of toluene-Triton X-100 scintillation fluid (2 parts of toluene and 1 part of Triton X-100, 8.0 g of 2,5-diphenyloxazole/liter, and 0.25 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene/liter).

Whatman diethylaminoethyl (DEAE, type DE32) and carboxymethyl (CM, type CM29) celluloses were from Reeve Angel and Co. Before use, DEAE-cellulose was washed successively in 0.5 N HCl, distilled water until pH 4 was obtained, 0.5 N NaOH, distilled water,
and then the starting buffer for the chromatography until the pH and conductivity of the eluate matched that of the buffer. CM-cellulose was washed successively in 0.5 N NaOH, distilled water until pH 8 was obtained, 0.5 N HCl, distilled water, and then washed with starting buffer similar to the DEAE-cellulose. All ion exchange cellulosics were packed under gravity.

Amino Acid Analysis—Amino acid analysis was performed on a Beckman model 119 analyzer using single column methodology. Standards containing 0.1 to 0.2 mg of protein in 1 ml of 6 N HCl were flushed with N₂ six times and sealed under vacuum. Samples were hydrolyzed at 110°C for 22 h. After hydrolysis, norleucine was added as an internal standard and the sample was dried by rotary evaporation. Samples (0.5 ml) were applied to a Beckman Autosampler. The peak areas were computed by an Infotronics CRS 110 integrator. Analyses are corrected for the destruction of serine and threonine, 15% and 8%, respectively.

The identification of the radioactive amino acids in the hydrolysate was made by using a Technicon amino acid analyser fitted with a stream splitter.

**RESULTS**

The time course of in vivo [3H]proline incorporation into the soluble fraction of parotid glands of rats treated with isoproterenol for 10 days is shown in Fig. 1. Animals given an injection of [3H]proline (250 μCi) were killed at the time periods indicated. Each time point represents the radioactivity incorporated by the parotid glands of a single animal. In four such in vivo labeling studies, tritium incorporation was always maximum at 3 h with only 10 to 30% of the label remaining after 8 h. Between 1 and 2% of the injected [3H]proline was incorporated into proline-rich proteins within 3 h. The specific activity (counts per min per mg of soluble protein) in extracts of parotid glands of rats treated for 10 days with isoproterenol was about 6-fold greater than similar preparations from normal animals.

In two experiments using ³H-labeled glands (six animals in each experiment), particulate, membranous materials were washed and sonicated to determine the distribution of radioactivity incorporated into the soluble and particulate cell fractions. More than 90% of the tritium in the gland homogenate was recovered in the combined soluble fractions from the first and second washes. The pellet, after three washes, contained less than 3% of the tritium of the crude homogenate.

The isolation and subsequent identification of proteins rich in proline from parotid glands were greatly facilitated by the in vivo incorporation of [3H]proline. The elution profile of the 100,000 × g supernatant fluid from parotid glands of rats injected with [3H]proline (Fig. 2) shows most of the tritium eluting as a symmetrical peak (Ipr-l) at an apparent molecular weight of about 70,000. More than 90% of the tritium recovered from the column was in this peak and more than 99% of the tritium incorporated into Fraction Ipr-1 was identified as [3H]proline by amino acid analysis after acid hydrolysis. A small amount (<3%) of radioactivity was always detected eluting with the salt volume of the column. This was determined to be free proline.

The profile of 280 nm absorbance shows protein peaks characteristic of human salivary secretions (29). There was little 280 nm absorbance eluting with the major radioactive peak. However, the radioactive peak (Ipr-1) eluted coincidental with the major 280 nm absorbance. Assuming that all soluble parotid gland proteins have similar molar extinction coefficients at 290 nm, the radioactive peak (Ipr-1) eluted with the most abundant protein peak.

To determine whether the decrease of [3H]proline incorporation after 3 h (Fig. 1) resulted from glandular secretion, the main ducts from the parotid glands were cannulated and pilocarpine was injected intravenously as a secretagogue. Pilocarpine was injected intravenously as a secretagogue. The profile of 280 nm absorbance shows protein peaks characteristic of human salivary secretions (29). There was little 280 nm absorbance eluting with the major radioactive peak. However, the radioactive peak (Ipr-1) eluted coincidental with the major 280 nm absorbance. Assuming that all soluble parotid gland proteins have similar molar extinction coefficients at 290 nm, the radioactive peak (Ipr-1) eluted with the most abundant protein peak.

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The abbreviations used are: Ipr, isoproterenol; Ipr used as a prefix, such as Ipr-1B, indicates specific protein fractions isolated from isoproterenol-treated rats; FRP, proline-rich protein.

**Fig. 1.** Time course of in vivo incorporation of [3H]proline into rat parotid gland after isoproterenol treatment.
ocarpine stimulated the secretion of about 50% of the radioactivity from parotid glands of rats labeled with [\(^{3}H\)]proline. The amount of [\(^{3}H\)]proline remaining in the glands was determined by removal of the glands and counting an aliquot of the crude homogenate. More than 95% of the radioactivity secreted upon stimulation with pilocarpine chromatographed on Sephadex G-100 similar to Fraction Ipr-1 (Fig. 2).

Dialysis of Ipr-1 against 50 mM sodium acetate at pH 4.7 for either 24 or for 48 h left more than 98% of the tritium within the dialysis bag. Flocculent material which appeared was pelleted by centrifugation and contained less than 1% of the tritium. It was important to use low molecular weight cutoff dialysis membranes (approximately 3,000) since losses of up to 15% of radioactive protein were observed in the dialysis medium after 24 h using standard dialysis membranes with about 12,000 molecular weight cutoff. The relatively high axial ratio of greater than 25 (19) could possibly contribute the ability of these asymmetric proteins to permeate the dialysis bag.

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Fraction Ipr-1A was applied to a column of DEAE-cellulose. Radioactivity eluted at an ionic strength of 0.11 and was coincident with 230 nm and 280 nm absorbing material (Fig. 4). Recovery of tritium in this peak was greater than 95%. This material (Ipr-1A) was desalted using Sephadex G-25. About 70 to 80% of the tritium eluted in the void volume (Ipr-1A) as expected, while the remaining 20 to 30% was retarded and eluted as a broad peak (Ipr-1Aa) (Fig. 5).

Fractions Ipr-1B1, -1B2, -1B3, and -1B4 were rechromatographed separately on CM-cellulose columns and elution profiles of Ipr-1B1, -1B2, and -1B3 are shown in Fig. 6. Each fraction eluted as a single symmetrical peak with radioactivity and 230 nm absorbance coincident and at the same salt concentrations as observed in the first CM-cellulose separation. Fractions Ipr-1B1 and -1B2 were combined and rechromatographed (data not shown). Two peaks were observed eluting at the same sodium acetate concentrations as in the first CM-cellulose chromatography. No 280 nm absorbance was detected eluting from the column between 0.1 and 0.3 M sodium acetate (Fig. 3). The relative amounts of these six basic proteins, primarily Ipr-1B1 through Ipr-1B6, varied with different preparations but each protein eluted at the same salt concentration.

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in Fig. 3. More than 90% of the tritium in Ipr-1 absorbed to the column and material not absorbed was designated Ipr-1A. At least six protein peaks (Ipr-1B, through Ipr-1B6), as detected by radioactivity and 230 nm absorption, were eluted from the column by a linear gradient of sodium acetate. In all experiments, the 230 nm absorbing peaks were coincident with radioactivity. No compounds absorbing at 280 nm were detected eluting from the column between 0.1 and 0.3 M sodium acetate (Fig. 3). The relative amounts of these six basic proteins, primarily Ipr-1B1 through Ipr-1B6, varied with different preparations but each protein eluted at the same salt concentration.

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was detected in any radioactive peak with recoveries of tritium greater than 90% from all columns.

Initial attempts to desalt Fractions Ipr-1B through -1B9 by dialysis against distilled water resulted in poor recovery (<50%). The fractions were, however, successfully desalted using Sephadex G-25, but there was a considerable retardation of all fractions. Apparently the relative affinities to Sephadex G-25 are affected by the basicity of the proteins when equilibrated with water and the more basic proteins are retarded. When Ipr-1B1 was chromatographed on Sephadex G-25 equilibrated with 25 mM ammonium bicarbonate at pH 7.4, radioactivity and 230 nm absorbance eluted at the void volume of the column. These data suggest a relatively strong interaction between these basic proteins and the Sephadex matrix.

The reproducibility of the isolation procedure was confirmed by dividing the crude homogenate of parotid glands from one isoproterenol-treated rat labeled with [3H]proline into two equal amounts and carrying out the isolation procedure on each portion separately. Sephadex G-100 and CM-cellulose column chromatographic profiles were essentially identical for each preparation. The single peaks obtained upon rechromatography of the individual proteins on CM-cellulose columns (Fig. 6) would seem to exclude interconversion, or a precursor-product relationship, among these proteins.

The possibility of proteolysis occurring during the isolation procedure was examined both by homogenizing parotid glands in the presence of a serine protease inhibitor, phenylmethylsulfonylfluoride, and by allowing a portion of the crude homogenate to incubate at 30°C for 30 min. Again, in each case, the elution profiles on both columns were similar to previous results suggesting that the multiple proline-rich protein species do not result from proteolysis during the isolation procedure. However, the possibility of proteolysis at some phase of synthesis cannot at this time be excluded.

The extent of the biological variability for the [3H]proline-rich proteins from parotid glands of rats was investigated by isolating proteins from individual rats labeled for 1, 3, and 4 h after a single injection of [3H]proline. All previous experiments for isolating proline-rich proteins utilized parotid glands from four to six rats of which two to three were labeled for 3 h with [3H]proline. The chromatographic profiles from these experiments were essentially the same except for the increase in tritium incorporation with time.

Results of the isolation procedure are summarized in Table 1.

![Fig. 5. Desalting of Ipr-1A. Fraction Ipr-1A was lyophilized and dissolved in 5 ml of H2O. The sample was loaded onto a Sephadex G-25 column (2.5 x 30 cm) equilibrated with H2O. Fractions of 3 ml were collected. The indicated fractions were pooled.](https://example.com/Fig5.jpg)

![Fig. 6. CM-cellulose chromatography of Ipr-1B1 through Ipr-1B9. Fractions Ipr-1B1, -B2, -B3 were diluted with water to reduce the salt concentration to between 50 to 100 mM sodium acetate. Each of the diluted fractions was chromatographed on a CM-23 cellulose column. The columns were washed with 10 to 40 ml of 50 mM sodium acetate at pH 4.7 and eluted with a linear gradient of 0.05 to 0.4 M sodium acetate. Fractions of 2 ml were collected.](https://example.com/Fig6.jpg)

**Table 1**

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* Protein determined from duplicate amino acid analysis.

<sup>b</sup> 100,000 x g supernatant.

* Assumed 90% of the radioactivity was incorporated into proline-rich proteins with a specific activity of 81,000 dpm/mg of protein.

* Assumed all of the radioactivity was incorporated into proline-rich proteins with a specific activity of 81,000 dpm/mg of protein.

* The radioactivity and protein concentration were determined after desalting on Sephadex G-25 columns.
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FIG. 7. Sephadex G-100 chromatography of extracts from submandibular glands. Submandibular glands from six isoproterenol-treated rats, three of which were labeled with 250 µCi of [3H]proline for 3 h, were homogenized and centrifuged (see "Experimental Procedures"). The 100,000 x g supernatant was fractioned on Sephadex G-100.

FIG. 8. Sephadex G-100 chromatography of pancreas extracts. Pancreas from six isoproterenol-treated rats, three of which were labeled with 250 µCi of [3H]proline for 3 h, were homogenized and centrifuged. The 100,000 x g supernatant was fractioned on Sephadex G-100.

FIG. 9. Sephadex G-100 chromatography of extracts of normal parotid glands. Parotid glands from rats labeled with [3H]proline for 3 h were homogenized and centrifuged. The 100,000 x g supernatant was fractioned on Sephadex G-100.

FIG. 10. CM-Cellulose chromatography of Fraction N-1. Fraction N-1, after dialysis against 50 mM sodium acetate, pH 4.7, was loaded on a CM-23 cellulose column and eluted as previously described. Small amounts of radioactive material eluted from the column coincidental with 230 nm absorbance and at the same salt concentration as Peaks Ipr-1B1 and Ipr-1B2 (Fig. 3).

DISCUSSION

This study shows a dramatic increase in a series of proline-rich proteins occurring concomitantly with hyperplasia and hypertrophy of the parotid glands of rats treated with isoproterenol. Other studies (23, 24) show a 6- to 10-fold increase in the specific activity of UDP-galactose:N-acetylgalcosamine galactosyltransferase, but no change in the activity of UDP-galactose:N-acetylgalactosamine galactosyltransferase. Presently, the relationship between these events is only a temporal involvement and biological inter-relationship must await further study. However, the purification of these unusual proteins was necessary in order to establish their chemical and physical properties.

Initial experiments on the changes in protein compositions of parotid glands of rats treated with isoproterenol clearly showed the presence of either a new protein, or a large increase in a constitutive component, which was identified as a proline-rich glycoprotein (10). This compound was acidic as judged by migration on polyacrylamide gels and contained no aro-
motic or sulfur-containing amino acids. Destaining of the gels resulted in a rapid loss of the band attributed to this proline-rich protein and in vivo labeling with radioactive proline was used in an attempt to label in a specific manner this protein. As a result several[^1^H]proline-rich proteins were detected, the acidic protein and at least six highly basic proteins with isoelectric points higher than 10 (19). More than 90% of the[^1^H]proline found in the soluble fraction of parotid gland homogenates was incorporated into the proline-rich proteins.

Apparently the series of basic PRPs (Ipr-1B, through Ipr-1B₃) are all distinct proteins since interconversions or precursor-product relationships have not been observed. The relative amounts of Ipr-1B through Ipr-1B₃ changed, but each protein in this series eluted at the same ionic strength from CM-cellulose. It should be noted that with all of the basic PRPs less than 5% contamination with other proteins was present as determined by polyacrylamide gel electrophoresis. When 100 µg of each PRP fraction was electrophoresed and destained, no bands staining for protein were observed. Controls indicated that < 2 pg of serum albumin could be detected. Additional evidence for purity is given in the accompanying paper (19).

Following isoproterenol treatment, PRPs account for more than 50% of the total soluble protein in homogenates of rat parotid glands. A comparison of PRPs in glands of normal and isoproterenol-treated animals suggests that glands from the isoproterenol-treated animals exhibit a greater than 10-fold increase of PRPs on a wet weight basis. In isoproterenol-treated animals, the ratio of the radioactivity of the basic protein to the acidic protein (Ipr-1A) is about 20:1.

Stopping isoproterenol treatment results in a rapid decrease of the PRPs, which reach normal patterns in about 7 days. The biological role of these proteins remains to be determined. A structural role in the zymogen granule membrane (10) and a role in dental maintenance (27) have been proposed.

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