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Acetylation of Prostaglandin Synthetase by Aspirin. Purification and Properties of the Acetylated Protein from Sheep Vesicular Gland†

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ABSTRACT: We previously presented evidence that aspirin (acetylsalicylic acid) inhibits prostaglandin synthetase by acetylating an active site of the enzyme. In the current work, we have labeled the enzyme from an acetone–pentane powder of sheep vesicular gland using [*acetyl*-^{3}H]aspirin and purified the [^{3}H]acetyl-protein to near homogeneity. The final preparation contains protein of a single molecular weight (85 000) and an amino-terminal sequence of Asp-Ala-Gly-Arg-Ala. The [^{3}H]acetyl-protein contained 0.5 mol of acetyl residues per mol of protein based on amino acid composition but only a single sequence was found.

The first enzyme of prostaglandin biosynthesis is a membrane-bound dioxygenase prostaglandin synthetase (prostaglandin cyclooxygenase, prostaglandin synthetase) (Samuelsson, 1972). The enzyme catalyzes the conversion of an essential fatty acid precursor, arachidonic acid (20:4), to a cyclic endoperoxide intermediate, prostaglandin G_2 (PGG_2) (Nugteren and Hazelhof, 1973; Hamberg and Samuelsson, 1973; Hamberg et al., 1974). Several groups have published studies concerning purification of the enzyme (Samuelsson et al., 1967; Miyamoto et al., 1974, 1976; Chan et al., 1975; Rome and Lands, 1975). Most recently, Miyamoto and co-workers reported a 750-fold purification of the enzyme from bovine vesicular gland microsomes and studied the effects of activators such as hematin and tryptophan on the activity of the enzyme (Miyamoto et al., 1976).

The anti-inflammatory drug, aspirin (acetylsalicylic acid), inhibits prostaglandin synthetase in a highly specific manner at micromolar concentrations (100 μM) within minutes (60 min) (Smith and Lands, 1971; Hamberg and Samuelsson, 1974; Hamberg et al., 1974; Roth et al., 1975; Roth and Majerus, 1975). Aspirin is an acetylating agent (Pinckard et al., 1968; Hawkins et al., 1969) and our studies indicate that the drug acetylates prostaglandin synthetase (Roth et al., 1975; Roth and Majerus, 1975). Furthermore, acetylation by aspirin is highly specific and proceeds under the same conditions as the drug's inhibitory effect on enzyme activity (μM concentration within min) (Roth et al., 1975; Roth and Majerus, 1975). Based on time course, substrate inhibition and copurification experiments, we have concluded that aspirin acetylates an active site of prostaglandin synthetase (Roth et al., 1975; Roth and Majerus, 1975; Rome et al., 1976) and that only active enzyme is susceptible to acetylation by aspirin. Therefore, [*acetyl*-^{3}H]aspirin can be used to label the enzyme specifically, providing an easily identifiable, covalently bound tritium marker for purification work. The acetylated portion of the enzyme has a molecular weight of 85 000 (Roth et al., 1975; Roth and Majerus, 1975).

In the current work, we have purified the aspirin-acetylated portion of prostaglandin synthetase from sheep vesicular gland and determined the amino acid compostion and amino-terminal sequence.

Experimental Procedure

Materials. Nonidet P40 (NP40)[1] was obtained from Particle Data Laboratories, Elmhurst, Ill. DEAE[2]-cellulose (DE52) was obtained from Whatman, and agarose (Bio-Gel A-1.5m 200–400 mesh) was obtained from Bio-Rad. Sodium dodecyl sulfate was purchased from British Drug House, and Ampholine carrier ampholyte was purchased from LKB. All other chemicals not previously described (Roth et al., 1975; Roth and Majerus, 1975) were reagent grade.

Enzyme Source. Sheep vesicular glands were kindly pro-

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[1] Shell trademark for polyoxyethylene glyco(9)-*p*-*tert*-octylphenol.

[2] Abbreviations used: PGG_2, prostaglandin G_2; NP40, Nonidet P40; DE52, DEAE-cellulose; Pth, phenylthiohydantoin; TLC, thin-layer chromatography; DEAE, diethylaminoethyl.

vided by Dr. Donald P. Wallach of Upjohn Co., Kalamazoo, Mich. Acetone–pentane powders of the tissue were prepared and used as the starting material for purification (Wallach and Daniels, 1971).

[Acetyl-^{3}H]Aspirin. [*acetyl*-^{3}H]Aspirin (180 Ci/mol) was synthesized from [^{3}H]acetic anhydride (New England Nuclear) and salicylic acid as described (Roth et al., 1975; Roth and Majerus, 1975). The preparation contained 97% aspirin and 3% salicylic acid by weight, and gas–liquid radiochromatographic analysis indicated that all tritium radioactivity was present in aspirin.

Enzyme Content. Prostaglandin synthetase content was measured by a sodium dodecyl sulfate gel electrophoresis method (Roth et al., 1975; Roth and Majerus, 1975). Preparations containing active enzyme were incubated with [*acetyl*-^{3}H]aspirin (180 Ci/mol) and, following complete acetylation (100 μM aspirin, 90 min, 37 °C), proteins were subjected to sodium dodecyl sulfate gel electrophoresis. A radiochromatogram of each gel was obtained by extracting labeled protein from gel slices and measuring radioactivity by scintillation counting (Roth et al., 1975; Roth and Majerus, 1975). Molecular weight was estimated by the migration of the labeled protein in sodium dodecyl sulfate gels in comparison with that of proteins of known molecular weight (Roth and Majerus, 1975). Enzyme content was determined as the amount of [^{3}H]acetate radioactivity bound to the 85 000 molecular weight protein.

Assuming that [*acetyl*-^{3}H]aspirin (180 Ci/mol) acetylates a single residue per mole of protein, the fully acetylated protein would contain 4.7×10^6 dpm/mg under our assay conditions. In highly purified enzyme preparations (after isoelectric focusing), essentially all tritium radioactivity was protein-bound, permitting assay of enzyme content by direct measurement in a scintillation counter.

Amino Acid Analysis. The final enzyme preparations were dialyzed against distilled water for 4 h, and aliquots were assayed for radioactivity by scintillation counting in Bray's solution (Bray, 1960). The samples (150 μg of protein) were lyophilized and then hydrolyzed in 6 N HCl in sealed evacuated tubes for 24 or 48 h. Amino acid analysis was performed using a Beckman 120C amino acid analyzer adapted for single column methodology.

Amino-Terminal Sequence Analysis. A sample (200 pmol of [^{3}H]acetyl protein) was lyophilized and subjected to amino-terminal analysis on a Beckman 890C sequencer by the method of Jacobs and Niall (1975). The coupling reactions were performed with 2% (v/v) phenyl [^{35}S]isothiocyanate (Amersham/Searle), 5 mCi/mmol, in heptane. Following identification of phenylthiohydantoin (Pth) derivatives by thin-layer chromatography (TLC) and autoradiography (Jacobs and Niall, 1975), Pth derivatives were quantitated by scraping the silica gel region containing radioactivity into Bray's solution (Bray, 1960) and measuring ^{35}S radioactivity by scintillation counting. Counting efficiency was 94% in this system. Spots were scraped from the one-dimensional TLC system (Jacobs and Niall, 1975) for quantitation of the Pth amino acids. One-half of the total sample was chromatographed in this system. A correction for background radioactivity was determined by scraping equivalent spots from tracks of other cycles corresponding to the identified amino acid (i.e., glycine background was determined by scraping the gel region corresponding to glycine from cycle 1 where Asp is the identified residue. The radioactivity in the "background" spot was subtracted from the radioactivity found in the glycine spot from cycle 3). Background radioactivity was 40% for glycine, and less than 20% for the other identified residues. Confirming identification of each residue was obtained by chromatography of the other half of the sample of each cycle in the appropriate two-dimensional TLC system (Jacobs and Niall, 1975). Arginine at cycle 4 was determined in the "basic" TLC system (Jacobs and Niall, 1975).

Purification Procedure. Acetylation. One gram of acetone–pentane powder was suspended by sonication in 50 mL of 0.1 M sodium phosphate buffer, pH 7.4, containing 3 mM reduced glutathione and 5 mM epinephrine bitartrate. Five milliliters of [*acetyl*-^{3}H]aspirin in distilled water was added to give a final concentration of 100 μM aspirin, and following incubation for 90 min at 37 °C, the suspension was centrifuged at 200 000*g* for 45 min at 4 °C. The supernate was discarded, and the pellets were suspended in water by sonication.

Solubilization. Sodium phosphate buffer, 0.5 M, and NP40 (20% v/v) were added to give a solution containing 0.01 M sodium phosphate, pH 7.0, and 1.25% NP40 in 40 mL, and, following incubation for 20 min at 37 °C, the suspension was centrifuged at 200 000*g* for 30 min at 4 °C. The supernatant solution containing the solubilized enzyme was saved.

DEAE-Cellulose Step. The solubilized enzyme (40 mL) was applied to a column (1.5 × 12 cm) of DEAE-cellulose equilibrated with 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1% NP40 at 4 °C, and the column was washed with the same buffer. The first 10 mL of eluate was discarded, and the next 80 mL of eluate, termed "DEAE eluate", was collected and dialyzed for 18 h at 4 °C against 0.0025 M sodium phosphate buffer, pH 7.0, containing 0.1% NP40.

Isoelectric Focusing. Isoelectric focusing was performed at 4 °C using a LKB 8101 (110 mL) column with a linear sucrose gradient containing 2% LKB Ampholine carrier ampholytes (0.4% pH 3.5 → 10, 1.6% pH 5 → 8). The entire DEAE eluate from the previous step was used to prepare the sucrose gradient (0 to 45%). Focusing was performed at 500 V for 48 h, and, at completion, current had fallen to a constant level of 1.4 mA. Fractions of 3 mL were collected from the column and those containing the peak of radioactivity (pH 6.6 → 7.2) were pooled and dialyzed for 18 h at 4 °C against 0.01 M sodium phosphate buffer containing 0.1% NP40.

Agarose Gel Filtration. The dialyzed pooled fractions were adjusted to contain 0.25 M sucrose, and the protein was precipitated by the addition of 4 volumes of acetone at 0 °C. The cloudy precipitate was stirred for 18 h at 4 °C and then collected by centrifugation at 16 000*g* for 10 min at 4 °C. The precipitate was solubilized by heating at 100 °C for 10 min in 2.0 mL of 0.01 M sodium phosphate buffer, pH 7.0, containing 1% sodium dodecyl sulfate and 0.1 M 2-mercaptoethanol. The sample was applied to a column (1.5 × 55 cm) of Bio-Gel A-1.5m equilibrated with 0.01 M sodium phosphate buffer, pH 7.0, containing 0.05% sodium dodecyl sulfate, 1 mM dithiothreitol, and 1 mM sodium azide at room temperature. The sample was eluted with the same buffer, and fractions (1.5 mL) containing the peak of radioactivity were pooled, concentrated, and rechromatographed on Bio-Gel A-1.5m in the same manner as outlined. All ^{3}H radioactivity from the isoelectric focusing column step appeared to be protein bound since all radioactivity applied to the agarose column eluted as a single peak just after the void volume.

The pooled peak fractions from the second agarose column constituted the final preparation and were analyzed for amino acid composition and amino-terminal sequence.

Protein was measured by the method of Lowry using a bovine serum albumin standard (Lowry et al., 1951).

Sodium Dodecyl Sulfate Slab Gel Electrophoresis. Sodium dodecyl sulfate slab gel electrophoresis was performed by the method of Laemmli (1970) using a 4% stacking gel and a 10%

TABLE I: Purification of Aspirin-Acetylated Protein from Sheep Vesicular Gland.

Fraction	Volume (mL)	Total protein (mg)	Total [*acetyl*-^{3}H] protein (dpm × 10^{-5})	Spec [*acetyl*-^{3}H] protein content (dpm × 10^{-4}/mg)	Recovery (%)	Purification (fold)
Starting material	56	640	84	1.3	100	
Solubilized fraction	40	69	61	8.8	72	6.8
DEAE eluate	80	18	42	23	50	18
Peak from isoelectric-focusing column	19	*a*	13		15	
Peak from second agarose column	6	0.47	7.2[b]	150	8.6	120

[a] Ampholyte interferes with protein determination. [b] Acetylated protein content of final preparation was determined directly in a scintillation counter. The content in other fractions was determined by a sodium dodecyl sulfate gel method (Roth et al., 1975; Roth and Majerus, 1975). The same results are obtained by the two methods when applied to highly purified samples (after isoelectric focusing).

TABLE II: Amino Acid Composition of Aspirin-Acetylated Protein.[a]

Amino acid	Moles/mole of [^{3}H]acetate: 24-h hydrolysate	48-h hydrolysate	Average
Lys	69	75	72
His	80	81	80
Arg	85	86	86
Asp	129	127	128
Thr	80	80	80
Ser	98	92	104[c]
Glu	174	156	165
Pro	117	111	114
Gly	145	145	145
Ala	84	84	84
Cys	ND	ND	
Val	69	85	85[d]
Met	37	36	37
Ile	52	69	69[d]
Leu	159	166	163
Tyr	60	64	62
Phe	90	93	91
Trp	ND	ND	
Total	1529	1550	
Mol wt	(171 400 g)	(172 800 g)	
Mol of [^{3}H]acetate/mol of protein[b]	0.5	0.49	

[a] Amino acid composition was determined as described in Experimental Procedure. Calculations were based on the amount of [^{3}H]acetate in the prehydrolysis sample; see Table I. [b] The calculation assumes a single acetylated residue per 85 000 molecular weight acetylated protein. [c] Extrapolated at zero time of hydrolysis. [d] 48-h value. ND, not determined.

separating gel. Proteins were stained by Coomassie blue.

Results

The results of a typical purification are summarized in Table I. Similar results were obtained on three occasions with the same final specific [^{3}H]acetyl-protein content in each preparation. Removal of soluble protein and solubilization of the membrane-bound enzyme with non-ionic detergent gave a 6.8-fold purification. The ion-exchange chromatography step is similar to that described by Miyamoto et al. (1974) for purification of prostaglandin synthetase from bull vesicular gland. The acetylated enzyme did not bind to the column under the conditions used, and the entire nonadherent fraction was used for the next step. The results of the isoelectric focusing step were similar to those found by Miyamoto et al. using active enzyme (Miyamoto et al., 1974, 1976). The acetylated enzyme was found between pH 6.6 and pH 7.2, compared with pH 6.9–7.3 for the active enzyme from bovine vesicular gland under slightly different conditions (Miyamoto et al., 1976). A visible precipitate formed after 6–12 h at the anode (lower pole) in our experiments and was associated with approximately 60% of the applied radioactivity. About two-thirds of the acetylated enzyme was lost during the isoelectric focusing step, perhaps due to protein precipitation. Whether the precipitated protein represents a different form of prostaglandin synthetase cannot be determined.

Agarose gel filtration was used for further purification of the acetylated protein and for the removal of ampholytes. The sample from the isoelectric focusing column eluted as a single major peak from the first agarose column. The A_{280} peak from the second agarose column corresponded closely to the peak of radioactivity suggesting that radioactive-labeled protein constituted the bulk of the final preparation.

Sodium dodecyl sulfate slab gel electrophoresis was performed on samples from various purification steps. A single major band of stained material was found in the sample from the isoelectric focusing step, and only one similar band was found in the samples from the agarose gel filtration steps. The band contained all of the radioactivity applied to the gel as determined by scintillation counting of protein extracted from gel slices and has an estimated molecular weight of 85 000. The migration of the protein in sodium dodecyl sulfate gels was identical whether or not the protein was reduced in 2-mercaptoethanol suggesting that the protein does not contain disulfide-linked subunits.

Amino Acid Analysis. The results of the amino acid analyses are summarized in Table II for 24- and 48-h hydrolysates of the final preparation from the second agarose column. Results are expressed as moles of amino acid per mole of tritiated acetyl group. This protein contains traces of both glucosamine and galactosamine which could not be quantitated accurately in the 24-h hydrolysates but represent less than 2% of the total protein.

Amino Acid Sequence. As shown in Figure 1 and Table III, the sequence of the first five amino-terminal residues is Asp-Ala-Gly-Arg-Ala. Because only small amounts of protein were available for analysis, we used one-half of the sample from each step for analysis by thin-layer chromatography. The identified residues were scraped from this plate and counted as described in Experimental Procedure. Recovery of each residue is shown in Table III. There is marked variability in recovery of individual residues, although it appears that approximately 2 mol

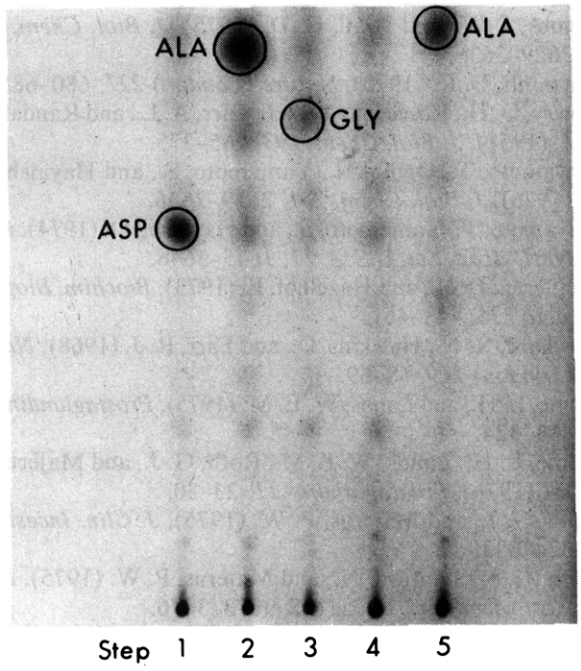

FIGURE 1: Autoradiograph of one-dimensional thin-layer chromatogram (Jacobs and Niall, 1975) of [^{35}S]Pth derivatives from amino acid sequence analysis. Solvent system is ethylene dichloride–glacial acetic acid (30:7). The film was exposed for 6 days. The arginine in cycle 4 was identified on a separate "basic" TLC chromatogram of the aqueous phase (Jacobs and Niall, 1975).

of protein is being sequenced/mol of acetyl group. The variability in quantitation of the individual Pth derivatives may be expected due to the extremely small amount of material available for analysis (tenfold less than previously studied using these methods) (Jacobs and Niall, 1975). Since previous studies have not used such small amounts of protein, we have no basis for deciding which of our identified residues might be most reliable for indicating the quantity of protein being sequenced. Thus individual values may be spuriously low because of adsorption to tubing or the cup of the sequencer or, conversely, spuriously high because of contamination with traces of radioactivity from other derivatives of the reagent. Additionally, the quantitation is only approximate since it is calculated using the stated specific activity of the phenyl isothiocyanate. We have reported our actual apparent recoveries since useful sequence information was obtained from trace amounts of protein even in the presence of excess sodium dodecyl sulfate. We emphasize that a single amino acid sequence was present since at each cycle of degradation only a single radioactive Pth residue, rising greater than 4-fold above background radioactivity, was identified except for cycle 3 where a 2.5-fold rise was seen.

Discussion

The specific [^{3}H]acetyl-protein content of 1.5×10^6 dpm/mg of the final preparation (Table I) can be compared with the calculated specific content of pure [*acetyl*-^{3}H]protein, 4.7×10^6 dpm/mg. The discrepancy can be explained in part by the fact that the extinction coefficient for the protein in the Lowry reaction is unknown (Lowry et al., 1951), and also by the presence of nonacetylated protein of 85 000 molecular weight in the final preparation. The nature of this nonacetylated protein is discussed below.

The fraction obtained following DEAE chromatography was analyzed by both sodium dodecyl sulfate gel electrophoresis and agarose gel filtration (data not shown) and was found to

TABLE III: Amino Acid Sequence Analysis of Aspirin-Acetylated Protein.[a]

Residue position	Residue	dpm	Recovery (mol/mol [^{3}H]acetate)
1	Asp	1110	1.5
2	Ala	2706	3.7
3	Gly	655	0.9
4	Arg	1570	2.1
5	Ala	1868	2.5

[a] A sample of the final preparation containing 200 pmol (8.7×10^4 dpm) of [*acetyl*-^{3}H]protein was analyzed (Jacobs and Niall, 1975). One-half of the sample from each cycle was fractionated by thin-layer chromatography as shown in Figure 1. Spots were scraped from the plate and counted as described in Experimental Procedure.

contain proteins of fairly uniform molecular weight (approximately 85 000). Therefore, the subsequent isoelectric focusing step provided considerable purification (Table I) by the removal of nonenzyme proteins of similar molecular weight. The majority of material from the isoelectric focusing step elutes from the first agarose column as a single peak (A_{280}) corresponding to the peak of tritium radioactivity. Only a small amount of lower molecular weight, nonlabeled protein is present. The results of the amino acid analysis (Table II) suggest that only half of the protein in the final preparation is acetylated. The sequence data indicate a single major peptide chain in the final preparation, implying that the material is relatively homogeneous with only half the molecules being acetylated. An alternative possibility, not excluded by our results, is that the enzyme is composed of two nonidentical subunits of similar molecular weight, one with a blocked amino-terminal residue.

After this work was completed, a paper appeared (Hemler et al., 1976) which described purification of the active form of cyclooxygenase from sheep seminal vesicles. These authors used a purification scheme similar to ours and achieved a 230-fold purification of enzyme from an acid precipitin fraction of the tissue. This compares with our 120-fold purification of acetyl–protein from an acetone–pentane powder of this same acid precipitin fraction. The activity of the pure enzyme was 46 000 nmol of O_2 consumed per mg of protein per min. In a collaborative study (Rome et al., 1976), we measured the acetylation by aspirin of the post-DEAE fraction in this purification and found 0.14 pmol of acetyl group/nmol of O_2 consumed. Therefore, the pure preparation would correspond to 6.4 nmol of acetyl group/mg of protein or 0.54 mol of acetyl group per 85 000 g of protein, a result in agreement with the current findings. Additionally, these authors established that only one-sized subunit was required for activity. They reported the molecular weight as 70 000 compared with our value of 85 000.

There are several possible explanations for the finding of less than 1 acetyl group per mol of protein. First, inactive enzyme if present in the starting material may copurify with acetylated protein. Second, hydrolysis and loss of protein-bound [^{3}H]acetyl groups may have occurred leading to a reduction in the recovery of the acetylated protein. However, the acetyl–protein bond is resistant to 2 M neutral hydroxylamine for 6 h at 37 °C, and therefore the acetyl group appears to be in a relatively stable linkage. Third, incomplete acetylation of susceptible sites during the initial incubation with [*acetyl*-^{3}H]aspirin could explain the finding. However, the time course of aspirin-mediated acetylation and enzyme inhibition shows greater than 90% acetylation and inhibition by 100 μM aspirin

at 90 min (Roth et al., 1975) and furthermore labeling of the enzyme after solubilization did not result in increased labeling. Finally, it is possible that there are traces of several contaminating proteins in the preparation, none of which is present in great enough quantity to be detected in our sequence experiment.

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