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Isolation and Covalent Structure of the Aspirin-Modified, Active-Site Region of Prostaglandin Synthetase†

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ABSTRACT: Aspirin (acetylsalicylic acid) inhibits prostaglandin synthesis by acetylating a single internal serine residue of the initial enzyme in the biosynthetic pathway, prostaglandin synthetase. In this study, the region of the enzyme that is modified by aspirin has been isolated, and its amino acid sequence has been determined. Sheep vesicular gland [*acetyl*-^{3}H]prostaglandin synthetase was purified following treatment with [*acetyl*-^{3}H]aspirin and digested with pepsin. An acetyl-^{3}H-labeled peptic peptide of approximately 25 residues was isolated by high-pressure liquid chromatography, and its amino acid sequence was determined to be Ile-Glu-Met-Gly-Ala-Pro-Phe-Ser-Leu-Lys-Gly-Leu-Leu-Gly-Asn-Pro-Ile-Glu-Ser-Pro-Glu-Tyr. The acetylated serine residue was located at position 8 in this sequence. The current study marks this polypeptide sequence as a region related to an active site of the enzyme.

Prostaglandin (PG)[1] synthetase (cyclo-oxygenase, prostaglandin endo-peroxide synthetase) catalyzes the first step in prostaglandin synthesis: the oxygenation of arachidonic acid to PGG_2 and the subsequent peroxidase-mediated reduction of PGG_2 to PGH_2 (Sammuelsson, 1972; Nugteren & Hazelhof, 1973; Miyamoto et al., 1976; Van der Ouderaa et al., 1977). The enzyme has been purified and characterized as a labile, membrane-bound, heme protein, M_r 70000, which requires an indole or phenol cofactor for full enzymatic activity in vitro (Rollins & Smith, 1980; Roth et al., 1981; Ohki et al., 1979; Smith & Lands, 1971).

Aspirin (acetylsalicylic acid) inhibits the oxygenase activity of cyclo-oxygenase by acetylating a single internal serine residue located somewhere within the polypeptide chain (Roth et al., 1975, 1980; Roth & Siok, 1978). Van der Ouderaa et al. have isolated a dipeptide, Phe-Ser, that contains the acetylated serine residue (Van der Ouderaa et al., 1980), but no other published sequence information is available for this region of the enzyme. In this study, we have used pepsin digestion to obtain a peptide of approximately 25 residues that contains the acetylated serine at position 8. The sequence data from this study document the fact that the acetylated serine is an internal residue and serves to mark this portion of the polypeptide chain as an active-site region of the enzyme.

Experimental Procedures

Preparation of Acetyl-^{3}H-Labeled Peptic Peptide. Prostaglandin synthetase was purified in the acetylated, ^{3}H-labeled form as described (Roth, 1982) through the first DEAE-

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[1] Abbreviations: PG, prostaglandin; HPLC, high-pressure liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; PTH, phenylthiohydantoin.

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cellulose step. Frozen ram seminal vesicles (Wellington Abattoir, Wellington, New Zealand), 25–35 g, were homogenized in buffer, and microsomes were obtained by centrifugation. The microsomal enzyme was suspended in buffer and treated with 100 μM [*acetyl*-^{3}H]aspirin, and the preparation was recentrifuged. The resulting microsomes were solubilized in 2% Tween-20, and following centrifugation, the supernatant material was subjected to DEAE-cellulose chromatography to give a highly purified enzyme preparation (80–90% of protein is [*acetyl*-^{3}H]prostaglandin synthetase). The eluate from the first DEAE-cellulose column was concentrated 6-fold by absorption dialysis with propylene glycol and mixed with appropriate volumes of deoxycholate and Triton X-100 to give a final concentration of 3% deoxycholate and 2% Triton X-100. To remove the nonionic detergent, the concentrated sample (4 mL) was applied to a column of Sephadex G-100 equilibrated with 0.02 M Tris–acetate buffer, pH 8.1/0.2% deoxycholate. The eluate from this gel-filtration step constituted the final preparation of acetyl-^{3}H-labeled enzyme and was used for pepsin digestion to obtain an acetyl-^{3}H-labeled peptide. Four such enzyme preparations, each from 30 g of seminal vesicles, were pooled to give approximately 250 nmol of [*acetyl*-^{3}H]prostaglandin synthetase for pepsin digestion.

Pepsin Digestion and Sample Preparation. The purified acetyl-^{3}H-labeled enzyme (250 nmol in 10 mL of Tris–acetate) was treated with β-mercaptoethanol, 5% final concentration, for 15 min at 37 °C. Ice-cold acetone, 20 volumes containing 24 mM HCl, was then added with stirring over several minutes, and the mixture was incubated for 1 h at 0 °C. The precipitate was collected by centrifugation and dissolved in 0.5 mL of 88% formic acid at room temperature.

A 20-fold volume of an acidified solution of pepsin, 0.05 mg/mL in 1 mM HCl, was added with stirring, and the mixture was incubated for 2 h at room temperature (Allen, 1981). The pepsin digest of approximately 15 mL was diluted with an equal volume of water, frozen, and lyophilized. The lyophilized material was dissolved in 5 mL of acetonitrile/water (3:1 v/v), and the organic solvent was removed under a stream of nitrogen. The sample was injected on a reverse-phase bed (SEP-PAK C_{18} cartridge, Waters Assoc., Milford, MA), and the bed then washed with water followed by acetonitrile/water (1:4 v/v). The ^{3}H-containing material was eluted with a 2-mL volume of acetonitrile/water (3:1 v/v, containing 0.1% trifluoroacetic acid), and the organic solvent was removed under nitrogen. The resultant acidified aqueous sample was used for isolation of the acetyl-^{3}H-labeled peptic peptide. The yield of acetyl-^{3}H-labeled peptic peptide was approximately 50% through the pepsin digestion and sample preparation steps.

Isolation of Acetyl-^{3}H-Labeled Peptic Peptide. The predominant peptide(s) containing the [^{3}H]acetate label was (were) isolated by means of three successive, high-pressure liquid chromatography (HPLC) steps. The same reverse-phase column (μBondapak C_{18}, Waters Assoc.) was used for each step. Either a linear or an exponential gradient was developed with a LDC gradient maker (Model 1601, Laboratory Data Control, Riviera Beach, FL) with two solvents termed A and B: solvent A consisting of 20 mM potassium phosphate pH 5.7 or 2.5, and solvent B consisting of 60% acetonitrile and 40% solvent A. The sample, 0.1 mL, was injected on the column, and peptide elution was followed by absorbance at 214 nm. The eluted peptides were analyzed for ^{3}H by scintillation counting. Successive fractions or peaks containing the bulk of the eluted ^{3}H were pooled, concentrated by use of the SEP-PAK cartridge as described above, and concentrated further under a stream of nitrogen. The conditions for each HPLC step are described in the legend for Figure 1. Because of the large amount of material present in the initial sample following pepsin digestion, two identical HPLC runs were required to separate the peptides of the starting sample (Figure 1A). Approximately 80% of the injected tritium was collected in the pooled fractions eluting around 60% solvent B (36% acetonitrile, v/v) as noted by the bar symbol in Figure 1A. In a typical preparation, approximately 100 nmol of acetyl-^{3}H-labeled peptide(s) was available after the first HPLC step. Following sample concentration, this material was subjected to a second HPLC step at pH 2.5 (Figure 1B), giving a predominant series of closely spaced acetyl-^{3}H-labeled peptides eluting at approximately 55% solvent B (33% acetonitrile) and containing about 35% of the injected [^{3}H]acetate, corresponding to approximately 30 nmol of acetyl-^{3}H-labeled peptide(s). The conditions for the third HPLC step were similar to those of the second step and gave a series of acetyl-^{3}H-labeled peptic peptides. The pooled fractions contained about 80% of the injected ^{3}H, corresponding to about 25 nmol of acetyl-^{3}H-labeled peptide. These peaks could not be easily resolved by HPLC methods, and in fact, amino acid analyses of the leading and trailing portions of this series of peptides were similar. Therefore, the major peaks of this series were pooled and used as the final preparation of purified acetyl-^{3}H-labeled peptic peptide. The sample was subjected to acid hydrolysis and analyzed for amino acid composition on a Beckman 121 amino acid analyzer (Spackman et al., 1958). Automated sequence analysis was performed on a Beckman 890C Sequanator with 0.1 M Quadrol program 030176, in the presence of 3 mg of polybrene, which, together with 33 nmol of glycylglycylglycine, was subjected to three precycles of Edman degradation (Tarr et al., 1978). Conversion of the sequencer product was carried out in-line with a Sequamat P-6 autoconverter with 1.5 N acetyl chloride in methanol (Horn & Bonner, 1977). The amino acid composition of the peptic peptide is shown in Table I, and the sequence analysis is shown in Table II.

Table I: Amino Acid Composition of Acetyl-^{3}H-Labeled Peptic Peptide(s)

amino acid	nmol of amino acid [a]	residues found [b]
lysine	1.26	1
aspartic acid	1.06	1 (asparagine)
serine	2.05	2
glutamic acid	2.21	3
proline	3.17	3
glycine	3.02	3
alanine	0.94	1
methionine	0.89	1
isoleucine	1.78	2
leucine	3.39	3
tyrosine	1.08	1
phenylalanine	1.16	1

[a] 1 nmol of purified acetyl-^{3}H-labeled peptic peptide was subjected to amino acid analysis. [b] Sequence data are given in Table II.

Trypsin Digestion. The purified acetyl-^{3}H-labeled peptic peptide(s) was (were) treated with trypsin, and the resultant peptides were separated by HPLC and assayed for amino acid content and ^{3}H. The acetyl-^{3}H-labeled peptic peptide (8 nmol) was dried under nitrogen, dissolved in 8 M urea/0.1 M NH_4HCO_3, and then diluted with water to give 2 M urea. Trypsin (0.16 nmol in 1 mM HCl) was added and the mixture incubated for 16 h at 22 °C. The tryptic cleavage at lysine

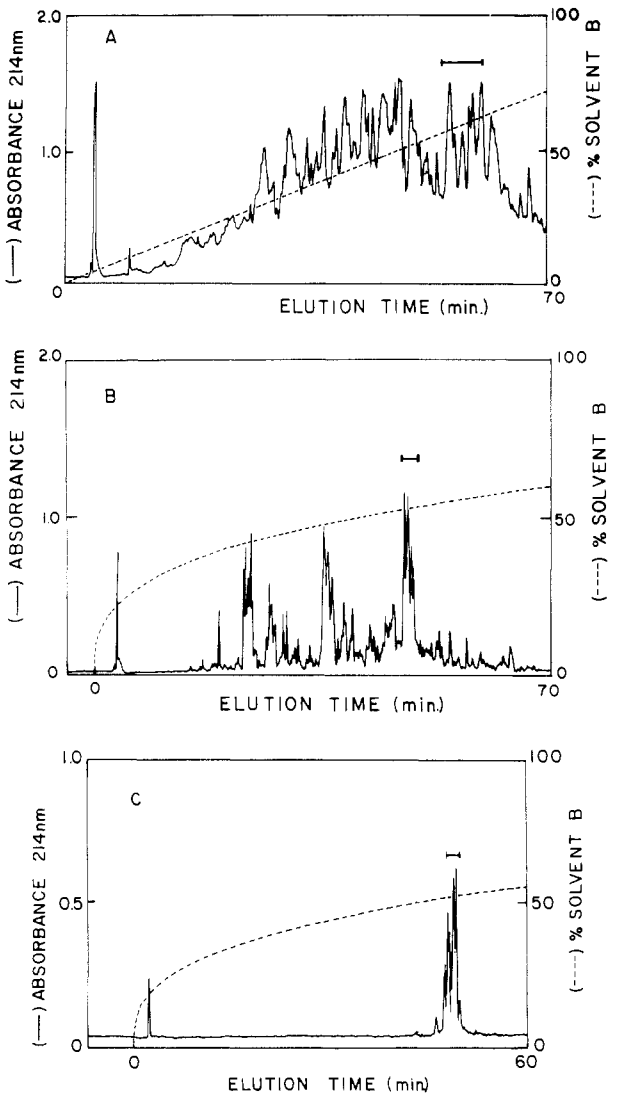

FIGURE 1: (A) Separation by HPLC of peptic peptides derived from [*acetyl*-^{3}H]prostaglandin synthetase. An aqueous sample (0.1 mL), containing peptides from 50 nmol of enzyme, was injected on the reverse-phase column. A linear gradient (---), 1 mL/min, over 70 min, from 100% solvent A (20 mM $KHPO_4$, pH 5.7) to 30% solvent A/70% solvent B (solvent B contains 40% solvent A and 60% acetonitrile) was used for peptide elution, indicated by absorbance at 214 nm (—). Fractions (2 mL) were collected and assayed for ^{3}H. Most (85%) of the ^{3}H eluted at approximately 55% solvent B. These fractions, indicated by the horizontal bar, were pooled, concentrated, and used as the sample for the separation noted in (B). (B) Separation by HPLC of acetyl-^{3}H-labeled peptic peptides from (A). An aqueous sample (0.1 mL) containing approximately 80 nmol of [^{3}H]acetate, derived from two pooled HPLC runs, as noted above in (A), was injected on the reverse-phase column. An exponential gradient (---), 1.5 mL/min, 90 min, setting 0.3 on LDC gradient maker, from 100% solvent A (20 mM H_3PO_4, pH 2.5) to 30% solvent A/70% solvent B (solvent B contains 40% solvent A and 60% acetonitrile) was used for peptide elution, indicated by absorbance at 214 nm (—). Individual peaks were collected and assayed for ^{3}H content. Approximately 35% of the ^{3}H eluted at 50% solvent B. These fractions, noted by the horizontal bar, were pooled, concentrated, and used as the sample for the separation noted in (C). (C) Separation by HPLC of acetyl-^{3}H-labeled peptic peptides from (B). An aqueous sample, (0.1 mL) containing 30 nmol of [^{3}H]acetate, obtained as noted in (B), was injected on a reverse-phase column. An exponential gradient (---), 2.0 mL/min, 90 min, setting 0.3 on LDC gradient maker, from 100% solvent A (20 mM H_3PO_4, pH 2.5) to 40% solvent A/60% solvent B (solvent B containing 40% solvent A and 60% acetonitrile) was used for peptide elution, indicated by absorbance at 214 nm (—). Individual peptide peaks were collected and assayed for ^{3}H. The middle peaks, noted by the horizontal bar, were pooled, concentrated, and analyzed as noted in the text.

Table II: Automated Sequencer Analysis of Acetyl-^{3}H-Labeled Peptic Peptide, 18 nmol[a]

cycle	amino acid identified	yield (nmol)
1	isoleucine	20
2	glutamic acid	16
3	methionine	12
4	glycine	5.7
5	alanine	7.3
6	proline	4.2
7	phenylalanine	5.8
8	serine[b]	
9	leucine	4.8
10	lysine	3.7
11	glycine	2.3
12	leucine	3.6
13	leucine	4.4
14	glycine	2.1
15	asparagine	1.8
16	proline	1.4
17	isoleucine	1.4
18	glutamic acid	1.4
19	X[c]	
20	proline	0.7
21	glutamic acid	1.0
22	tyrosine	0.8
23	blank	
24	blank	

[a] PTH derivatives were identified by reverse-phase chromatography on a μBondapak C_{18} column developed with 5–45 linear gradient of 0.1% aqueous acetic acid/methanol. Identification was confirmed on an alkylphenyl column with a 18–54 linear gradient of 0.06% aqueous propionic acid/methanol gradient. [b] Serine phenylthiohydantoin derivative was identified as a dehydroalanyl residue. [c] Serine was assigned at cycle 19 from the amino acid composition. Limited quantity percluded the back-conversion by hydrolysis in hydroiodic acid vapor.

Table III: Amino Acid Composition of Tryptic Peptides[a]

peptide A		peptide B	
amino acid	mol/mol of [^{3}H]acetate	amino acid	nmol[b]
lysine	0.8	aspartic acid	1.0
serine	0.6	serine	1.0
glutamic acid	0.7	glutamic acid	0.9
glycine	0.7	glycine	1.9
alanine	0.6		
isoleucine	0.6	isoleucine	1.0
leucine	0.7	leucine	2.0
phenylalanine	0.6	tyrosine	0.7
methionine	trace		
proline[c]	+	proline[c]	+

[a] Tryptic peptides derived from purified acetyl-^{3}H-labeled peptic peptide(s) were isolated by HPLC. [b] Sample of 1 nmol, estimated by comparing recovery to that of ^{3}H in peptide A. Peptide B did not contain [^{3}H]acetate. [c] Present but not quantitated.

(10th residue of the peptide) resulted in two peptides, termed A and B. The material was injected directly on the HPLC columns under conditions identical with those described for Figure 1A except that the pH was 2.5 rather than 5.7. Peptide A, which contained ^{3}H, eluted at 45% solvent B, and peptide B, which did not contain ^{3}H, eluted at 55% solvent B. Amino acid compositions of the peptides are given in Table III. The HPLC chromatogram is not shown.

Results and Discussion

The active site of prostaglandin synthetase, which interacts with aspirin, can be selectively labeled with ^{3}H at a single serine residue by use of [*acetyl*-^{3}H]aspirin. Some preliminary sequence information was available prior to this study, namely,

a Phe-Ser dipeptide (Van der Ouderaa, 1980) and a Ser-Leu-Lys sequence from our own preliminary unpublished work, using a trypsin–chymotrypsin cleavage of the intact enzyme, HPLC to isolate a ^{3}H-containing peptide, and amino acid analysis to identify the amino acids present. Therefore, we were expecting a Phe-Ser-Leu-Lys sequence within this active-site region.

Pepsin proved to be useful in cleaving a peptide(s) that included 7 residues to the NH_2 side of the modified serine and at least 14 residues to the COOH side. This acetyl-^{3}H-labeled peptide could be isolated by HPLC as shown in Figure 1. The chromatogram in Figure 1C shows several peaks, all of which contained tritium. Attempts to separate these peaks by varying pH, flow, and solvents in the described HPLC system were unsuccessful in that the peptides could not be resolved in adequate yield. Furthermore, these peptides appeared quite similar in amino acid composition. Accordingly, the major peaks shown in Figure 1C were pooled and subjected to amino acid analysis (Table I), giving discrete results for the amino acids noted. Furthermore, the sample gave a single, clearly defined NH_2-terminal residue, isoleucine, and contained a single unequivocal sequence through 22 residues as indicated in Table II. Amino acid analysis of the peptic peptide and one tryptic peptide gave unexplained low values for glutamic acid (Table I, 2.21 instead of 3 nmol; Table III, 0.9 instead of 2 nmol).

Several possible explanations for the heterogeneity of the peptide sample shown in the HPLC chromatogram in Figure 1C can be given. First, the peptide sample probably had some heterogeneity at the COOH terminus. A small amount of histidine was seen in some of the amino acid analyses, and this finding would be consistent with a population of peptides with two or more COOH-terminal extensions but with a single NH_2 terminus. Second, the methionine at position 3 in the peptide may have undergone partial oxidation during preparation. Third, the peptide(s) may have retained variable amounts of detergent despite the acid/acetone precipitation step and the succeeding HPLC column elutions. We conclude that HPLC methods have considerable resolving power for peptides and will separate closely related peptides that share considerable segments of sequence homology.

The purified peptic peptide contains two serine residues, either of which could be the aspirin-acetylated site. The data of Van der Ouderaa et al. (1980) and our own preliminary data noted above indicate that the Phe-Ser-Leu-Lys sequence at residues 7–10 contains the acetylated serine. This assignment was confirmed by tryptic digestion of the purified peptic peptide. Trypsin digestion gave two major peaks that were easily separated by HPLC. The amino acid compositions of the peptides are given in Table III. Only peptide A contained ^{3}H, and its amino acid composition was consistent with the first 10 residues of the intact peptic peptide. Alternatively, the second tryptic peptide had no tritium content and had an amino acid composition consistent with residues 11–22 of the starting peptide.

A further experiment was conducted to ascertain the position of the acetylated residue. Earlier, we observed that conversion of the sequencer product to the more stable PTH derivative led to complete cleavage of the *O*-acetyl bond in the acetylated serine. Therefore, the purified peptic peptide (20 nmol) was subjected to automated Edman degradation, but only the first three cycles were derivatized and identified (Ile-Glu-Met), to verify that the correct peptide was present. The next nine cycles were collected without derivatization, in order to prevent complete cleavage of the *O*-acetyl bond in the acetylated serine residue, and simply assayed for tritium. Each cycle from 4 to 12 contained a small amount of retained tritium. However, cycle 8 contained the maximum amount of tritium, and each cycle thereafter contained 40% or less compared to that found in cycle 8. The result indicates that pH changes during Edman degradation do lead to considerable cleavage of the *O*-acetyl bonds in the acetylated serine at position 8 but that a small amount of *O*-acetyl groups nevertheless do remain in the peptide and were localized at position 8.

Prostaglandin synthetase has a number of properties such as a single heme-binding site, a capacity to bind to lipid bilayers, and an ability to interact with intracellular reductants (Strittmatter et al., 1982). These properties obviously relate to the amino acid sequence of the enzyme. However, other than the NH_2-terminal sequence of 14 residues (Roth et al., 1980) and the work of Van der Ouderaa et al. (1980), the present study provides the only additional information on the primary structure of the enzyme. In the event that the complete amino acid sequence of the enzyme is determined, either by conventional sequence analysis or by gene-cloning techniques, the current study serves to mark the region of the protein that interacts with aspirin in a covalent fashion.

Registry No. Prostaglandin synthetase, 9055-65-6; cyclo-oxygenase, 39391-18-9; aspirin, 50-78-2.

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