Formation of Thiol Conjugates of 9-Deoxy-$\Delta^9,\Delta^{12}(E)$-prostaglandin D_2 and $\Delta^{12}(E)$-Prostaglandin D_2†

Jacob Atsmon,‡,§ Brian J. Sweetman,‡ Steven W. Baertschi,∥ Thomas M. Harris,∥ and L. Jackson Roberts, II*,‡,§

Departments of Pharmacology, Medicine, and Chemistry and Centers in Clinical and Molecular Toxicology, Vanderbilt University, Nashville, Tennessee 37232

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ABSTRACT: Albumin catalyzes the transformation of prostaglandin D_2 to 9-deoxy-$\Delta^9,\Delta^{12}(E)$-prostaglandin D_2 and to isomeric prostaglandin D_2 compounds including $\Delta^{12}(E)$-prostaglandin D_2. Both of these compounds are α,β-unsaturated ketones, which should render them susceptible to nucleophilic addition. We therefore examined the ability of the compounds to form conjugates with thiols glutathione and cysteine. During incubation with excess glutathione, both 9-deoxy-$\Delta^9,\Delta^{12}(E)$-prostaglandin D_2 and $\Delta^{12}(E)$-prostaglandin D_2 formed a conjugate. Conjugation of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-prostaglandin D_2 occurred very rapidly; approximately 70% was conjugated within 2 min. In contrast, conjugation of $\Delta^{12}(E)$-prostaglandin D_2 with glutathione proceeded at a much slower rate; only 38% was conjugated at 60 min. The formation of both conjugates was enhanced by glutathione *S*-transferase. Conjugation of both compounds with cysteine was found to occur more rapidly than with glutathione. This effect was more pronounced with $\Delta^{12}(E)$-prostaglandin D_2 in which 60% conjugated with cysteine within 2 min. These differences are likely attributed to greater steric hindrance for conjugation across the Δ^{12} double bond compared to that across the Δ^9 bond. Analysis by fast atom bombardment mass spectrometry confirmed the formation of the glutathione conjugate of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-prostaglandin D_2. Following prolonged incubation of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-prostaglandin D_2 with excess glutathione in the presence of glutathione *S*-transferase, a small quantity of a bis conjugate of this compound was also detected by mass spectrometry. ^1H NMR analysis determined that in the mono glutathione conjugate of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-prostaglandin D_2 the glutathione was conjugated at C-9. To assess the formation of conjugates in vivo, tritiated 9-deoxy-$\Delta^9,\Delta^{12}(E)$-prostaglandin D_2 was injected intravenously in a rat, and urine and bile were collected for 4 h. Of the recovered radioactivity, 90% was excreted into the bile. Essentially all of the radioactivity in the bile was present in the form of a polar conjugate. HPLC analysis of the bile revealed a major radioactive peak with an elution volume characteristic for that of the glutathione conjugate of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-prostaglandin D_2, suggesting that conjugation of this prostaglandin with glutathione may also occur in vivo. In addition, in view of the fact that glutathione is ubiquitous and present in most cells, it may be important to consider the possibility that conjugation of these prostaglandins with glutathione may play a role in modulating their biological actions.

Prostaglandin (PG)[1] D_2 is an unstable molecule by virtue of the fact that it is a β-hydroxy, β,γ-unsaturated ketone which renders protons α to the carbonyl acidic and easily removable under mild alkaline conditions, leading to facile loss of the hydroxyl group. Serum albumin has been shown to catalyze dehydration of PGD_2 in vitro, which has been attributed to interaction of the prostaglandin with alkaline binding sites on albumin (Kikawa et al., 1984; Fitzpatrick & Wynalda, 1983). We have recently demonstrated that albumin stereoselectivity catalyzes exchange of the C10 β proton and the C12 proton of PGD_2 (unpublished experiments). As a consequence of proton abstraction at C12 and reprotonation, albumin also catalyzes isomerization of PGD_2.

One of the major isomers formed in the presence of albumin is $\Delta^{12}(E)$-PGD_2 (unpublished experiments), and the major dehydration product formed is 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 (Kikawa et al., 1984; Fitzpatrick & Wynalda, 1983). A characteristic of both of these compounds is that they are α,β-unsaturated ketones. This renders these molecules highly reactive and susceptible to nucleophilic addition reactions. Thiols such as glutathione (GSH), which is ubiquitous in the body, readily conjugate with α,β-unsaturated ketones (Mannervick & Danielson, 1988), including A-series prostaglandins (Chaudhari et al., 1978; Cagen et al., 1976). Therefore, we have examined the ability of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 and $\Delta^{12}(E)$-PGD_2 to conjugate with GSH and cysteine in vitro and investigated whether formation of polar conjugates is a prominent pathway of metabolic disposition of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 in vivo in the rat.

Experimental Procedures

Chemicals. Unlabeled PGD_2 was the generous gift of John Pike of Upjohn Co. [5,6,8,9,12,14,15-^3H]PGD_2 (100 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Human serum albumin prepared from fraction V albumin, cysteine hydrochloride, glutathione, and glutathione *S*-transferase from rat liver were from Sigma Chemical Co. (St. Louis, MO). The glutathione *S*-transferase preparation

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*To whom correspondence should be addressed.

‡Department of Pharmacology.

§Department of Medicine.

∥Department of Chemistry and Centers in Clinical and Molecular Toxicology.

[1] Abbreviations: PG, prostaglandin; FAB, fast atom bombardment mass spectrometry; HPLC, high-performance liquid chromatography; GC, gas chromatography; MS, mass spectrometry; GSH, glutathione.

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presumably contains a mixture of isoforms of the enzyme. Solvents were purchased from Burdick and Jackson (Muskegen, MI).

Generation of $\Delta^{12}(E)$-PGD$_2$ and 9-Deoxy-$\Delta^9,\Delta^{12}(E)$-PGD$_2$. PGD$_2$ was incubated overnight at 37 °C with constant stirring in a buffered sodium phosphate solution (50 mM, pH 7.5) of human serum albumin (50 mg/mL). Following incubation, the mixture was acidified to pH 3 with 1 N HCl and applied to a C-18 Sep-Pak cartridge (Waters Associates, Milford, MA). The column was washed sequentially with 10 mL of H_2O and 10 mL of heptane, and the prostaglandins were then eluted with 10 mL of ethyl acetate. The ethyl acetate eluate was dried under nitrogen and subjected to reverse-phase HPLC on an Alltech C-18 column, using the solvent system acetonitrile/H_2O/acetic acid (29:71:0.01 v/v/v), 1 mL/min, 1-mL fractions. $\Delta^{12}(E)$-PGD$_2$ and 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD$_2$ characteristically elute at 20 and 40 mL, respectively. The peaks were detected either by monitoring absorption at 245 nm or by assaying an aliquot of collected fractions by scintillation counting. The collected fractions were then extracted with ethyl acetate, dried under nitrogen, and redissolved in ethanol. Analysis of the compounds by electron ionization gas chromatography/mass spectrometry (GC/MS) as a methyl ester, *O*-methyloxime, trimethylsilyl ether derivative and ^{1}H NMR confirmed the structures of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD$_2$ and $\Delta^{12}(E)$-PGD$_2$. The compounds were quantified by UV absorbance as described elsewhere (Kikawa et al., 1984; Bundy et al., 1983).

In Vitro Conjugation of 9-Deoxy-$\Delta^9,\Delta^{12}(E)$-PGD$_2$ and $\Delta^{12}(E)$-PGD$_2$ with GSH and Cysteine. Tritiated 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD$_2$ and $\Delta^{12}(E)$-PGD$_2$ were incubated at 37 °C with GSH or cysteine in phosphate-buffered saline (50 mM, pH 7.5). The molar ratio of the prostaglandin to the thiol was 1:10. An aliquot of the mixture was removed at various time points, acidified immediately to pH 3 by addition of 1 N HCl to prevent further conjugation, and then extracted with ethyl acetate. Unconjugated prostaglandins readily extract into ethyl acetate at acidic pH, whereas prostaglandins conjugated with thiols will not extract into organic solvents due to the highly polar nature of the thiol moiety (Chaudhari et al., 1978; Cagen et al., 1976). Therefore, the formation of a GSH or cysteine conjugate was assessed by determining the percent of radiolabeled PG that did not extract into ethyl acetate at pH 3. Because a small amount of water is taken up by the organic phase when water and ethyl acetate are mixed, a small percent (approximately 10%) of GSH or cysteine conjugate appears in the ethyl acetate extract.

In experiments in which large quantities of GSH conjugates of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD$_2$ were required for structural analysis, the prostaglandin and GSH were incubated as described above for 45 min, acidified to pH 3, and applied to a preconditioned C-18 Sep-Pak cartridge (Waters Associates). The cartridge was then washed sequentially with 10 mL of 50 mM ammonium acetate (pH 3) and 10 mL of heptane, and the compounds were then eluted with 10 mL of ethanol. The compound was then purified by reverse-phase HPLC on a C-18 Alltech column (4.6 × 250 mm), using a solvent system of acetonitrile/aqueous ammonium acetate (50 mM, pH 3) (25/75 v/v), 1 mL/min, 1-mL fractions. The compound was detected by assaying an aliquot of the collected fraction by scintillation counting or by absorption at 245 nm. The GSH adduct of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD$_2$ characteristically eluted at 19–21 mL. Although the ability of this HPLC system to separate various conjugates of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD$_2$ has not been extensively evaluated, it has been found to chromatographically separate GSH, cysteinylglycine, and cysteine conjugates of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD$_2$ in which the C11 keto and Δ^{12} double bond have been reduced (Atsmon et al., 1990).

Effect of GSH S-Transferase on GSH Conjugation. Tritiated prostaglandins were incubated with GSH in phosphate-buffered saline (pH 6.5) to which 30 units of GSH *S*-transferase was added. At designated time points, aliquots were withdrawn, acidified immediately to pH 3, and extracted with ethyl acetate. Again, the formation of a GSH conjugate was assessed by determining the percent of radioactivity that did not extract into ethyl acetate. Control incubations were carried out without the presence of the enzyme.

Analysis of Prostaglandin Conjugates by Fast Atom Bombardment Mass Spectrometry. Fast atom bombardment mass spectra (FAB-MS) were obtained by using a VG 70-250 HF GC/MS instrument, equipped with a standard unheated VG FAB ion source and saddle field gun (Ion-Tech Model B11N) producing a beam of xenon atoms at 8 KeV and 1 mA. Glycerol was used as the matrix. The mass spectrometer was adjusted to a resolving power of 2500, and spectra were obtained at 10 s/decade by using an accelerating voltage of 6 KV. Instrument parameters were optimized for the production and detection of negative ions. Sequential scans were averaged and matrix-subtracted to maximize spectral quality.

^{1}H NMR Analysis of Prostaglandin Conjugates. ^{1}H NMR spectra were obtained on either a Bruker AM-400 or NR-300 at 400 and 300 MHz, respectively. Samples were dissolved in either [$^2H_4$]methanol or $CDCl_3$ and sealed under nitrogen. Chemical shifts are assigned relative to either the residual methanol–CHD_2 signal at 3.30 ppm or the $CHCl_3$ signal at 7.26 ppm. Parameters were 16K of sampling and data points, 60° pulse, and a 2.0-s relaxation delay. Assignment of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD$_2$ was made by using a COSY spectrum acquired with the standard Bruker microprogram. A total of 256 1K spectra were recorded with a 90–τ–60 pulse sequence. Sine-bell apodization, magnitude calculation, and symmetrization were employed.

Metabolic Fate of [^{3}H]-9-Deoxy-$\Delta^9,\Delta^{12}(E)$-PGD$_2$ in the Rat. A male Sprague-Dawley rat was anaesthetized with sodium pentobarbital, and a polyethylene cannula (PE-50) was inserted into the common bile duct. [^{3}H]-9-Deoxy-Δ^9,Δ^{12}-(E)-PGD$_2$ (3.5 μCi) in 0.5 mL of normal saline was injected over 5 min into the tail vein. The bile was collected in microcentrifuge tubes containing 100 mM sodium citrate buffer (pH 3) and placed on ice to prevent deconjugation ex vivo at alkaline pH. Microtubes were changed every 10 min, and an aliquot was immediately extracted with ethyl acetate. Urine was also collected during the course of the experiment, including the residual volume retained in the bladder after the rat was sacrificed. Three milliliters of blood was drawn at the end of the experiment into a tube containing heparin, and the red blood cells were separated from plasma by centrifugation. Both urine and blood components were acidified to pH 3 by 1 N HCl and extracted with ethyl acetate. A sample of the collected bile was also extracted by using a C-18 Sep-Pak and then subjected to reverse-phase HPLC as described previously.

Results

Conjugation of 9-Deoxy-$\Delta^9,\Delta^{12}(E)$-PGD$_2$ and $\Delta^{12}(E)$PGD$_2$ with GSH and Cysteine in Vitro. In contrast to unconjugated prostaglandins which readily extract into ethyl acetate at pH 3, GSH and cysteine adducts will not extract into ethyl acetate due to the highly polar characteristics of the charged groups in the thiol moiety. Therefore, we initially assessed the time course of conjugation of GSH and cysteine with radiolabeled 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD$_2$ and $\Delta^{12}(E)$-PGD$_2$ by determining

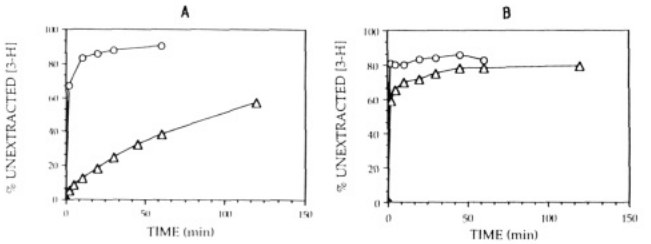

FIGURE 1: Time course of conjugation of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 and $\Delta^{12}(E)$-PGD_2 with GSH and cysteine. Tritiated 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 (○) and $\Delta^{12}(E)$-PGD_2 (△) were incubated with excess GSH or cysteine. An aliquot was removed at designated time points, acidified to pH 3, and immediately extracted with ethyl acetate. Formation of polar thiol conjugates was monitored over time and expressed as the percent of total radioactivity that did not extract into the organic phase. (A) Conjugation with GSH; (B) conjugation with cysteine. Results are the mean of three experiments. The SD are equal to or smaller than the symbols.

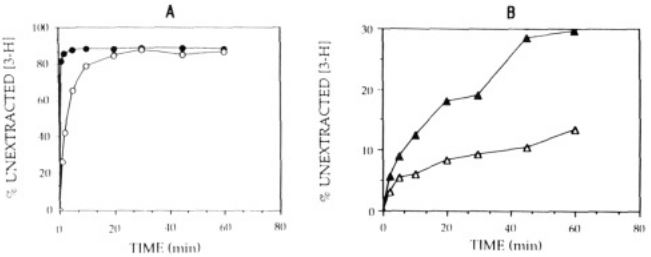

FIGURE 2: Effect of GSH *S*-transferase on conjugation of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 and $\Delta^{12}(E)$-PGD_2 with GSH. (A) Tritiated 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 was incubated with GSH as described in Figure 1, in the presence (●) or absence (○) of GSH *S*-transferase. (B) Tritiated $\Delta^{12}(E)$-PGD_2 was similarly incubated with GSH in the presence (▲) or absence (△) of the enzyme. The results are the mean of three experiments. The SD are equal to or smaller than the symbols.

the percent of radioactivity that does not extract into ethyl acetate during incubation of these thiols with the prostaglandins.

As shown in Figure 1 the amount of unextractable tritium increased over time during incubation of the prostaglandins with either GSH or cysteine. Within 2 min, approximately 70% of the 9-deoxy compound was conjugated with GSH and the conjugation was essentially complete at 10 min. In contrast, only approximately 38% of the $\Delta^{12}(E)$-PGD_2 was conjugated with GSH at 60 min (Figure 1A). Both prostaglandins conjugated more rapidly with cysteine than with GSH (Figure 1B). Approximately 85% of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 was conjugated with cysteine within 2 min, and approximately 60% of $\Delta^{12}(E)$-PGD_2 was conjugated at this time point. The increased rate of conjugation with cysteine compared to GSH, especially with $\Delta^{12}(E)$-PGD_2, may be attributed to less steric hindrance associated with cysteine conjugation.

The effect of GSH *S*-transferase on conjugation of these prostaglandins with GSH was then examined. GSH *S*-transferase was found to enhance significantly the rate of conjugation of GSH with both 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 ($p < 0.01$ at 10 min) (Figure 2A) and $\Delta^{12}(E)$-PGD_2 ($p < 0.001$ at 60 min) (Figure 2B). As may be noted, the rate of conjugation of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 and $\Delta^{12}(E)$-PGD_2 with GSH in the control incubations in the absence of GSH *S*-transferase in these experiments is somewhat less than that shown in Figure 1. This is attributed to the fact that the experiments in Figure 1 were performed at pH 7.5, whereas the control experiments assessing the effect of GSH *S*-transferase were performed at pH 6.5, where this enzyme exhibits greatest activity. However, nonenzyme conjugation is also pH dependent due to the fact that it is the thiolate anion

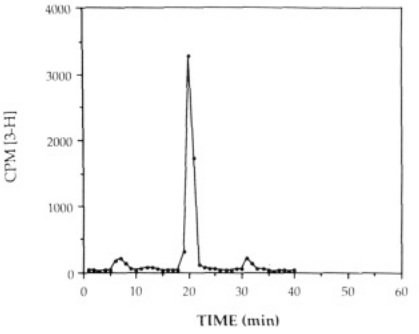

FIGURE 3: RP-HPLC analysis of products formed following incubation of radiolabeled 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 with GSH for 45 min in the absence of GSH *S*-transferase. Tritiated 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 was incubated in vitro with GSH for 45 min and subjected to RP-HPLC with a solvent system of acetonitrile/water/acetic acid (29:71:0.01 v/v/v), 1 mL/min, 1-mL fractions. A single prominent peak eluted with a retention volume of 19–21 mL.

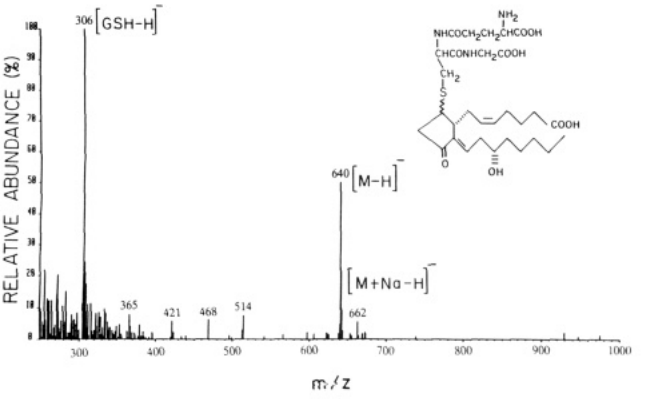

FIGURE 4: Negative ion FAB mass spectrum of the major compound with an elution volume of 19–21 mL in Figure 3.

which conjugates, and thus the rate of conjugation decreases with decreasing pH.

FAB-MS and ^1H NMR Analysis of the GSH Conjugate of 9-Deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2. The putative GSH conjugate of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 was analyzed by FAB-MS and ^1H NMR. Prior to these analyses, the conjugate was purified by RP-HPLC following incubation of radiolabeled 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 with GSH for 45 min. RP-HPLC analysis yielded a single major peak eluting at retention volume of 19–21 mL (Figure 3). The negative ion FAB mass spectrum of the material in that peak is shown in Figure 4. This was characterized by two prominent peaks at m/z 640 $[M - H]^-$ and m/z 306 $[GSH - H]^-$. This was interpreted as consistent with a mass spectrum of a mono GSH conjugate of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2. A similar mass spectrum was also obtained when the incubation mixture was only extracted by using a C-18 Sep-Pak and not purified by HPLC. FAB-MS analysis was also carried out after prolonged incubation of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 with GSH for 4 h in the presence of GSH *S*-transferase followed by C-18 Sep-Pak extraction. In addition to the same prominent ions at m/z 640 and 306, additional prominent ions of lesser intensity were present at m/z 947 and 929 (Figure 5). These were interpreted as the $[M - H]^-$ and $[M - H_2O - H]^-$ ions, respectively, of a bis-GSH conjugate, indicating the formation of a small quantity of a bis conjugate after prolonged incubation with GSH.

As shown previously (Figure 1), GSH conjugates with $\Delta^{12}(E)$-PGD_2 at a much slower rate than with 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 and cysteine conjugates with $\Delta^{12}(E)$-PGD_2 much more rapidly compared to GSH. A likely explanation for this is that there is less steric hindrance for conjugation at the Δ^9 double bond compared to that at the Δ^{12} double bond.

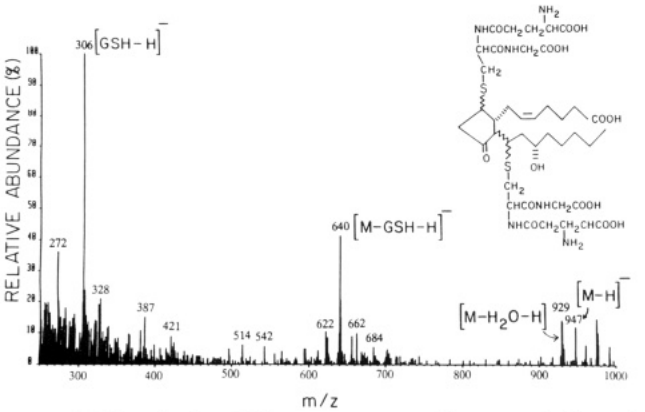

FIGURE 5: Negative ion FAB mass spectrum of compounds formed following prolonged incubation of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 with GSH in the presence of GSH *S*-transferase.

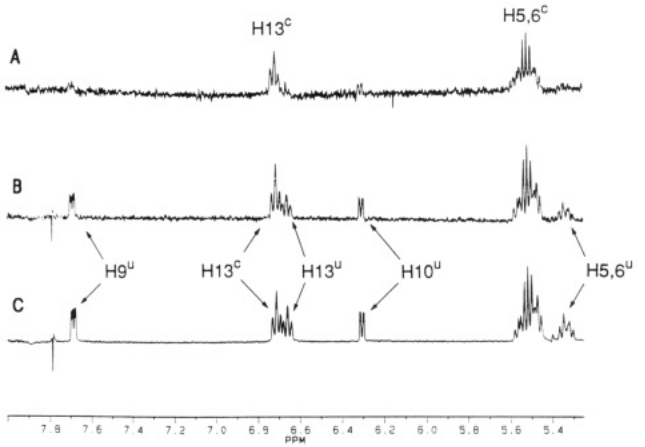

FIGURE 6: Analysis of the mono-GSH conjugate of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 by ^1H NMR. The mono-GSH conjugate of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 was prepared and purified as described in Figure 3 and then immediately analyzed by ^1H NMR. (A) Initial NMR spectrum obtained showing the absence of resonances from the Δ^9 double bond; (B) NMR spectrum obtained 1 h later showing the beginning of the appearance of resonances of H9 and H10 protons, indicating that part of the GSH conjugate had become unconjugated; (C) NMR spectrum obtained after 3 h indicating that approximately 35% of the initial GSH conjugate had become unconjugated. Superscripts c and u denote assignments for the conjugated and unconjugated protons, respectively.

Therefore, the finding that the mono GSH conjugate of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 forms more rapidly than the bis conjugate is consistent with the hypothesis that GSH is attached at C9 of the mono conjugate. Since the FAB-MS analysis did not provide information regarding the site of covalent attachment of GSH, ^1H NMR analysis of the mono GSH conjugate was carried out in an attempt to obtain this information. Because of the small amount of sample and interference from ammonium acetate present, we were unable to assign the upfield portion of the spectrum. We were able, however, to observe the olefinic region of the spectrum (5.25–8.0 ppm) (Figure 6). The only resonance clearly visible in this region is a triplet at 6.71 ppm, which we have assigned as the C13 proton by comparison with the spectrum of the 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2. The resonances for the olefinic protons on C9 and C10 are notably absent in the initial spectrum (Figure 6A). This indicates that GSH is adding across the Δ^9 double bond rather than at the Δ^{12} double bond. Thus, GSH is presumably linked to C9 resulting from a 1,4 Michael addition to the α,β-unsaturated system. From repeated NMR analysis of the conjugate over time, we observed that the addition is apparently reversible and the compound

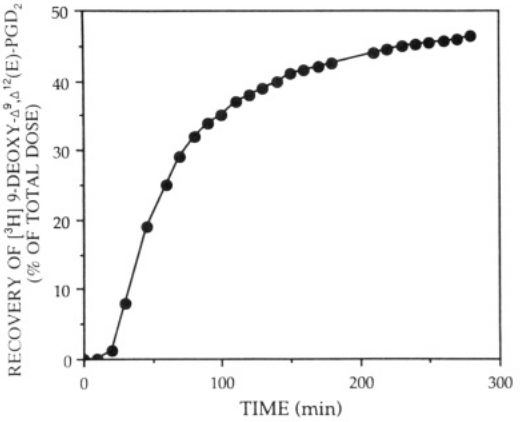

FIGURE 7: Time course of excretion of radioactivity into the bile following intravenous administration of radiolabeled 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 in the rat. Following intravenous administration of tritiated 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 in a rat, bile samples were collected over time and assayed for radioactivity. Data are expressed as the cumulative radioactivity excreted as a percent of the total dose administered.

deconjugates. As the GSH deconjugates, it is oxidized to GSSG by dissolved oxygen, thereby preventing reconjugation. After 1 h, resonances begin to appear for the C9 proton and unconjugated H10 and H5,6 (Figure 6B). After 3 h, approximately 35% of the initial GSH conjugate had deconjugated (Figure 6C).

Metabolic Fate of Intravenous Radiolabeled 9-Deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 in the Rat. Because conjugation of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 with GSH occurred very rapidly in vitro even in the absence of GSH *S*-transferase, the possibility that conjugation may represent a major pathway of metabolic disposition of this PG in vivo was examined. Following intravenous injection of 3.5 μCi of [^3H]-9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 into a rat, approximately 0.1 mL of bile was collected every 10 min from the cannulated common bile duct. The cumulative radioactivity recovered in the bile, expressed as a percent of the total radioactivity injected, is shown in Figure 7. Excretion of radioactivity into the bile was detected within 20 min. Approximately 45% of the administered radioactivity was recovered in the bile within 4 h. In contrast, only 5% of the administered radioactivity was recovered in the urine, and no radioactivity was detected in red blood cells or plasma at 4 h. Essentially all of the radioactivity in the bile failed to extract into ethyl acetate at pH 3, indicating that this material was present in the form of a polar conjugate. Approximately 50% of the radioactivity recovered in the urine also did not extract into ethyl acetate.

To examine the nature of the radiolabeled material excreted into the bile, the bile was subjected to RP-HPLC analysis using the same conditions as were employed for purification of the GSH conjugate of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 formed in vitro. This analysis revealed a single major radioactive peak eluting at retention volume (19–21 mL) characteristic of that of the mono GSH conjugate of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 as shown in Figure 3.

Discussion

This study demonstrates that GSH and cysteine readily form conjugates with 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 and $\Delta^{12}(E)$-PGD_2 by nonenzymatic nucleophilic addition. Although the nonenzymatic conjugation reaction is rapid, especially with 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2, GSH *S*-transferase was found to further enhance the rate of conjugation of GSH with both PGs.

The finding that the formation of cysteine conjugates occurred much more rapidly than the formation of GSH con-

jugates suggested that steric influences may be involved in the conjugation of GSH with these prostaglandins. This possibility seems to be further supported by the finding that GSH conjugated with $\Delta^{12}(E)$-PGD_2 at a much slower rate compared with 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2. Conjugation across the Δ^{12} double bond would be expected to be associated with greater steric hindrance than conjugation across the Δ^9 double bond. Consistent with this was the finding that following incubation of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 with GSH for 45 min, essentially only a mono conjugate was formed when analyzed by HPLC and FAB-MS. ^1H NMR analysis established the site of attachment of GSH at C9. More prolonged incubation (4 h) in the presence of GSH *S*-transferase, however, resulted in the formation of detectable quantities of a bis GSH conjugate.

Because 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 readily conjugates with GSH in vitro, two important questions arise: (a) whether conjugation may represent an important pathway of metabolic disposition of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 in vivo and (b) whether GSH conjugates of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 may play a role in the biological actions of this eicosanoid.

In experiments aimed at addressing the first question, it was found that following intravenous injection of radiolabeled 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 into a rat, 90% of the recovered radioactivity was excreted into bile and 10% into urine after 4 h. Essentially all of the radiolabeled material in the bile was in the form of a polar conjugate. HPLC analysis of the bile revealed a major radioactive peak with an elution volume characteristic of that of the in vitro GSH conjugate of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2. These data indicate that the formation of polar conjugates of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 is a major pathway of metabolic disposition of the PG in vivo in the rat. Although not conclusive, the HPLC analysis of the bile suggests that the moiety to which 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 was conjugated may be GSH.

GSH conjugates of xenobiotics are usually excreted into the urine only after being further converted to mercapturic acids (Chasseaud, 1976). Therefore, although the nature of the conjugated material recovered in the rat urine is unknown, it may be a mercapturic acid. Enterohepatic recirculation may allow this further metabolism of GSH-derived conjugates, as occurs in many elimination processes involving conjugation with GSH (Reed & Meredith, 1984). In our study in which the common bile duct was cannulated, further metabolism of GSH conjugates via enterohepatic recirculation was prevented. Thus, because of the experimental conditions employed in this study, the relative amount of polar conjugates that normally may appear in the urine may be underestimated.

Although species differences exist, since formation of polar conjugates represents a major pathway of metabolic disposition of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 in the rat, it is likely that this also occurs in humans. Hirata et al. recently identified unconjugated 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 in human urine using a GC/MS assay, suggesting that it was formed in vivo (Hirata et al., 1988). This finding implicating that PGD_2 undergoes dehydration in vivo is consistent with our previous identification of a 15-deoxy metabolite of PGD_2 in the monkey and in humans (Ellis et al., 1979; Roberts & Sweetman, 1985). However, in these studies of PGD_2 metabolism, 9-deoxy metabolites of PGD_2 were not identified. This may be attributed to the fact that only approximately 40% of radiolabeled metabolites present were identified. Alternatively, however, the majority of 9-deoxy metabolites may have been present in the form of a polar conjugate. Because these conjugates are involatile, they cannot be analyzed by GC/MS, which was employed in these studies, and, therefore, they would not have been identified. If this is the case, it seems possible that the unconjugated 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 detected in human urine by Hirata et al. may only represent a small fraction of the total 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 present, the majority being present as a polar conjugate. It is possible that 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 may also be formed in the kidney and in the genitourinary tract and hence escape conjugation to a large extent. We have also observed that GSH conjugates of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 will deconjugate over time (Figure 6). Therefore, it is also possible that the 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 that was detected in human urine may have been excreted into the urine initially as a conjugate and subsequently became deconjugated in part prior to analysis by GC/MS. Further studies will be required to address these possibilities.

Approximately 40% of the administered radiolabeled dose of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 could not be accounted for in urine and bile at 4 h. However, as shown in Figure 7, the biliary excretion of radioactivity had plateaued at 4 h. As shown in our studies, 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 conjugates readily not only with GSH but also with cysteine. Therefore, it seems possible that 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 could accumulate in tissues conjugated with thiol-containing proteins and thereby escape biliary and urinary excretion.

In regard to the question of whether conjugation may also play a role in the biological actions of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2, we have recently obtained evidence that conjugation of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 with GSH does in fact modulate one of the known biological actions of this PG, which is its ability to inhibit tumor cell proliferation in vitro. 9-Deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 is known to inhibit proliferation of a variety of tumor cell lines, and intracellular uptake of the compound is necessary for it to exert this action (Narumiya & Tukushima, 1986). Because 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 readily conjugates with GSH, we examined the extent to which this may occur intracellularly. In two tumor cell lines, rat hepatoma tissue culture cells and Chinese hamster ovary cells, we found that intracellular 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 exists almost entirely in the form of a GSH conjugate. Furthermore, we found that depletion of intracellular GSH in these cells markedly enhances its antiproliferative activity (Atsmon et al., 1990). Thus, in regard to the antigrowth activity of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2, conjugation with intracellular GSH appears to be an important process that modulates this biological action.

In summary, this paper describes the finding that 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 rapidly conjugates in vitro nonenzymatically with GSH and cysteine. $\Delta^{12}(E)$-PGD_2 also rapidly conjugates with cysteine but conjugates with GSH at a relatively slower rate. GSH *S*-transferase further enhances the rate of conjugation of both PGs with GSH. Formation of polar conjugates of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2, which may comprise GSH conjugates or its derivatives, also appears to be a major pathway of metabolic disposition of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 in vivo in the rat. These findings suggest that efforts to assess endogenous production of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 might best be directed toward identification and quantification of polar conjugates of the compound or its metabolites. In addition, formation of GSH conjugates of 9-deoxy-$\Delta^9,\Delta^{12}(E)$-PGD_2 should be considered a potentially important process that can modulate the biological activity of this compound.

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Sequence Determinants for H1 Binding on *Escherichia coli lac* and *gal* Promoters†

Sylvie Rimsky* and Annick Spassky
Unité de Physicochimie des Macromolécules Biologiques, Institut Pasteur, CNRS:UA1149, 75724 Paris Cedex 15, France
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ABSTRACT: The H1 protein is a likely candidate for structuring DNA in the bacterial nucleoid. We have studied determinants leading to its binding to DNA (and in particular to *Escherichia coli lac* and *gal* promoters) in vitro through the pattern of attack of both DNaseI and the copper–*o*-phenanthroline complex [$(OP)_2Cu^+$]. The binding of H1 depends on the primary sequence of DNA. H1 also associates with recognition sites for specific proteins, in particular with the Pribnow box and the CRP binding site. Binding of H1 to the Pribnow box of the wild-type *lac* promoter does not change the pattern of nucleolytic digestion with $(OP)_2Cu^+$. In contrast, binding of H1 to the strong *lac* promoter mutants Ps and UV5 appears to change the conformational state of this DNA. Similar changes in accessibility of the minor groove surrounding the respective binding sites were observed for both H1–DNA and CRP–DNA complexes.

H1 is a low molecular weight protein (15 500) originally described by Jacquet et al. (1971). It was found to be associated with the nucleoid of *Escherichia coli* by several authors (Varshavsky et al., 1977; Bakaev, 1981). The gene for the protein H-NS, which is probably identical with H1 protein, has recently been cloned from several organisms (La Teana et al., 1989). A few years ago, we showed that this protein is able to form complexes with DNA in vitro and that, for different sizes and sequences of linear or circular DNA, in such a complex, its binding to DNA resulted in a tight compaction of the DNA accompanied by a very small topological change in the superhelicity (Spassky et al., 1984).

In this work, we study the binding of H1 protein to several known DNA sequences through the pattern of attack of two nucleases: DNaseI[1] and the artificial nuclease *o*-phenanthroline–cuprous complex [$(OP)_2Cu^+$]. The endonuclease DNaseI produces footprints from which the position of the bound H1 protein may be deduced with respect to the DNA sequence. Cleavage with $(OP)_2Cu^+$ yields information about the conformational state of the DNA. $(OP)_2Cu^+$ rapidly cleaves DNA through an oxidative pathway at physiological pH and temperature (Sigman et al., 1979). This reagent attacks B-DNA in the minor groove (Kuwabara et al., 1986), implying that a noncovalent intermediate forms in the minor groove. Thus, changes in the accessibility of the minor groove should be expected to have an effect on the observed reactivity of the reagent. This is precisely what is seen in non-B-DNA, e.g., Z- or A-DNA, where the minor groove has a very different conformation from that of the canonical B form; in this case, the $(OP)_2Cu^+$ nuclease does not cleave the DNA well (Pope & Sigman, 1984). The pattern of variation with $(OP)_2Cu^+$ is different from that observed with DNaseI. Whereas DNaseI has preferred sites of attack distributed along the length of the sequence, hyperreactive sites to $(OP)_2Cu^+$ attack are clustered in different regions, reflecting a sequence-dependent variation of minor-groove geometry (Kuwabara et al., 1986; Sigman, 1986).

We investigated H1 binding within the *lac* and *gal* control regions; in these regions binding sites for H1 generally overlap the specific DNA binding sites of CRP, RNA polymerase, and *lac* repressor.

In order to determine whether the positioning of H1 on DNA was only a function of the primary sequence or a consequence of monomer–monomer interactions, we compared the binding of H1 to the wild-type sequence and to the same sequence containing an insertion of two base pairs. We also investigated binding of H1 to the Pribnow box of the wild-type *lac* promoter and two mutants: Ps (C-T at position −9) and UV5 (A-T at position −8, and C-T at position −9). We find that H1 makes sequence-specific contacts and that the final

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[1] Abbreviations: DNaseI, deoxyribonuclease I; CRP, cAMP receptor protein (presence of cAMP bound to protein is implied); bp, base pair; $(OP)_2Cu^+$, 2:1 1,10-phenanthroline–cuprous ion complex.

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