Lipoxygenation Activity of Purified Prostaglandin-Forming Cyclooxygenase†

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ABSTRACT: Purified cyclooxygenase, a single enzyme which catalyzes the formation of endoperoxide from arachidonic acid (20:4) in a bis(dioxygenase) reaction, is capable of oxygenating eicosadienoic acid (20:2) at C-11 in a single dioxygenase reaction. The partial oxygenation of 20:2 resembles the formation of prostaglandin from 20:4, with both oxygenation reactions exhibiting similar pH optima, substrate K_m values, and cofactor effects including a need for peroxide and an absolute requirement for heme. In addition, those processes known to destroy 20:4 oxygenase activity, such as heat inactivation, inactivation with anti-inflammatory drugs, and turnover-mediated inactivation, have equally destructive effects on 20:2 oxygenase activity. Thus, both oxygenations are catalyzed by one enzyme. All of the above similarities for 20:2 and 20:4 oxygenation demonstrate that C-11 oxygenation is an integral rate-limiting step of cyclooxygenase action rather than a separate reaction resembling that of plant lipoxygenase.

The endoperoxide, prostaglandin G (PGG),[1] is the first isolable product (Hamberg et al., 1974) recognized in the biological synthesis of prostaglandins, thromboxanes, and prostacyclin from polyunsaturated fatty acids containing three or more *cis*-methylene-interrupted double bonds. PGG is formed by the cyclooxygenase-catalyzed addition of 2 molecules of oxygen to the fatty acid (for reviews, see Samuelsson, 1969; 1972; Van Dorp, 1971; Samuelsson et al., 1975; Lands et al., 1977) in what may be a controlled free radical reaction (Samuelsson et al., 1966; Nugteren et al., 1966a). Enrichment of the 13-L-^{3}H in substrate during oxygenation indicated that the first detectable covalent step for the 20-carbon substrates may be abstraction of the 13-L-hydrogen (Hamberg & Samuelsson, 1967b). The latter authors proposed that one molecule of oxygen adds to carbon 11 and then probably forms a peroxy radical which attacks carbon 9, causing cyclization concomitant with the addition of the second molecule of oxygen to carbon 15. Indirect support of the concept that the initial oxidation may occur at carbon 11 came from formation of 11-hydroxy 20:2 (Nugteren et al., 1966a,b) during incubation of 20:2 (*n*-6) with crude cyclooxygenase preparations. Furthermore, significant amounts of 11-hydroxy 20:3 were found in addition to prostaglandins when crude preparations were incubated with 20:3 (*n*-6) (Hamberg & Samuelsson, 1967c).

An attempt was made to determine if the oxidation at carbon 11 could follow that at carbon 15 by incubating the cyclooxygenase with 15-hydroperoxy or 15-hydroxy 20:3, but since no prostaglandins were formed, the results were reported to support the concept that the first molecule of oxygen adds to the 11 position (Hamberg & Samuelsson, 1967b). In a different approach, glutathione peroxidase was added to reaction mixtures to trap hydroperoxy intermediates, but this resulted in total inhibition of the reaction (Lands et al., 1971) in a manner similar to that noted with lipoxygenase (Smith & Lands, 1972a). The similar roles for peroxide and the ability of both soybean lipoxygenase and cyclooxygenase to oxygenate at carbon 11 facilitate the speculation that non-heme plant lipoxygenase could be a model enzyme which might mimic one of the steps during the bis-dioxygenation of arachidonate: there could be a rate-limiting lipoxygenation reaction at carbon 11 or 15 (involving the 13-hydrogen) followed by a separate cyclizing oxygenation to produce PGG.

Interpretations based on the above-mentioned results should, however, be considered with caution since they were developed using crude preparations containing the complex of enzymes referred to as "prostaglandin synthetase" as well as numerous other enzymatic activities. Recently, the cyclooxygenase from sheep (Hemler et al., 1976) and bovine (Miyamoto et al., 1976) vesicular glands has been purified to homogeneity. The enzyme from sheep showed the properties of a tetramer containing 2 non-heme iron atoms and 2 molecules of bound heme when fully active (Hemler & Lands, 1977). Possibly the two forms of iron in cyclooxygenase may act separately in the two different oxygenations that occur. Lipoxygenase is a non-heme iron enzyme (Chan, 1973; Roza & Francke, 1973) and lipoxygenation of eicosadienoate might involve only a non-heme iron lipoxygenase activity without requiring other cofactors (e.g., heme) that appear to be needed for the cyclizing oxygenation that occurs with arachidonate.

Using the purified cyclooxygenase, we have conducted experiments which show that a single enzyme catalyzes the addition of one molecule of oxygen to 20:2 and two molecules of oxygen to 20:4. Also, we have verified that the cyclooxygenase oxidizes 20:2 (*n*-6) at carbon 11. We have found that this lipoxygenation of 20:2 was unlike the reaction catalyzed by the plant lipoxygenase and it had to some degree every recognized characteristic of the 20:4 cyclooxygenase reaction.

Materials and Methods

Materials. Sheep vesicular glands and flurbiprofen (U-27182) were generously donated by the Upjohn Co., Kalamazoo, Mich., PGG_2 was kindly supplied by Gustaf Graff (University of Minnesota), indomethacin was a gift of the

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[1] Abbreviations used: PGG_2, PGH_2, and $PGF_{2\alpha}$, prostaglandins G_2, H_2, and $F_{2\alpha}$; DDC, sodium diethyl dithiocarbamate; GSP, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; MalNEt, *N*-ethylmaleimide.

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Merck Co., West Point, Pa., eicosa-5,8,11,14-tetraenoic acid and eicosa-11,14-dienoic acid were purchased from Nuchek Prep, Elysian, Minn., hemin chloride was from Calbiochem, and sodium diethyl dithiocarbamate (DDC), glutathione (oxidized and reduced), and myoglobin were obtained from Sigma Chemical Co., St. Louis, Mo.

Bovine liver catalase (10 800 Sigma units/mL) was obtained from Sigma and assayed spectrophotometrically (ΔA_{240} per min; Beers & Sizer, 1952) and polarographically (oxygen released from 10 mM H_2O_2). Glutathione peroxidase was prepared from erythrocytes by L. DeFilippi (University of Michigan) and assayed as previously described (Cook & Lands, 1975a). Globin (4.3 mg/mL) was prepared from myoglobin by the method of Teale (1959) and then dialyzed in distilled water. All other chemicals were reagent grade purchased from common commercial sources.

Preparation of Cyclooxygenase. Cyclooxygenase was purified from sheep vesicular glands through the DEAE-cellulose chromatography step as previously described (Hemler et al., 1976). Enzyme fractions (~20 mL) from DEAE-cellulose were chromatographed at 4 °C on Bio-Gel A-1.5 M (2.2 × 44 cm) previously equilibrated with 20 mM Tris-Cl (pH 8.0) containing 10 mM DDC, 20% ethylene glycol, and 0.2% Tween 40. Active fractions were combined and dialyzed at 4 °C in 50% glycerol for further purification by isoelectric focusing (Hemler et al., 1976). Active fractions from isoelectric focusing were chromatographed at 4 °C on Sephadex G-200 (2.5 × 22 cm) previously equilibrated with the same buffer used on the above Bio-Gel A-1.5 m column.

To obtain a cyclooxygenase preparation that was significantly depleted of heme, DEAE-purified cyclooxygenase (70 mL, 641 000 units) was mixed with globin (30 mL, 4.3 mg/mL) and then chromatographed on Bio-Gel A-1.5m at 4 °C. The column (5.0 × 73.5 cm) was previously equilibrated with 0.05 M sodium phosphate (pH 7.0) containing 0.01 M DDC and 20% ethylene glycol and then preloaded with 120 mL of globin (22 mg/mL) in 0.01 M sodium phosphate (pH 7.0) containing 20% glycerol. The active fractions eluted with equilibration buffer were pooled and dialyzed in 50% glycerol for isoelectric focusing, after which the enzyme was either used directly or dialyzed in 0.02 M sodium phosphate (pH 7.0) containing 30% glycerol, or desalted on a Bio-Gel P-30 column (22 × 2.5 cm) equilibrated with 0.05 M potassium phosphate (pH 8.0) in 20% ethylene glycol. All of the purified cyclooxygenase was routinely stored at −70 °C, and only preparations of greater than 90% purity (by disc gel electrophoresis) were used in subsequent studies.

Measurement of Enzyme Activity. Enzyme activity (1 nmol oxygen/min equals 1 unit) was determined polarigraphically (Rome & Lands, 1975a) in reaction vessels containing 50–100 μM arachidonic acid or 20–80 μM eicosadienoic acid, 0.67 μM hematin, and 0.67 mM phenol or 0.1 to 0.33 mM DDC in a total volume of 3 mL of 0.1 M Tris-Cl (pH 8.5) at 30 °C.

Arachidonate Products. Using standard conditions, 50 μL of cyclooxygenase (0.35 mg/mL, 10 000 units/mg) was added to [1-^{14}C]arachidonate (23 μM) in the presence and absence of 0.67 mM phenol or 0.33 mM DDC. After oxygen consumption was monitored polarigraphically for 1 min, the 3-mL reaction mixtures were each mixed with 10 mL of precooled (−78 °C) diethyl ether and then acidified with 0.25 mL of 1 M citric acid and shaken vigorously. When the aqueous phase was frozen (−78 °C bath), the ether layer was filtered through an ether-washed Kimwipe and reduced in volume (to 0.5 mL) with a rotary evaporator, then a stream of nitrogen. A portion (50 μL) of the extract from a reaction mixture containing no phenol or DDC was reduced with triphenylphosphine (100 μL, 1% in diethyl ether) for 1 h. Samples from each reaction mixture and the reduced sample (stored on dry ice) were chromatographed on plates of silica gel H at ambient temperature and developed with ethyl acetate–isooctane–acetic acid (50:50:0.5, with no paper lining curtains). Relative abundances and migrations of products were analyzed with a Berthold LB 2760 radioscanner and compared with standard prostaglandins D, E, F, G, and H.

Stoichiometry. The stoichiometry of oxygen addition to 20:4 was determined from the amount of oxygen consumption (based on 232 μM O_2 initially present) divided by the amount of [1-^{14}C]-20:4 converted to oxygenated prostaglandin products as quantitated from TLC radioscanner recordings.

Eicosadienoate Products. Eicosadienoic acid (87 μM) was reacted with cyclooxygenase (30 000 units/mg; 17 μg/mL) in the presence of 0.6 μM hematin and 0.67 mM phenol at 30 °C for 1 min and then the mixture was added to 10 mL of ice-cold diethyl ether and acidified to pH 4.0 with 1 M citric acid. The aqueous layer was twice reextracted with 2 mL of diethyl ether; the ether extracts were combined and dried over sodium sulfate and then evaporated to 0.5 mL in vacuo and applied to a silica gel H plate. The plate was developed in diethyl ether–petroleum ether–acetic acid (50:50:1) and scanned with a radioscanner using standards of [1-^{14}C]-18:2-OOH and [1-^{14}C]-18:2-OH to locate the hydroxy and hydroperoxy regions. The reaction products, combined with the appropriate hydroxy or hydroperoxy standard, were scraped from the plate and extracted by washing with 2 mL of diethyl ether and twice with 2 mL of diethyl ether–methanol (1:1), and the solvent was removed under a gentle stream of nitrogen. The residue was treated with 0.5 mL of diazomethane in diethyl ether–methanol (9:1) for 30 min at room temperature, and, after the diazomethane was removed with a stream of nitrogen, both samples were incubated overnight in 200 μL of trimethyl phosphite–diethyl ether (1:9). Following trimethyl phosphite removal under nitrogen, incubation with 100 μL of bis(trimethylsilyltrifluoroacetamide) in 1% trimethylchlorosilane (Regasil) 30 min at 65 °C and removal of the silating reagent under nitrogen, the residue was dissolved in CS_2 for quantitation by gas chromatography.

Isomeric Composition of Products. All reactions from which the oxygenated products were characterized by mass spectrometry were carried out at 30 °C in 3.0 mL of 0.1 M sodium phosphate (pH 9.2). The concentrations of the various components were: 20:4, 46 μM; 20:2, 43 μM; glutathione or phenol, 0.67 mM; and hematin, 0.7 μM. The reactions were initiated by the addition of 100 μL of purified prostaglandin cyclooxygenase (10 000 units/mg, 0.25 mg/mL) or lipoxygenase (7 μmol of O_2 per min per mg, 2 mg/mL), terminated after 1–7 min, and extracted by the above procedure used for eisocadienoate. After 1 mL of freshly prepared diazomethane in diethyl ether–methanol (9:1) was added to the 0.5-mL ether extracts, the mixture was incubated for 30 min at room temperature, the volume was reduced under a stream of nitrogen to approximately 0.1 mL and a 500-fold molar excess of trimethyl phosphite in diethyl ether (10 v/v) was added to reduce the hydroperoxy group to the hydroxyl form. The sample was then taken to dryness under N_2, dissolved in diethyl ether, and stored at −20 °C.

Hydrogenation and Silylation. Aliquots of each sample were hydrogenated in 0.5 mL of hexane for 30 min under 5 lb of pressure using 1 mg of 5% palladium on carbon as a catalyst. The catalyst was removed by filtration and washed twice with 1 mL of chloroform:methanol (1:1). Both the unsaturated and hydrogenated samples were silylated 30 min at 65 °C with 50 μL of bis(trimethylsilyltrifluoroacetamide) + 1% trimethyl-

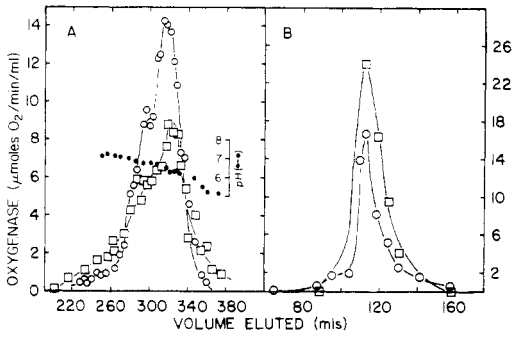

FIGURE 1: (A) Coelectrofocusing of 20:2 (□—□) and 20:4 (○—○) oxygenase activities. Isoelectric focusing was performing using a 440-mL LKB 8101 Ampholine column (Hemler & Lands, 1977) and oxygenase activity was assayed as described in Materials and Methods. The values for oxygenase activity with 20:2 have been multiplied by 10 to facilitate comparisons. (B) Cochromatography of 20:2 and 20:4 oxygenase activities. Pooled enzyme fractions from an isoelectric focusing experiment were chromatographed on Bio-Gel P-300 previously equilibrated with 0.01 M Tris-Cl (pH 8.0) containing 5 mM DDC, 20% ethylene glycol, and 0.2% Tween 40. Dilution of 20-μL aliquots for assay of oxygenase activity with 20:2 (□—□) and 20:4 (○—○) gave 0.033 mM DDC in the assay mixture. As in A, the values of 20:2 activity were multiplied by 10.

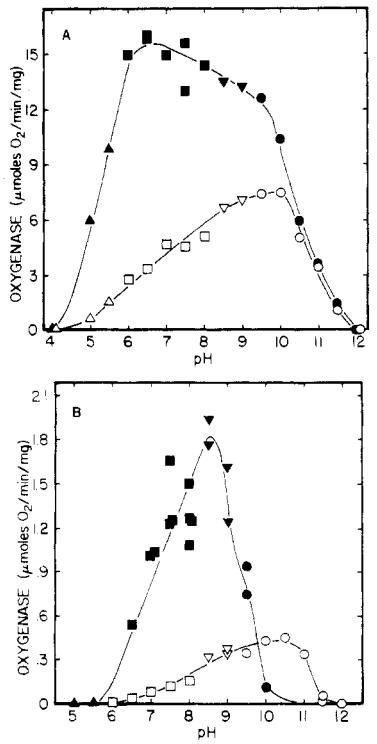

FIGURE 2: (A and B) Similar effects of pH on oxygenation of 20:4 and 20:2. The activity of cyclooxygenase (2.6 μg/mL) was determined in reaction chambers containing 20:4 (A) or 20:2 (B) in the absence (open figures) or presence (closed figures) of 0.67 mM phenol. Constant ionic strength (μ = 0.1) buffers including acetate (▲), phosphate (■), and Tris-Cl (▼), and 0.1 M glycine-NaOH (●) were used at the indicated pHs.

chlorosilane (Regasil) and injected directly into the gas chromatograph–mass spectrometer or taken to dryness under N_2 and dissolved in CS_2 before injection.

Gas Chromatographic and Mass Spectrometric Analysis. Gas chromatographic analysis was conducted with a Varian 2700 equipped with a flame detector, and mass spectra were obtained on a Finnigan 3200; in both cases the separation of the reaction products was achieved on 1% SE-30 in glass columns. In the former, the temperature of the injector was 230

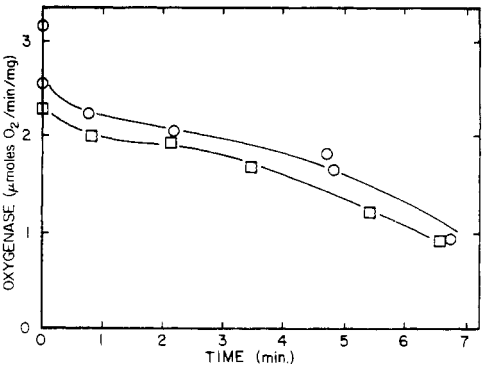

FIGURE 3: Heat inactivation of oxygenation activity for 20:4 and 20:2. Oxygenase (~3.4–6.8 μg/mL) in the presence of 0.17–0.33 mM DDC was allowed to stir at 30 °C in oxygen electrode chambers for various time intervals. Then oxygenation was initiated by the addition of 20:4 (○—○) or 20:2 (□—□) except at zero time, when substrate was added prior to enzyme. As in Figure 1, the values for 20:2 have been multiplied by 10.

TABLE I: Time-Dependent Inactivation of Cyclooxygenase Reaction with 20:2 and 20:4 by Indomethacin.[a]

Substrate	Indomethacin	Time (min)	Act. (nmol min^{-1} mg^{-1})	% act.
20:4	+	0	40 000	100
	+	0.3	16 900	42
	+	0.6	11 900	30
	+	1.5	8 935	22
	−	2.0	35 500	(89)
20:2	+	0	5 700	100
	+	0.3	2 500	44
	+	0.5	1 400	25
	−	1.5	6 300	(110)

[a] After cyclooxygenase (55 μg/mL) was preincubated with or without 2.1 μM indomethacin for the indicated time interval, the enzyme solution was diluted 40–80-fold and assayed for oxygenase activity in the presence of 0.13–0.27 mM DDC.

°C, the column oven 190 °C, and the detector 250 °C, while in the latter the injector was maintained at 215 °C, column at 185 °C, and the separator at 230 °C.

Mass spectra (100–500 amu) at 30 eV were taken every 4 s, and the data were processed by the Finnigan 6100 program using a Computer Automation Alpha 16 computer. For structure determination, mass spectra from the top of the eluted peaks were used after subtracting a background spectrum taken prior to the peak. The isomeric composition of the hydroxy acids was determined by scanning the total ion chromatogram for the fragment with 11 or 15 carbons from the hydrogenated methyl ester and comparing the measured areas of the peaks having an equivalent chain length of 22.1.

Results

Copurification of oxygenase activity for 20:4 and 20:2 occurred at each step in the purification of the cyclooxygenase. As shown in Figure 1A, for example, the oxygenation activity with both substrates cofocused with an isoelectric point of 6.3–6.7, as previously described for the cyclooxygenase (Hemler & Lands, 1977). Also, gel filtration on Bio-Gel P-300 (Figure 1B) indicated that both oxygenation reactions were catalyzed by enzymes(s) of the same molecular weight. In each case the oxygenation of 20:4 was several fold faster than that of 20:2, although the presence of DDC (also, see below) appeared to increase the ratio of 20:2 oxygenation rate relative

TABLE II: Isomeric Products from 20:2.[a]

Reaction conditions	11-OH (287) (%)	15-OH (343) (%)
Lipoxygenase	6	94
Cyclooxygenase	93	7
Cyclooxygenase + hematin	96	4
Cyclooxygenase + phenol	94	6

[a] Reaction products were extracted and analyzed for isomeric composition by GC/MS as described in materials and methods. The values are expressed as the percent distribution between ions of *m/e* 287 and 343.

TABLE III: Effect of Additions on Cyclooxygenase Reactions.[a]

Addition	20:4 K_m (μM)	20:4 V_{max} (nmol min^{-1} mg^{-1})	20:2 K_m (μM)	20:2 V_{max} (nmol min^{-1} mg^{-1})
None	2.3 (6)	13 000	2.3 (8)	1200
DDC	2.4 (8)	22 000	2.5 (7)	5700
Phenol	2.8 (7)	27 000	20 (8)	7900

[a] Cyclooxygenase (1.7–2.8 μg/mL) was assayed at pH 8.5 with 20:4 and 20:2, and phenol (0.67 mM) and DDC (0.33 mM) were included as indicated. The number of assays used to obtain each K_m and V_{max} is included in parentheses.

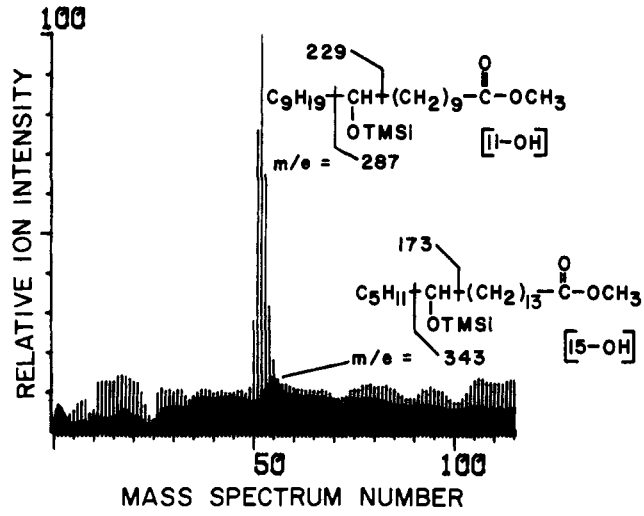

FIGURE 4: Scan of *m/e* 287 and 343 of the total ion chromatogram obtained from GC/MS analysis of the hydrogenated derivatives extracted from the reaction of 20:2 and purified cyclooxygenase.

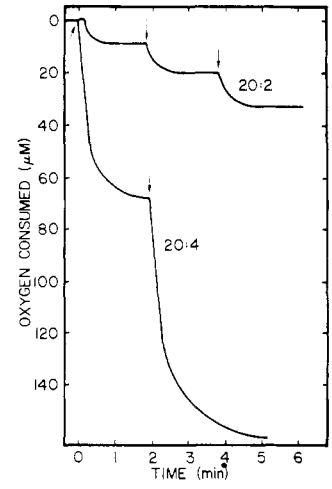

FIGURE 5: Oxygen consumption during oxygenation of 20:4 and 20:2. Reactions were initiated (arrows) by the addition of aliquots of a stock solution of cyclooxygenase (0.19 mg/mL; 20 000 nmol of O_2 per min per mg) to reaction chambers as described in Materials and Methods. The enzyme stock solution contained 10 mM DDC and 80 μL, 50 μL, and 50 μL were used for the 20:2 reactions, while 50 μL then 50 μL was used for the 20:4 reactions.

to 20:4 oxygenation rate. This ratio was significantly higher in Figure 1B (24/16.5) than in Figure 1A (8.7/14) as DDC was presumably removed during isoelectric focusing.

The oxygenation of both substrates was affected in a similar manner by pH showing broad pH optima for 20:4 (pH 8.5–10; Figure 2A) and 20:2 (pH 8.5–11; Figure 2B). When 0.67 mM phenol was included as a cofactor, the pH optima were significantly more acidic for both acids: 20:4 (6–9.5; Figure 2A) and 20:2 (8.5; Figure 2B). Also, phenol stimulated the peak oxygenase activity for 20:4 (twofold) and 20:2 (fourfold). When 0.33 mM DDC was present (data not shown), stimulation of oxygenation of 20:4 (twofold) and 20:2 (fourfold) again was observed but without a shift in the pH optima or shape of the pH curves.

Oxygenase activity with either 20:2 or 20:4 was gradually lost when purified cyclooxygenase was subjected to heat inactivation at 30 °C in the presence of heme. Significantly, the losses occurred in parallel (Figure 3) with half lost in about 6 min. Indomethacin (Table I) and flurbiprofen (data not shown) inhibited the oxygenation of both 20:4 and 20:2 in a time-dependent manner and activity with both acids decreased at similar rates.

The predominant product formed from [1-^{14}C]-20:4 in the absence of cofactors comigrated with authentic PGG_2 standard, and, upon treatment with triphenylphosphine, it comigrated with standard $PGF_2\alpha$. When 0.67 mM phenol was present, the product comigrated with standard PGH_2, and, in the presence of 0.33 mM DDC, the predominant product comigrated with $PGF_2\alpha$.

To verify that the observed oxygen uptake was due to oxygenation of 20:4, the amount of oxygen consumed after 1 min of reaction was compared to the amount of [1-^{14}C]-20:4 consumed. An O_2/fatty acid ratio of 2.0 was observed when active cyclooxygenase was assayed in the absence of added hematin, or in the presence of 0.67 μM hematin, or 0.33 mM DDC. When 0.67 mM phenol was present, the ratio was 2.2.

Gas chromatographic analysis of the trimethylsilyl ether methyl ester of the unsaturated hydroxy acid formed from 20:4 by soybean lipoxygenase showed an equivalent chain length (ecl) of 21.3 on 1% SE-30, whereas the derivatized product formed from 20:2 by soybean lipoxygenase or the purified prostaglandin cyclooxygenase had an ecl value of 21.8. Although the relative intensities of the peaks differed, the mass spectra for the unsaturated products of 20:2 formed by either lipoxygenase or cyclooxygenase exhibited peaks at 410 (m), 395 (m − 15), 379 (m − 31), 363 (m − 47), 339 (m − 71, C_5H_{11}), 320 (m − 90), 249 (m − (71 + 90)), and 225 ($(CH{=}CH)_2 - CH(TMSiO^+) - C_5H_{11}$), 15-OH isomer; ($TMSiO^+CH - (CH{=}CH)_2 - C_5H_{11}$), 11-OH isomer; both (m − 185). Showing very close analogy to 20:2 oxygenated products, the mass spectrum of the product of 20:4 formed by lipoxygenase had peaks at 406 (m), 391 (m − 15), 375 (m − 31), 359 (m − 47), 335 (m − 71), 316 (m − 90), 245 (m − (71 + 90)), and 225 (see above).

After hydrogenation, the three hydroxy acid derivatives had an ecl value of 22.1 and the mass spectra of the products of li-

TABLE IV: Glutathione Peroxidase Inhibition of 20:4 and 20:2 Oxygenations.[a]

Substrate	Addition	GSP (units/mL)	Time to optimum (s)	Velocity (nmol min^{-1} mg^{-1})	Act. restored by NEM nmol min^{-1} mg^{-1})
20:4	None	0	12	30 000	0
	GSH	0	13	40 100	0
	GSH	2000	17	27 600	9 000
	GSH	4000	18	15 500	18 000
	GSH	6000	20	14 900	19 800
	GSH	8000	36	3 000	28 200
	None	8000	13	30 000	0
	GSH, GSSG	0	13	44 400	0
20:2	GSH	0	14	8 500	0
	GSH	1000	16	6 000	800
	GSH	2000	19	3 100	3 200
	GSH	4000	50	1 200	5 700
	GSH, GSSH	0	14	8 000	0

[a] Cyclooxygenase (0.6–1.1 μg/mL) was assayed in oxygen electrode chambers containing GSH (0.67 mM), GSSG (67 μM), and GSP as indicated. Reactions were initiated by addition of enzyme and the time to optimum is the interval between reaction initiation and the attainment of optimum velocity (Cook & Lands, 1975b). After 1.5 min, when the initial reaction had subsided, 1 mM NEM was added and the velocity of any further reaction was determined.

TABLE V: Requirement for Heme for the Oxygenation of 20:2 and 20:4.[a]

Hematin added	Act. (nmol min^{-1} (mL of enzyme)$^{-1}$) 20:4	20:2
+	4700	480
− (+)	0 (4900)	0 (730)

[a] Cyclooxygenase (~1.4 μg/mL) was assayed in the standard assay mixture plus 0.67 mM phenol in the presence or absence of 0.67 μM hematin. If hematin was not present initially, it was then added after 20–25 s to initiate oxygenation giving the rate noted in parentheses.

poxygenase with either 20:2 or 20:4 were identical, with peaks at 399 (m − 15), 383 (m − 31), 367 (m − 47), 343 (m − 71), and 173 ($CH_3(CH_2)_4$ − CH-OTMSi$^+$) and small peaks at 229 ($CH_3(CH_2)_8$ − CH-OTMSi$^+$) and 287 (CH_3-O-C(=O)-$(CH_2)_9$ − CH-OTMSi). The mass spectra of 20:2 and purified prostaglandin cyclooxygenase (under all conditions) showed peaks at 399 (m − 15), 383 (m − 31), 367 (m − 47), 287, and 229 (see above) and small peaks at 343 and 173. The above assignments of the various fragments follow those described by Hamberg & Sammuelsson (1974a,b).

The areas of the 287 and 343 peaks in the total ion chromatogram (shown in Figure 4) were used to determine the isomeric compositions of the products formed by the reaction of 20:2 (*n*-6) with lipoxygenase or cyclooxygenase as presented in Table II. The presence of phenol or excess hematin did not affect the isomeric composition of the products.

In agreement with the results shown in Figure 2, results in Table III showed that 0.67 mM phenol or 0.33 mM DDC significantly stimulated the 20:2 and 20:4 oxygenation rates (5–7-fold and 2-fold, respectively) under standard assay conditions. The greater stimulation of 20:2 helps to interpret Figure 1 in which 20:4 was oxygenated at a rate that is approximately 11-fold higher than 20:2 when DDC was absent (Figure 1A) and only 3.5- to 4-fold higher than 20:2 when DDC was present (Figure 1B). In most subsequent studies of 20:2 oxygenation, phenol or DDC was routinely included in order to obtain the higher reaction rates. The apparent K_m values for each substrate were similar in the presence or absence of DDC. Phenol significantly raised the apparent K_m for 20:2 (from 2.3 to 20 μM) while not greatly affecting that for 20:4 (~2.3 μM).

When saturating amounts of 20:2 or 20:4 were oxygenated by the purified cyclooxygenase (regardless of cofactors present), there was a burst of activity, then a rapid inactivation within 1–2 min after reaction initiation (Figure 5). Reactive substrate was still present since oxygenation could be reinitiated by the addition of further aliquots of cyclooxygenase (arrows) or soybean lipoxygenase (data not shown). Conversely, further additions of each substrate after inactivation had occurred caused no further reaction.

The presence of increasing amounts of glutathione peroxidase (GSP) caused increased inhibition of the oxygenation of both 20:2 and 20:4 as shown in Table IV. Increased times to the attainment of optimum velocity were associated with lower velocities and the peroxidase inhibition was dependent upon the presence of the GSP cofactor, glutathione (GSH). This requirement for the glutathione cofactor is further evidenced by the rapid acceleration of the slowed reaction rate immediately after addition of the sulfhydryl scavenger, *N*-ethylmaleimide (MalNEt). For example, when 20:4 was oxygenated in the presence of 8000 units/mL of glutathione peroxidase (GSP), a maximum reaction rate of only 100 nmol min^{-1} mL^{-1} of enzyme was attained. When MalNEt was added, GSP was no longer effective, and the reaction velocity rapidly accelerated to a maximum of 980 nmol min^{-1} mL^{-1} of enzyme. The stability of the cyclooxygenase in the presence of GSP is indicated by the sums of the inhibited and subsequently released velocities since these sums were nearly identical with uninhibited controls regardless of the amount of GSP used to inhibit. In control experiments, oxidized glutathione, a product of the peroxidase reaction, and GSP alone had no inhibitory effect whereas reduced glutathione (GSH) appeared to stimulate rather than inhibit. Catalase (11 units/mL) did not inhibit the oxygenation of either substrate in the presence or absence of cofactors.

Partial removal of bound heme was obtained by treatment of cyclooxygenase from DEAE-cellulose with globin before and during chromatography on Bio-Gel A-1.5m. This resulted in the partial conversion of enzyme to an apoenzyme form as

evidenced by a greater requirement for added heme to attain maximal activity. During subsequent manipulations, the activity of holoenzyme (which already contained heme) was completely lost while the apoenzyme remained stable. Thus, cyclooxygenase preparations were obtained which were totally unable to catalyze the oxygenation of either 20:2 or 20:4 unless hematin was added to the reaction (Table V). This result was obtained in several separate experiments. In control experiments, the heme-initiated reaction with both acids was inhibited in the presence of 16 μM flurbiprofen and there was no oxygen reaction with hematin plus substrate alone or hematin plus enzyme alone (results not shown). Also, further addition of hematin after the reaction had stopped due to "self-catalyzed inactivation" caused no further reaction.

Discussion

Using modifications of previous procedures (Hemler et al., 1976), we have demonstrated that oxygenase activities for both 20:4 and 20:2 copurify through all purification steps, and the final homogeneous enzyme preparation can oxygenate both substrates. For comparative purposes the mass spectrum of the unsaturated product of 20:4 oxygenation by lipoxygenase (exclusively the C-15 isomer; Hamberg & Samuelsson, 1967a) was obtained, and the oxygenation of 20:2 by cyclooxygenase or lipoxygenase resulted in products with unsaturated mass spectra that were closely analogous to the 20:4 product. Since unsaturated C-11 and C-15 isomers split into fragments of equal mass (due to allylic cleavage), isomeric composition was not determined until the products were hydrogenated. The validity of analyzing total ion scans of saturated products as a means for determining the percent distribution of each isomer was clearly demonstrated by the results obtained with products of 20:2 or 20:4 with soybean lipoxygenase. Following the pattern of *n*-6 specificity established for 20:4 and other soybean lipoxygenase substrates (Hamberg & Samuelsson, 1967a), 20:2 like 20:4 was oxygenated predominantly at the 15 position. However, in marked contrast to soybean lipoxygenase, the pure cyclooxygenase oxygenated the 11 position (*n*-10) in agreement with the result reported (Nugteren et al., 1966a,b) for a crude microsomal preparation of cyclooxygenase. This specificity is also clearly distinct from platelet lipoxygenase which oxygenated at carbon-12 (Nugteren, 1975), and lipoxygenase activity reported for polymorphonuclear leucocytes (at carbons 5 or 8; Borgeat et al., 1976). There are apparently many lipoxygenases, and a specificity for the *n*-10 position has been reported for corn (Gardner & Weisleder, 1970), potato (Galliard & Phillips, 1971), and tomato (Matthew et al., 1977) lipoxygenases.

Oxygenation of 20:4 yielded the expected hydroperoxy product, PGG_2, and formed the hydroxy derivative, PGH_2, when a reductant such as phenol was present. Whereas PGH_2 formation is apparently due to peroxidase activity of the cyclooxygenase (Miyamoto et al., 1976), the further reduction of PGF_2 that we observed in the presence of DDC was probably nonenzymic as noted by Chan et al. (1975). The mixture of hydroxy and hydroperoxy products formed from 20:2 suggests that phenol can serve as a cosubstrate for the peroxidase activity which reduces the C-11 hydroperoxide of 20:2 in addition to reducing the C-15 hydroperoxy group of PGG_2. The peroxidatic cofactors, phenol and DDC, also stimulated the overall rate of 20:2 and 20:4 oxygenation. Since the stoichiometry of 2 mol of O_2 per mol of arachidonate converted to prostaglandin did not significantly vary in the presence or absence of phenol, DDC, or hematin, the observed stimulatory effects represented enzyme-catalyzed oxygenation rather than a cooxidation or autoxidation or other artifactual stimulation of oxygen consumption.

Phenol stimulation could possibly be caused by an increased rate of free radical formation of fatty acid substrate or intermediate(s) or by facilitated interconversion of intermediate redox states of the heme iron (Yamazaki, 1974). Since the reaction with 20:2 is subject to greater stimulation by phenol than the 20:4 reaction, the enzyme may be more likely to spend time in an unproductive intermediate form with the former fatty acid. The stimulatory effect of DDC is unusual since DDC is mainly known for its inhibitory properties at higher concentrations that may involve metal chelation (LeTellier et al., 1973) or peroxide removal (Ishimura & Hayaishi, 1973). Another alternative (suggested by Ouderaa et al., 1977) is that phenol or DDC facilitated peroxide removal may retard destruction of the needed heme and thus promote higher initial reaction rates. Thus 20:2 oxygenation which occurred at a lower rate, perhaps due to greater destruction (by 20:2-OOH), was more stimulated by DDC and phenol, whereas 20:4 oxygenation appeared to be less affected by its extremely unstable peroxide product (PGG_2) and was thus less stimulated by DDC and phenol.

Besides being similarly affected by peroxidatic cofactors, the 20:4 and 20:2 oxygenation reactions had many other features in common. As might be expected for controlled free radical reactions (Samuelsson et al., 1966; Nugteren et al., 1966a; Porter & Funk, 1975), both oxygenations showed broad pH optima in the absence of peroxidatic cofactors similar to that reported for 20:4 oxygenation by crude cyclooxygenase (Egan et al., 1976). The more acidic pH optima, obtained as a result of phenol stimulation (between pHs 6.0 and 9.5), are more in agreement with previously reported studies using crude (bovine, Yoshimoto et al., 1970; Takeguchi et al., 1971; Flower et al., 1973; and ovine, Wallach & Daniels, 1971; Raz et al., 1975) enzyme preparations. These earlier assays all had been conducted in the presence of phenolic or indole cofactors and showed pH optima near 8.0. From the pH profiles, it appears that the mode of action of phenol stimulation is clearly distinct from that of DDC, which did not shift the pH optima.

In crude preparations of bovine (Takeguchi et al., 1971; Yoshimoto et al., 1970; Flower et al., 1973) and ovine (Egan et al., 1976) cyclooxygenases, arachidonate concentrations of 30–100 μM gave half-maximal velocities, whereas we found that the purified cyclooxygenase had apparent K_m values that were approximately tenfold lower for 20:4 and 20:2. Presumably, there was inhibition due to excessive binding of fatty acid to contaminating proteins in the crude preparations. Phenol was again distinct from DDC in causing a higher apparent K_m for 20:2 relative to 20:4. This phenol effect suggests a weaker interaction for 20:2 with the cyclooxygenase.

Time-dependent, irreversible inhibition by indomethacin and flurbiprofen occurred with the purified enzyme in a manner similar to that observed with crude systems (Smith & Lands, 1971; Rome & Lands, 1975b). Thus it appears that these drugs directly modify the cyclooxygenase to an inactive form. The parallel loss of activity with both substrates indicated that the same active site was essential for both oxygenation reactions.

The parallel inactivation of both activities upon incubation at 30 °C (Figure 3) further supports the concept that all of the essential rate limiting features of 20:4 oxygenation are common to 20:2 oxygenation. A third mode of inactivation, that which is turnover mediated, occurred during the oxygenation reactions as observed earlier for the crude cyclooxygenase (Smith & Lands, 1972b). The rate of enzyme destruction during the 20:2 oxygenation was comparable to that during 20:4 oxy-

genation, suggesting that a prosthetic group or part of the active site crucial for oxygenation at carbon 11 was being modified during the reaction.

The role of heme as a prosthetic group for bis-dioxygenation of arachidonate has been previously demonstrated (Hemler & Lands, 1977). We now see that oxygenation of 20:2 at carbon 11 also required the presence of heme, thus strongly suggesting that the catalysis of oxygen addition to position 11 with both substrates may need the heme prosthetic group. Since heme is labile in the presence of peroxides (formed in both oxygenation reactions), heme destruction may be a key event in turnover-mediated inactivation.

As previously described for the crude enzyme (Smith & Lands, 1972a,b; Lands et al., 1976), decreased reaction rates and increased lags, or induction periods, were observed with purified cyclooxygenase when peroxide was removed from reaction mixtures by added glutathione peroxidase. Because different levels of added peroxidase could stop reactions which had already proceeded at various rates, it appears that peroxide does not merely activate the enzyme at an initial stage of the reaction, but that it must be present above a minimal level (estimated at 0.1 μM, Lands et al., 1976) throughout the reaction. Since the 20:2 as well as the 20:4 oxygenation reaction could be stopped and then reactivated dependent upon peroxidase activity, it appears that peroxide, like heme, is needed for catalysis of the oxygenation at C-11. The lack of inhibition of either reaction by catalase suggests that the peroxide involved is a lipid peroxide (probably reactive products) rather than hydrogen peroxide.

In summary, although the cyclooxygenase-catalyzed oxygenation of 20:2 had some kinetic features characteristic of the none-heme iron soybean lipoxygenase activity, such as self-destruction and a requirement for peroxide (Smith & Lands, 1970, 1972a), it had many features uncharacteristic of lipoxygenase such as an absolute requirement for heme, phenol and DDC stimulations, and peroxidase activity, and anti-inflammatory drug inhibition. Also, the concept of "half-reaction" may be misleading as the 20:2 reaction was mechanistically similar to 20:4 in every respect and depended on the full enzyme capability of cyclooxygenase. Thus it appears that 20:2 undergoes oxygenation with perhaps further oxygen activation, just as 20:4, but unlike 20:4, it cannot form a final irreversible covalent cyclic derivative due to lack of needed π bonds (see cofactor discussion above). In support of this concept that an active intermediate undergoes spontaneous cyclization and oxygenation at C-15 (n-6), a nonenzymic model system was used to show that removal of peroxide hydrogen from the n-10 hydroperoxide derivative of linoleic acid could lead to both free radical cyclization and addition of another mole of O_2 at the n-6 position to form prostaglandin analogues (Porter & Funk, 1975).

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High Resolution Proton Magnetic Resonance Spectroscopy of Histones and Histone–Histone Complexes in Aqueous Solution†

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ABSTRACT: Low molecular weight histone complexes of H2A (≃dimer), H2B (≃tetramer), H3-H4 (≃tetramer), H2A-H2B (≃dimer), and H2B-H4 (≃dimer) have been prepared in 2 M NaCl and neutral pH at 4 °C. These materials are free of nonspecific aggregate and are suitable for study by high resolution proton magnetic resonance spectroscopy. Such spectra have been recorded in aqueous solution under conditions allowing a study of the exchangeable proton resonances of histone complexes for the first time and indicate that the structured regions are rich in hydrophobic amino acids, as well as arginine and some acidic amino acids. Most of the lysine and probably alanine residues remain in a motile, random coil-like state after formation of the complexes. It is suggested that arginine residues may be important in inter- and/or intra-subunit interactions in histone complexes.

Since the demonstration by Hewish & Burgoyne in 1973 that eukaryotic chromatin consists of subunits, there has been considerable interest in the structure of this subunit. It now appears that the repeating subunit (nucleosome or ν body) consists of ~140-200 base pairs of DNA double helix wound around the outside of an octameric histone core of two each of the histones H2A, H2B, H3, H4. (For recent reviews, see Lewin, 1975; Weintraub et al., 1976; Kornberg, 1977).

Recent work has also shown that, under appropriate conditions, acid extracted isolated histones can form secondary structures (see, e.g., Bradbury & Rattle, 1972; Bradbury et al., 1975; Pekary et al., 1975; Shih & Fasman, 1971; Adler et al., 1975a,b), relatively specific heterotypic histone-histone complexes (see, e.g., D'Anna & Isenberg, 1973, 1974a,b), and/or very high molecular weight aggregates (see, e.g., Sperling & Bustin, 1975; above references; as well as a review by Van Holde & Isenberg, 1975).

Assessment of the biological relevance of the various renatured forms of histones is a difficult task in the absence of specific enzyme-like functional assays. The only valid criterion for functional renaturation is the formation of nucleosomal structures when bound to DNA. Recent studies of Felsenfeld and co-workers (Camerini-Otero et al., 1976) have shown that acid extracted histones may renature and reassociate with DNA to form a nucleosome-like core.

Evidence is accumulating in support of specific small molecular weight histone complexes stable at high ionic strength and low temperature as the kinetic units responsible for nucleosome structure (Pardon et al., 1978; Weintraub et al., 1975). These histone complexes may reassociate with DNA to form chromatin-like material (Oudet et al., 1975; Fulmer & Fasman, results in progress).

Since it is probable that, to a large extent, nucleosome structure is determined by the structure of low molecular weight histone complexes rather than high molecular weight histone aggregates, it is of considerable importance to prepare homogeneous renatured complexes for physical studies. It has been found that the direct mixing of salt to histones in low ionic strength buffer, at ambient temperature, resulted in considerable aggregation (~30%) even at intermediate final con-

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