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Biochemistry

Identification and functional characterization of leukotriene B_4 20-hydroxylase of human polymorphonuclear leukocytes

(5-lipoxygenase)

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ABSTRACT **A single reaction product was formed during the incubation of 1.5 μM (5S,12R)-dihydroxy-6,14-*cis*-8,10-*trans*-[^{3}H]icosatetraenoic acid (leukotriene B_4, LTB_4) for 30 min at 37°C in 10 mM potassium phosphate buffer (pH 7.5) with 100 μM NADPH and the 150,000 × *g* supernatant of sonicated human polymorphonuclear leukocytes (PMN). The reaction product exhibited the same mobility on reversed-phase HPLC (RP-HPLC) and TLC as standard 20-hydroxy-LTB_4 (20-OH-LTB_4). When the ω-oxidation product of [^{3}H]LTB_4 was eluted from a Sep-Pak, resolved by RP-HPLC, and analyzed by GC/MS, its structure was determined to be solely 20-OH-LTB_4. The K_m of the 20-hydroxylase for [^{3}H]LTB_4 at its optimal pH of 7.5 was 0.22 ± 0.08 μM (mean ± SD, *n* = 4) and the V_{max} was 48 ± 11 pmol/min·mg of protein (mean ± SD, *n* = 4). When the concentration of [^{3}H]LTB_4 was fixed at 1.5 μM, the K_m for NADPH was 1.01 ± 0.59 μM (mean ± SD, *n* = 3). The location in the 150,000 × *g* supernatant of the LTB_4 20-hydroxylase distinguishes it from the cytochrome P-450 system of liver, lung, and kidney microsomes and from the NADPH oxidase–cytochrome b_{-245} system of the human PMN. The LTB_4 20-hydroxylase is either a unique cytochrome P-450 or other monooxygenase.**

Human polymorphonuclear leukocytes (PMN) stimulated with the calcium ionophore A23187 (1–3) or the chemotactic peptide f-Met-Leu-Phe (4) produce (5S,12R)-dihydroxy-6,14-*cis*-8,10-*trans*-icosatetraenoic acid (leukotriene B_4, LTB_4) and convert this compound to 20-hydroxy-LTB_4 (20-OH-LTB_4) and 20-carboxy-LTB_4 (20-COOH-LTB_4) (1–4). Exogenously added LTB_4 is also metabolized by intact PMN to these products (1–3). This ω-oxidation pathway results in the progressive deactivation of the chemotactic activity of LTB_4 (4). The enzyme(s) catalyzing these conversions has not yet been described in a cell-free system.

In this manuscript we describe a unique NADPH-dependent monooxygenase that converts LTB_4 solely to 20-OH-LTB_4. It is distinguished by its presence in the 150,000 × *g* supernatant of sonicated PMN from both the microsomal cytochrome P-450 systems known to catalyze the ω-oxidation of prostaglandins and long-chain fatty acids in a variety of tissues and from the NADPH oxidase–cytochrome b_{-245} system of the human PMN (5–15).

MATERIALS AND METHODS

Materials. Ethyl acetate, isooctane, acetic acid, and petroleum ether were from Fisher; silica gel 60-F254 TLC plates were from Scientific Products, Bedford, MA; NADPH, NADH, and 4β-phorbol 12β-myristate 13α-acetate (PMA) were from Sigma; and methanol, HPLC-grade, was from Burdick and Jackson, Muskegon, MI. Synthetic LTB_4 (16) was supplied by E. J. Corey (Department of Chemistry, Harvard University, Cambridge, MA), and [^{3}H]LTB_4 (32 mCi/mmol; 1 Ci = 37 GBq) was obtained from New England Nuclear. 20-OH-LTB_4 and 20-COOH-LTB_4 standards were prepared by the method of Hansson *et al.* (1) after the incubation of 30 μg of LTB_4 with intact human PMN.

Preparation of PMN and LTB_4 20-Hydroxylase. Four to 10 × 10^8 PMN were isolated from 500 ml of citrate-anticoagulated blood and purified to >97% purity by sequential dextran sedimentation, hypotonic lysis, and Ficoll-Hypaque gradient centrifugation (17). The PMN were washed in Hanks' balanced buffered saline without calcium or magnesium (GIBCO) and were resuspended at a concentration of 10^8 cells per ml in 0.34 M sucrose. The cells were sonicated at 0°C for two 30-sec intervals at a setting of 4 (50% pulse cycle) with a Branson sonifier. Unbroken cells and debris were sedimented at 400 × *g* for 5 min at 4°C, and the resulting supernatant, termed the sonicate, was centrifuged at 150,000 × *g* for 1 hr at 4°C. The resulting high-speed supernatant, representing the cytosol, was stored at −70°C and used as a source of LTB_4 20-hydroxylase. The cytosol was found to contain 3.4 ± 1.0 mg of protein per ml (mean ± SD, *n* = 4) by the method of Lowry *et al.* (18).

Assays of LTB_4 20-Hydroxylase. One-hundred-microliter portions (48–670 μg) of cytosol were routinely mixed with 100 μl of 10 mM potassium phosphate buffer (KP buffer) (pH 7.5), 1.5 μM [^{3}H]LTB_4 (40,000 cpm/nmol), and 100 μM NADPH for 30 min at 37°C. The reactions were stopped by the addition of 800 μl of ice-cold methanol. Each test tube was mixed in a Vortex and then centrifuged at 1000 × *g* at 4°C for 10 min. Eight hundred microliters of the supernatant was injected onto an Altex ODS 10-μm C_{18} reversed-phase HPLC (RP-HPLC) column. Elution was performed with one of three isocratic solvents of different proportions (vol/vol) of methanol/water/acetic acid, 65:35:0.1 (system A), 70:30:0.1 (system B), and 57:43:0.1 (system C), adjusted to pH 5.6 with ammonium hydroxide. The flow rate of each solvent was 1 ml/min, and 1-ml fractions were collected and added to 10 ml of Aquasol (New England Nuclear) for quantitation of radioactivity by β-scintillation counting in a Tracor Mark III β-scintillation counter (Tracor Instruments, Elk Grove, IL). The respective retention times of the LTB_4 and 20-OH-LTB_4 standards were determined with on-line monitoring at OD_{280} with a Hitachi model 100-40 spectrophotometer. In solvent system A, the retention time of LTB_4 standard was 20.7 min (mean, *n* = 2) and that of 20-OH-LTB_4 standard was 4.52 min (mean, *n* = 2). In

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Abbreviations: LTB_4, (5S,12R)-dihydroxy-6,14-*cis*-8,10-*trans*-icosatetraenoic acid; 20-OH-LTB_4, 20-hydroxy-LTB_4; 20-COOH-LTB_4, 20-carboxy-LTB_4; PMA, 4β-phorbol 12β-myristate 13α-acetate; PMN, polymorphonuclear leukocyte(s); RP-HPLC, reversed-phase HPLC.

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solvent system B, the retention time of LTB_4 standard was 12.05 ± 0.38 min (mean ± SD, n = 4) and that of 20-OH-LTB_4 standard was 4.12 min (mean, n = 2). In this assay of the enzyme the overall recovery of radioactivity from RP-HPLC resolution of substrate and product was 65% ± 6% (mean ± SD, n = 9). Alternatively, a comparable reaction was stopped after 30 min at 37°C by the addition of 80 μl of 2 M citric acid and 400 μl of ice-cold ethyl acetate. Each test tube was mixed in a Vortex and then centrifuged at 1000 × g for 5 min at 4°C. One hundred microliters of the upper ethyl acetate phase was spotted on a silica gel 60-F254 TLC plate and developed in the organic phase of ethyl acetate/isooctane/acetic acid/water, 110:50:20:100 (vol/vol). LTB_4 and 20-OH-LTB_4 standards were spotted in an adjoining lane and identified by iodine vapors; standard LTB_4 and 20-OH-LTB_4 had R_f values of 0.5 and 0.25, respectively. The corresponding areas of the silica gel were scraped into counting vials, 10 ml of Aquasol was added, and the radioactivity was quantitated by β-scintillation counting. The overall recovery of applied radioactivity in this assay method was 80% ± 21% (mean ± SD, n = 5).

Sample Preparation and Characterization by GC/MS. The product of the reaction of PMN cytosol and [^{3}H]LTB_4 was obtained by incubating 36 μg of [^{3}H]LTB_4 (8000 cpm/nmol) for 90 min at 37°C with 10 ml of cytosol (38.8 mg of protein) in 40 ml of 10 mM KP buffer (pH 7.5) in the presence of 100 μM NADPH. The incubation mixture was injected on a reversed-phase Sep-Pak that had been washed previously with 30 ml of ethanol followed by 30 ml of distilled water. The Sep-Pak was washed with 10 ml of distilled water, 10 ml of petroleum ether, 10 ml of ethyl acetate, and 10 ml of methanol. Twenty-microliter portions of each fraction were assayed for radioactivity in a β-scintillation counter; all of the radioactivity eluted in the ethyl acetate wash. The ethyl acetate was evaporated to dryness under nitrogen and the labeled reaction product(s) was resuspended in 1 ml of methanol/water/acetic acid (57:43:0.1, pH 5.6), applied to a C_{18} RP-HPLC column, and eluted isocratically in this same solvent at a flow rate of 1 ml/min. Optical density at 280 nm was monitored. One-milliliter fractions were collected and 10-μl aliquots of each were quantitated by β-scintillation counting. [^{3}H]LTB_4, which eluted (solvent system C) at a retention time of 31.5 min, was converted to one product eluting with the retention time of 5.65 min, as determined by ultraviolet monitoring and β-scintillation counting. RP-HPLC fractions containing the radiolabeled product were pooled for analysis by GC/MS.

Three micrograms of standard 20-OH-LTB_4 and 3 μg of the purified product of the reaction between [^{3}H]LTB_4 and PMN cytosol were each lyophilized to dryness, redissolved in 0.5 ml of methanol, and methylated by the drop-wise addition of diazomethane. Methanol and excess diazomethane were removed under a stream of nitrogen at room temperature. Trimethylsilyl ethers were prepared by adding 10 μl of bis(trimethylsilyl)trifluoroacetamide (Supelco, Bellefonte, PA) and 10 μl of acetonitrile and warming the mixture to 60°C for 15 min.

Catalytic hydrogenation was performed by dissolving 3 μg of the methyl esters of standard 20-OH-LTB_4 or of the purified reaction product in 0.5 ml of methanol, adding 5–10 mg of rhodium on alumina, and bubbling hydrogen through the solution for 4 min at room temperature. The solution was centrifuged and the supernatant was transferred to a clean tube and evaporated to dryness under a stream of nitrogen. Trimethylsilyl ethers were then prepared as described above.

Gas chromatographic separations were performed on a 60 × 0.2 cm glass column with 3% OV-101 on 80- to 100-mesh Supelcoport (Supelco) with a Finnigan 9500 gas chromatograph interfaced to a Finnigan 3200 quadropole mass spectrometer (Finnigan, Sunnyvale, CA). For fatty acid methyl ester equivalent chain length determinations, the GC oven temperature was programed to increase from 150°C to 300°C at a rate of 10°C/min. Mass spectra were obtained by electron impact ionization (70 eV).

RESULTS

In the initial studies the reactions of 250 μl of PMN sonicate, 1.21 mg of cytosol protein in 250 μl, and 1.71 mg of the 150,000 × g membrane pellet (resuspended to one-third the original volume of the sonicate) in 250 μl, respectively, with 1.5 μM [^{3}H]LTB_4 and 100 μM NADPH were compared after incubation of the mixtures in a final 500-μl volume at 37°C for 30 min in 10 mM KP buffer (pH 7.5). After resolution by RP-HPLC in solvent B, the substrate and an ω-oxidation product(s) with a retention time identical to that of the 20-OH-LTB_4 standard were found in the reaction mixture formed with the sonicate (Fig. 1*A*). The sonicate converted 50.7% of the added [^{3}H]LTB_4 to product (Fig. 1*A*), whereas none of the substrate was metabolized when NADPH was omitted from the reaction mixture; 100 μM NADPH was therefore included routinely in subsequent reactions. The cytosol converted 18.8% of the added substrate to ω-oxidation product (Fig. 1*B*), whereas 3.8% of the substrate was converted to product by the resuspended membrane pellet (Fig. 1*C*). For two experiments the sonicate converted a mean value of 40.3%, the cytosol converted a mean of 18.9%, and the membrane fraction converted a mean of 2.0% of the added [^{3}H]LTB_4. In a parallel experiment, using the same cells as the initial experiment, 10^8 cells per ml were activated with 1 μg of PMA per ml for 6 min at 37°C and were sonicated and sedimented to yield cytosol and pellet. After PMA activation 250 μl of the sonicate converted 42.3% of [^{3}H]LTB_4 to ω-oxidation product, 1.03 mg of the cytosol converted 18.5%, and 805 μg of the membrane fraction converted 3.2% of the substrate.

The products of the reaction of 1.5 μM [^{3}H]LTB_4 and cytosol supplemented with 100 μM NADPH were analyzed by isocratic elution from a 10-μm 0.46 × 25 cm C_{18} RP-

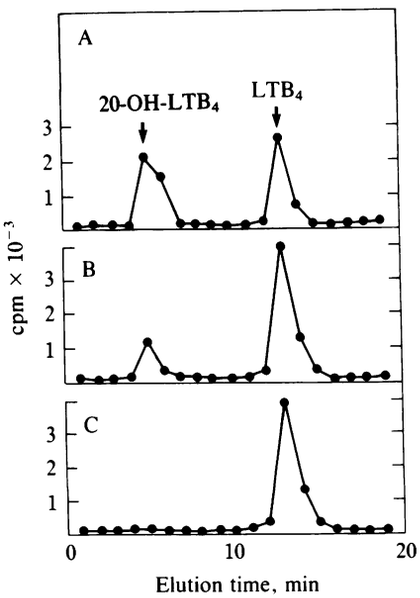

FIG. 1. RP-HPLC analysis of the ω-oxidation of [^{3}H]LTB_4 by subcellular fractions of human PMN. Two-hundred-fifty microliters of cell sonicate (*A*), 250 μl (1.21 mg) of cell cytosol (*B*), and 250 μl (1.71 mg) of resuspended membrane pellet (*C*) were incubated with 1.5 μM [^{3}H]LTB_4 and 100 μM NADPH for 30 min at 37°C. The retention times of the 20-OH-LTB_4 and LTB_4 standards are indicated.

HPLC column in a solvent of acetonitrile/water/acetic acid, 23:77:0.05 (vol/vol), for 50 min followed by a linear gradient to 100% acetonitrile over 1 hr. One-milliliter fractions were collected and the retention times of the radioactive peaks were compared to those of known standards 20-OH-LTB_4 and 20-COOH-LTB_4. At 30 min, when the [^{3}H]LTB_4 was 19% converted, and at 90 min when the conversion was complete, the sole reaction product eluted at 53 min at the retention time of the 20-OH-LTB_4 standard (53 min) and none eluted earlier in the position of the 20-COOH-LTB_4 standard (49 min).

GC/MS analysis of the methyl ester, trimethylsilyl ether derivative of the ω-oxidation metabolite of [^{3}H]LTB_4 revealed one major GC peak with an equivalent chain length of 26.6, identical to the equivalent chain length determined for the 20-OH-LTB_4 standard. The mass spectrum of the metabolite GC peak (Fig. 2) was also identical to the published mass spectrum of the 20-OH-LTB_4 derivative (1). The mass spectrum of the hydrogenated ester, trimethylsilyl ether derivative of the metabolite (equivalent chain length, 27.1) was in agreement with the mass spectrum (1) of the hydrogenated 20-OH derivative (equivalent chain length, 27.1). Selected ion scans for ions characteristic of 20-COOH-LTB_4, m/z 448, 507, 523, and 538 (1), indicated the absence of this product.

The ω-oxidation of 1.5 μM [^{3}H]LTB_4 by incremental amounts of cytosolic protein from 48 to 670 μg was linear during incubation for 20 min at 37°C in the presence of 100 μM NADPH in 10 mM KP buffer (pH 7.5), as assessed by quantitation of radiolabeled product after resolution by RP-HPLC in solvent B. The time course of ω-oxidation of 1.5 μM [^{3}H]LTB_4 by 280 μg of cytosol was linear from 0 to 45 min, as analyzed by RP-HPLC in solvent A. The pH dependence of the ω-oxidation of [^{3}H]LTB_4 was determined in three separate buffers by incubating 120 μg of cytosol for 20 min at 37°C with 1.5 μM [^{3}H]LTB_4 and 100 μM NADPH; the reaction products were analyzed by TLC. A pH optimum of 7.5–8.5 was observed, with 80% of maximal activity occurring at pH 9.0 and 20% occurring at pH 6.5 (Fig. 3).

To establish the cofactor dependence of the ω-oxidation of [^{3}H]LTB_4, cytosol was incubated for 30 min at 37°C with 1.5 μM [^{3}H]LTB_4 in 10 mM KP buffer (pH 7.5), alone and with 100 μM NADPH and 100 μM NADH, respectively, and analyzed by either TLC or RP-HPLC, using solvent A or B. In each experiment, a single protein concentration between 93 and 500 μg was used. The cytosol alone was 19.5% ± 3.5% (mean ± SD, n = 4) as active as the reaction mixture

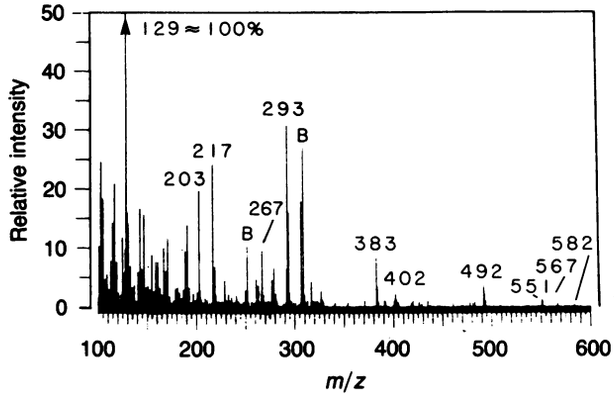

FIG. 2. Mass spectrum of the product of [^{3}H]LTB_4 and PMN cytosol. Three micrograms of the purified product was esterified with diazomethane, saturated by catalytic hydrogenation, converted to its trimethylsilyl ether derivative, and analyzed by GC/MS.

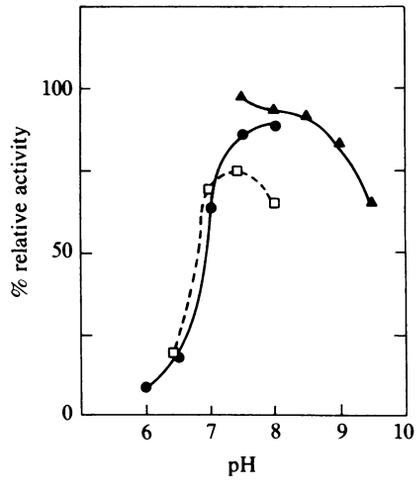

FIG. 3. pH dependence of LTB_4 20-hydroxylase. Data are expressed relative to 100 mM Tris·HCl buffer (pH 7.5), which is taken as 100%. □, 100 mM KP buffer; ●, 100 mM Hepes buffer; and ▲, 100 mM Tris·HCl buffer.

containing NADPH, and NADH showed 48% (mean, n = 2) of the cofactor activity of NADPH.

The kinetics of the conversion of incremental amounts of [^{3}H]LTB_4, 0.05–5 μM, to 20-OH-LTB_4 by 280 μg of cytosol in the presence of 100 μM NADPH is shown in Fig. 4. The reaction was carried out for 20 min at 37°C and was analyzed by TLC. In this experiment the K_m for [^{3}H]LTB_4 was found to be 0.17 μM and the V_{max} was 54.7 pmol/min·mg of protein. For four experiments in which the analyses were by TLC or RP-HPLC in buffer B, the K_m was 0.22 ± 0.08 μM (mean ± SD) and the V_{max} was 48 ± 11 pmol/min·mg of protein (mean ± SD). To determine the K_m for NADPH, the conversion of 1.5 μM [^{3}H]LTB_4 to 20-OH-LTB_4 in the presence of incremental amounts of NADPH from 0.05 to 5 μM was assayed for 10 min at 37°C in 10 mM KP buffer (pH 7.5) and was analyzed by TLC (Fig. 5). In this experiment with 368 μg of cytosol, the K_m for NADPH was 1.12 μM and for three experiments it was 1.01 ± 0.59 μM (mean ± SD).

DISCUSSION

The cytosol (Fig. 1*B*) of sonicated human PMN in the presence of NADPH catalyzed the ω-oxidation of [^{3}H]LTB_4 to 20-OH-LTB_4 in a time- and dose-dependent fashion and at a pH optimum of 7.5–8.5 (Fig. 3). The structure of the single reaction product was presumptively identified by its identical retention time on two RP-HPLC solvent systems and identical mobility on TLC to 20-OH-LTB_4. The structure was proven by GC/MS analysis (Fig. 2) and the lack of a

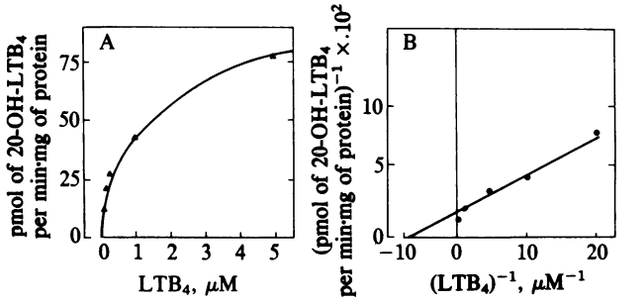

FIG. 4. Kinetics of LTB_4 20-hydroxylase for [^{3}H]LTB_4. Concentrations of [^{3}H]LTB_4 at 0.05–5 μM were assayed with 100 μM NADPH and 280 μg of PMN cytosol. (*A*) LTB_4 concentration versus velocity. (*B*) Lineweaver–Burk plot.

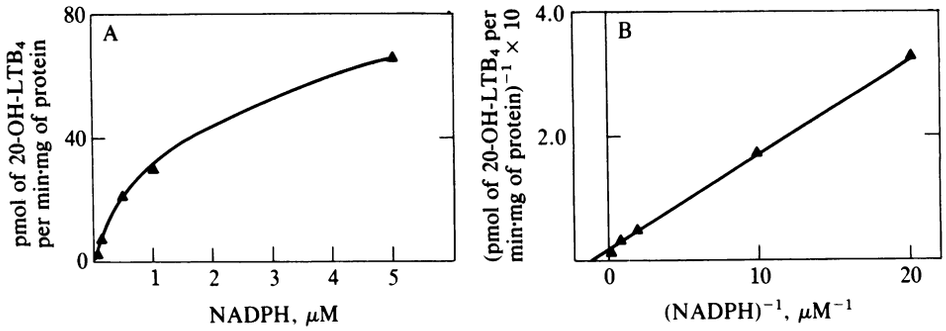

FIG. 5. Kinetics of LTB_4 20-hydroxylase for NADPH. Concentrations of NADPH at 0.1–5 μM were assayed with 1.5 μM [^{3}H]LTB_4 and 368 μg of cytosol. (*A*) NADPH concentration versus velocity. (*B*) Lineweaver–Burk plot.

20-COOH-LTB_4 contaminant was confirmed by GC/MS analysis of the derivatized, hydrogenated methyl ester with selected ion scans.

The subcellular distribution of the LTB_4 20-hydroxylase to the cytosol of the PMN sonicate is different from the microsomal P-450 systems known to catalyze ω-oxidations of fatty acids and prostaglandins in various other cell types (5–9) and from the cytochrome b_{-245} of human PMN, both of which require detergent for solubilization (5, 6, 12, 13). The inhibition of ω-oxidation of LTB_4 in intact human PMN by CO has led Shak and Goldstein (19) to suggest the possible involvement of a cytochrome P-450 in the ω-oxidation of LTB_4. The results presented here are consistent with the 20-hydroxylase being a unique form of a cytochrome P-450 system or a novel monooxygenase. The absence of a requirement for prior activation of PMN with PMA further distinguishes this enzyme from the NADPH oxidase–cytochrome b_{-245} system of human PMN (14, 15).

The identification of the LTB_4 20-hydroxylase in the 150,000 × *g* supernatant indicates that the subsequent oxidation of 20-OH-LTB_4 to 20-COOH-LTB_4 observed with intact cells (1–4) is catalyzed by an enzyme in the particulate fraction or requires the presence of an additional cofactor. The K_m of the 20-hydroxylase for [^{3}H]LTB_4 (0.22 μM) (Fig. 4) is approximately one order of magnitude below that reported for the ω-oxidation of LTB_4 by intact PMN (2). The V_{max} for [^{3}H]LTB_4, 48 pmol/min·mg of protein, represents a value of 16.3 nmol/min × 10^7 cells; this is 51.8% of the V_{max} reported by Powell (2) for the metabolism of exogenous LTB_4 by intact PMN. The concentration of NADPH in intact PMN is reported to be 100 μM (20), and this concentration of NADPH probably accounts for the conversion of some [^{3}H]LTB_4 to 20-OH-LTB_4 by some cytosol preparations in the absence of added NADPH. The K_m reported here for NADPH of the LTB_4 20-hydroxylase (1.01 μM) (Fig. 5) indicates that the availability of this cofactor may not be rate limiting for this reaction and that the generation of cytosolic LTB_4 in PMN may be the predominant rate-limiting step in the 20-hydroxylation of LTB_4.

Note Added in Proof. When human PMN are disrupted by homogenization in 10 mM Tris·HCl, pH 7.0/0.25 M sucrose/1 mM EDTA, or by nitrogen cavitation in an isotonic phosphate buffer, and are then subjected to sequential centrifugation at 10,000 × *g* for 10 min and at 105,000 × *g* for 1 hr, LTB_4 20-hydroxylase is localized to the 105,000 × *g* pellet.

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