Comparison of recombinant cyclooxygenase-2 to native isoforms: aspirin labeling of the active site

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Abstract The search for isoform-specific enzyme inhibitors has been the focus of much recent research effort. Towards this goal, human recombinant cyclooxygenase-2 (EC 1.14.99.1, prostaglandin H synthase) was expressed in insect cells and purified to >98% purity. Recombinant enzyme was characterized both by physical methods and activity measurements and shown to be fully active with kinetic properties similar to native COX-2 and COX-1. After detergent extraction, the enzyme had hydrodynamic properties indistinguishable from native bovine COX-1 and corresponded to the enzyme dimer as measured with size-exclusion chromatography. Peptide mapping via Lys-C protease identified a site of N-linked glycosylation and the aspirin covalent modification site. In the presence of heme, aspirin-specifically acetylated Ser-516. The enzyme will be suitable for biophysical studies and may lead to isoform-specific enzyme inhibitors.

Key words. Aspirin; Cyclooxygenase; Labeling; Expression; Purification

1. Introduction

The recent discovery [1,2] and cloning [3–8] of an inducible isoform of cyclooxygenase (prostaglandin G/H synthase) has initiated a reinvestigation of inflammatory prostanoid biosynthesis. We and several others [8–10] have put forward a model in which the constitutive form of cyclooxygenase (COX-1) plays a key role in cellular housekeeping functions while the inducible form (COX-2), when upregulated by mediators, such as IL-1β or TNF-α, produces pro-inflammatory amounts of cyclooxygenase products during an inflammatory response.

Most non-steroidal anti-inflammatory drugs (NSAIDs) are known to inhibit both isoforms of cyclooxygenase with only modest selectivity for one isozyme. The model presented previously, however, posits that drugs which are selective for COX-2 might give the anti-inflammatory benefits of NSAIDs while avoiding their deleterious side-effects. These side-effects, which include gastric ulceration and renal insufficiency, can reasonably be ascribed to inappropriate inhibition of COX-1. Therefore, the design of COX-2-specific inhibitors has attracted considerable interest among enzymologists and molecular pharmacologists.

Exploitation of the enzymatic and structural differences between the COX-1 and COX-2 isoenzymes constitutes one strategy to develop COX-2-specific inhibitors [11–13]. Structural information about COX-1 [14] indicates that the amino acid residues which are involved in binding heme and fatty acid substrates are highly conserved in the COX-2 sequence. The enzymologic properties of COX-1 are well-studied, as are the properties of certain COX-1 inhibitors. For COX-2, no structural information has been revealed and the enzymologic properties have been investigated only minimally.

Whereas COX-1 is known to be covalently modified by aspirin at Ser-530 [14–17], the site of covalent modification of COX-2 has not yet been definitively demonstrated (see also Lecomte et al. [18]). Aspirin inhibited prostaglandin synthesis in whole cells or in microsomal assays using native or recombinant enzymes [10,14,18–20]. However, COX-2 was shown to be different than COX-1 in these systems as it caused an increased production of an alternate product 15-HETE [18,20–23]. In this study, we employed recombinant human COX-2 produced in the baculovirus/insect cell expression system and purified by a two-step process. The resulting enzyme has been characterized for substrate preferences, heme stoichiometry and kinetic properties. Using this well-characterized enzyme and peptide mapping strategies, aspirin was demonstrated to label Ser-516 specifically and efficiently. The site is analogous to residue Ser-530 of COX-1.

2. Materials and methods

2.1. Chemicals

Chemicals obtained were ram seminal vesicle COX-1 and sheep placental COX-2 from Cayman (Ann Arbor, MI), Lys-C, endoglycosidase F and endoglycosidase H from Boehringer (Indianapolis, IN), material for SDS-PAGE and Western analysis from BioRad (Richmond, CA), chemiluminescent detection reagents from Amersham (Little Chalfont, UK). Normal-phase chromatography media was from Pharmacia (Uppsala, Sweden), reverse-phase columns from Alltech (Deerfield, IL) and HPLC equipment from Waters (Medford, MA) All other chemicals were the highest quality available and generally obtained from Sigma (St. Louis, MO).

2.2. COX-2 purification

All procedures were performed at 4°C unless otherwise indicated. The baculovirus expression system was a modification of the system described by Miller et al. [24]. HighFive cells (Invitrogen) were cultured in 1-l spinner flasks (Techne) in Grace's complete insect media containing 5% fetal bovine serum (Gibco) until the cell density was 1.8–2.2 $\times 10^6$ cells/ml. Cells, whose viability was at least 98% at this stage, were infected with baculovirus [24] at a multiplicity of infection (MOI) of 5 and harvested 72 h later. To obtain microsomal membranes, the pellets from 2.5-l cultures were thawed and resuspended in a total volume of 100 ml buffer A (100 mM $KHPO_4$, pH 7.2, 0.3 mM diethyldithiocarbamate, 1 mM EDTA). The suspension was sonicated at the maximum setting of a microtip probe (Branson Sonifier 250) 6 × for 5 s with 30-s intervals for cooling. Sonicates were spun at 1000 × g for 10 min and the pellets collected and the sonication procedure repeated. The combined supernatants were spun at 150,000 × g for 45 min and the waxy pellets resuspended with 30 strokes of a Dounce homog-

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enizer with tight-fitting pestle. Microsomal suspensions were aliquoted and frozen pending purification.

A microsome pellet corresponding to 2.5 l of cell culture and weighing ~10 g wet weight was used for a typical purification. Microsomes were centrifuged at 200,000 × *g* for 20 min and resuspended into 200 ml of TECP buffer (50 mM Tris, pH 8.0, 1 mM EDTA, 0.1 mM DDC, 0.1 M sodium perchlorate). The microsomes were again centrifuged at 200,000 × *g* for 20 min and the pellet resuspended into 100 ml of 2% *β*-octyl glucoside (BOG) in TE (20 mM Tris, pH 7.4, 0.1 mM EDTA) containing anti-proteases (pepstatin, aprotinin, leupeptin, chymostatin; all present at 1 μg/ml). The mixture was incubated with gentle shaking for 1 h then centrifuged at 200,000 × *g* for 1 h. The supernatant was diluted with TE to a final BOG concentration of 0.9% and then was loaded onto a DEAE-Sepharose (Fast Flow) column (1 × 20 cm) equilibrated with TEG (TE with 0.9% BOG) + 0.1 M NaCl The column was run at a flow rate of 1 ml/min with a linear gradient of NaCl from 0.1 to 0.35 M over 60 min and COX-2 eluted at ~0.20 M. The active fractions were pooled, adjusted to 2 mM calcium chloride and mixed in batch for 1.5 h with 20 ml of Lentil-Lectin-Sepharose (Pharmacia) equilibrated with a buffer of TEG supplemented with 2 mM calcium chloride and 150 mM NaCl. The gel was washed with 3 washes of 10 vols. of buffer, packed into a small disposable column and COX-2 eluted at room temperature with 0 5 M α-methyl pyranoside in the same buffer. The active pool was concentrated with an Amicon Centricon-30 membrane, separated into aliquots and frozen at −80°C.

For size-exclusion chromatography, a 90-ml Sephacryl S300 column (Pharmacia) was used in a buffer of TEG containing 0.15 M NaCl SDS-PAGE analysis was performed using standard procedures with BioRad 7.5, 10 or 12% acrylamide gels Gels were either stained with Coomassie brilliant blue or analysed by Western analysis using rabbit polyclonal antibodies either anti-chicken PGHS-2 (Oxford Biomedical Research, No. PG25) or anti-COX-2 peptide (Oxford Biomedical Research, No. PG26). Purity of COX-2 samples were judged by reverse-phase HPLC using a C4 Alltech column and a 30–70% acetonitrile gradient over 30 min and monitoring OD at 210 nm.

2 3 Peptide mapping

COX-2 was treated with endoglycosidase F (a mixture of 6 U endoglycosidase F and 150 U glycopeptidase F per vial; Boehringer Mannheim product 878740) overnight at 37°C in a total volume of 0.1 ml containing 0.3 U of the glycosidase and 100 μg COX-2. For digestion of native (non-denatured) COX-2, the buffer was 0.25 M sodium acetate, pH 6.5, 20 mM EDTA and 10 mM 2-mercaptoethanol. For digestion of denatured COX-2, the same buffer was supplemented with 10 mg/ml SDS and then diluted with 60 mg/ml NP40 as per the manufacturer's instructions. Endoglycosidase H was used according to the manufacturer's recommendations. COX-2 was treated with endoproteinase Lys-C (BM No. 1047 825) after reduction and carboxyamidation. COX-2 was diluted with 2 vols. of UBET buffer (0.1 M NH_4CO_3, 1 0 mM EDTA, 0.02% Tween 20, 25 mM methylamine, 9.0 M Urea), DTT was added to a final concentration of 10 mM and the mixture was incubated in the dark at 37°C for 30 min. Iodoacetamide was added to a final concentration of 20 mM and the mixture kept at 37°C for an additional 30 min. Lys-C was added to this reduced and alkylated COX-2 to a ratio of 1:100 and incubated at 37°C for 4 h. To stop the Lys-C digestion, 1/10 vol. of 10% TFA was added and the mixture analysed by HPLC using an Alltech macrosphere 300 C4 column with a 45 min linear gradient from 15% B (90% acetonitrile, 0.09% TFA)/85% A (5% acetonitrile, 0.9% TFA) to 45% B/55% A. Detection was performed by monitoring the eluent for absorption at 210 nm with a Waters 481 UV detector and monitoring fluorescence emission at 350 nm with a Waters 470 fluorescent detector using an excitation wavelength of 280. Samples were dried in a Savant rotary evaporator and analysed by either Harvard Microsequence Facility in Boston (MA) (mass spectral analysis) or at Commonwealth Biotechnology in Richmond (VA) (sequencing, amino acid analysis).

2 4. Miscellaneous methods

Cyclooxygenase activity was determined by an oxygen uptake assay using a Yellow Springs Instruments polarographic electrode (Yellow Springs, MD) with modifications of a reported method [25] Assays were done at 37°C in 0.1 M Tris, 4 mM EDTA, 0.05% Tween and 2 mM phenol, pH 8.0. Enzyme samples were preincubated on ice with 50 μM hematin and activation was initiated by injection of the enzyme into 3 ml of buffer containing arachidonic acid. Oxygen consumption was evaluated using a program (Labview, National Instruments) to measure maximal velocity, V_{opt}, as nmol oxygen/min/ml. Peroxidase was measured as described previously [24]. Heme titration was performed as described by Kulmacz and Lands [26].

Radiolabeled aspirin was made from [^{3}H]acetic anhydride (370 mCi/mmol; Amersham) based on the procedure of Ali [27] and purified twice by reverse-phase HPLC using a Metachem Intersil ODS-2 column and a mobile phase of 74:25 1 (v/v/v) methanol:water:acetic acid. Aspirin was stored in ethanol after HPLC purification. Aspirin labeling was performed using a modification of the method used by Chen et al. [19]. To a tube containing 16 μM COX-2 (1.2 mg in 500 μl buffer), 20 μM hematin and either 0 or 1 mM flurbiprofen, [^{3}H]aspirin was added to give a final concentration of 3 mM. After incubation at room temperature for 30 min, unreacted aspirin was removed by a desalting NAP-5 column (Pharmacia) equilibrated with 20 mM Tris, pH 8.0, 1 mM EDTA, 0.9% BOG buffer. Aliquots of enzyme samples were checked in oxygen uptake assays. Labeled enzyme was further evaluated by peptide mapping. Protein was determined by Lowry analysis using BSA as a standard and confirmed by amino acid analysis (Commonwealth Biotechnologies, Richmond, VA).

3. Results

The baculovirus expression system previously described was further refined by the use of HighFive insect cells which resulted in a 2-fold higher level of expression of COX-2 then previously reported [24]. Purification of the enzyme (Table 1) was achieved following detergent extraction of the microsomes. Inspection of SDS-PAGE gels of the purification steps (Fig. 1) revealed that the enzyme was visible in the crude microsomal fraction as a broad band of 73 kDa which represented ~4% of the total protein. BOG detergent was effective in extracting the enzyme from microsomes with good recovery of activity and with a noticeable enrichment of the 73-kDa band as judged by SDS-PAGE. A 68-kDa band was apparent in the non-extracted microsomal fraction (lane 2, Fig. 1a) which was recognized by anti-COX-2 antibodies using Western analysis (Fig. 1b). Since

Table 1
Purification of recombinant COX-2

Fraction	Total activity (μmol/min)	Total protein (mg)	Specific activity	Recovery (%)	Purification enrichment (fold)
Microsomes	494	511	1	100	1
Detergent extract	444	111	4	90	4
Insoluble microsomes	6	275	0	1	0
DEAE pool	300	52	6	61	6
Purified	188	8	25	38	26
Lentil-Lectin unbound	71	28	2	14	3

Purification COX-2 from insect cells is described in Section 2. Samples from each step were collected and analysed for protein by Lowry assay and for COX-2 activity by oxygen uptake (Section 2). Specific activity is from the oxygen consumption assay. The results are an average of four preparations with generally good reproducibility between runs. Peroxidase assay (not shown) gave results similar to oxygen consumption results.

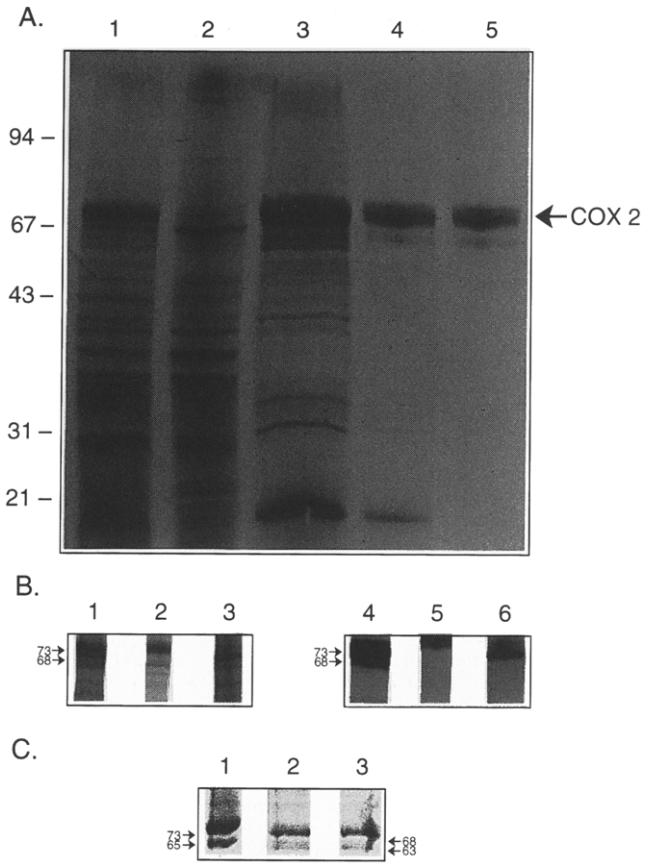

Fig 1 SDS-PAGE and Western analysis of COX-2 purified from baculovirus-infected HighFive cells. Recombinant COX-2 was purified from insect cells (Section 2) as outlined in Table 1. (A) Purification fractions were run on a 12% SDS-PAGE gel then stained by Coomassie brilliant blue. Lane 1, crude microsomes; lane 2, pellet after detergent extraction; lane 3, soluble phase of detergent extracted microsomes; lane 4, pool from DEAE-Sepharose column; lane 5, Lentil-Lectin-Agarose-purified enzyme. The purified enzyme in this preparation appears as a major band with a molecular weight of 73 kDa and a minor COX-2 band at 65 kDa (see text). (B) Identification of two COX-2 species in microsomes: three samples were run on 12% SDS-PAGE gels and tested: left, crude microsomes; center, detergent insoluble microsome pellets; and right, detergent extracts. The analysis was done either by staining gels with Coomassie brilliant blue (left) or by Western analysis with a COX-specific antibody (right). The two major bands of COX-2 are indicated as a 73-kDa band extracted by detergent and a 68-kDa insoluble band. (C) Endoglycosidase treatment of COX-2: purified enzyme was treated with endoglycosidase F and run on a SDS-PAGE gel and stained with Coomassie brilliant blue. Lane 1 was purified COX-2 control, lane 2 was COX-2 treated with endoglycosidase F in non-denaturing buffer and lane 3 was COX-2 treated with endoglycosidase F in a denaturing buffer containing SDS (Section 2). In this preparation, both bands of COX-2 shift to lower molecular weight; the 73 kDa shifting to 68 kDa and the minor 65 kDa shifting to 63 kDa. See the test for details The relative position for molecular weight standards is indicated in the figure as molecular weight $\times 10^{-3}$.

the band is not extracted from the microsomes by BOG and since no activity remains in the extracted membranes, the protein presumably represented an improperly folded or denatured COX-2 fraction. The non-extracted band has the same migration as COX-2 which had been treated with endoglycosidase to remove N-linked carbohydrate (see below). The overall yield from microsomes to pure enzyme was ~40%. It was essential to maintain the detergent concentration at 0.9% in order to prevent contaminating proteins from adhering to COX-2 and to prevent the enzyme from aggregating. The enzyme was stored at −80°C and little loss in activity was noticed over 6 months.

COX-2 activity migrated on a S300 Sephacryl size-exclusion column (Fig. 2, tubes 27–32) in a manner indistinguishable from native COX-1 (not shown) and similar to an aldolase standard (molecular weight 154 kDa). As discussed earlier [19,28], this migration corresponds to an enzyme dimer. Some higher molecular weight material eluted at the void volume (Tube 20–22). Rechromatography of the dimer pool (not shown) gave a profile similar to the original column run, containing both dimer and higher aggregate. The result underscores the tendency of COX-2 to aggregate even in detergent. Upon storage of enzyme samples at 4°C for up to 1 week, higher molecular weight bands became evident by SDS-PAGE. The slight tailing shoulder of the dimer peak in Fig. 2 may represent a small fraction of monomeric COX-2 and this tail was similarly evident upon rechromatography, indicating it was created from the dimer dissociating into monomers during or before the column run. Enzyme activity co-migrated principally with the dimer peak and a slight activity was seen in the higher molecular weight peak.

Sequence analysis of the purified COX-2 gave the predicted N-terminal of Ala-Asn-Pro-Cys-, indicating that the 17 amino-acid signal sequence had been efficiently removed. From the SDS-PAGE analysis we estimate the enzyme to be ~98% pure. The chief visible contaminant (typically <5% of the major band) was a band which ran just below the main COX-2 band, has an approximate molecular weight 65 kDa and is likely to be a proteolytic degradation product produced during purification. Endoglycosidase treatment of purified COX-2 with either endoglycosidase F or H resulted in a protein which migrated considerably faster as judged by mobility on SDS-PAGE (Fig. 1c) and having an approximate molecular weight of 68 kDa. (It is worth noting that the resulting band co-migrated with the COX-2 material not extracted by detergent from microsomes as seen in Fig. 1a, lane 3.) When COX-2 was treated with endoglycosidase F either in buffer or buffer supplemented with

Fig 2 Size Exclusion Chromatography

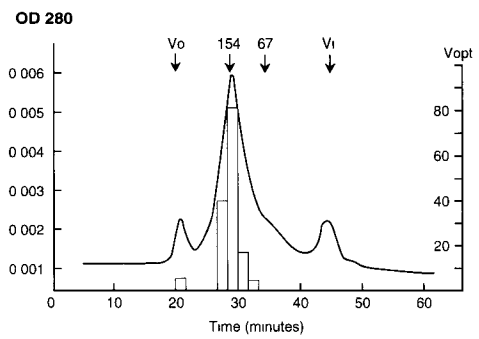

Fig. 2. S300 Sephacryl size-exclusion chromatography of COX-2. After purification of COX-2 by Lentil-Lectin-Agarose chromatography, the sample was concentrated with an Amicon filtration device then the enzyme was passed through a S300 Sephacryl gel filtration column. Fractions were analysed for activity as described in section 2. Activity is expressed in the bargraph as $V_{opt}/10$ μl of sample (nmol/min) Activity corresponded to the main peak with slight activity associated with the high molecular weight peak. The late-running peak is probably non-protein as no activity migrated with this peak.

Table 2
Peptide mapping of COX-2

Predicted fragment	Sequence		Length	Mass
F1	N–41		24@	2645@
F2	42–64	GP1	23	2642
F3	65–68		4	520
F4	69–82		14	1668
F5	83–123	w	41	4735
F6	124–152	GP2.w	29	3225
F7	153–154		2	203
F8	156–166		11	1199
F9	167–172		6	798
F10	173–197		25	3021
F11	198–201		4	500
F12	202–229		28	3078
F13	230–234		5	676
F14	235–237		3	318
F15	238–239		2	277
F16	240–253		14	1654
F17	254–303	w	50	5800
F18	304–328	w	25	2968
F19	329–344		16	1948
F20	345–346		2	259
F21	347–355		9	1122
F22	356–387	w	32	4000
F23	388–432	GP3	45	5126
F24	433–445		13	1478
F25	446–454		9	1234
F26	455–459		5	694
F27	460–471		12	1429
F28	472–497		26	2882
F29	498–518		21	2262
F30	519–532	w	14	1539
F31	533–559		27	2838
F32	560–575		16	1737
F33	576–598	GP4	23	2402
F34	599–604		6	734

Actual fragment	Fluorescence	Sequence	Mass	Assignment
A			1793*	F32
B				
C			4473*	F30/31
D				
E				
F	w			(F18)
G	w			F6 = GP2
H	w			
I	w		3999	F22
J	w		3077	F12
K	w			(F5 or F18/19)
L	w			
M		PIDP + EMSAE		F10 + F28/29
N	w	GLMGN		F30/(7)
O		EMSAE		F28/29
P	w		6249*	F30/31/32
Q				
R		EMSAE		F28/29

*Carboxyamidated
@After processing

Left: theoretical characterization of a Lys-C peptide map of COX-2. Peptides containing tryptophan residues are indicated with a 'W'. Right: peptides identified in Fig. 3 were collected and analysed by various methods including amino acid analysis, peptide sequencing and mass spectral analysis (MS) using electrospray or laser desorption. Peaks which could be positively identified (sequencing and MS) are noted in the last column. Certain peaks were tentatively identified either using MS alone or because parent ion mass was >2% from the predicted values and these peaks are listed in parentheses. Fragments of incomplete digestion are indicated with a '/' between peptides. Fragment F28/29 is noted in three peaks, none of which were fluorescent and, therefore, do not contain fragment F30. Variable posttranslational modification (myristoylation and phosphorylation) could account for the multiple bands.

SDS (see Section 2), the same 68-kDa band was produced, indicating that the sites of glycosylation were accessible in the native conformation. When COX-2 was treated with endoglycosidase in non-denaturing buffers, the enzyme did not loose activity (results not shown).

We developed a peptide map of purified COX-2 in order to probe for structural details. Lys-C protease was theoretically capable of hydrolyzing COX-2 into 35 fragments (Table 2, right side). Of these, 22 would be >10 amino acids in length and 6 would contain tryptophan residues. When analysed by C4-reverse-phase HPLC using various gradients, up to 20 fragments were resolved and ~8 were fluorescent indicative of the presence of tryptophan (Fig. 3). Through a combination of UV and fluorescent absorption, mass spectral analysis, amino acid analysis and peptide sequencing, 15 of these fragments were identified. A summary of the identification of these fragments is indicated in Table 2 (left side). As can be seen, several of the fragments were the result of incomplete digestion yielding non-terminal fragments.

The sequence of COX-2 predicts the existence of four potential glycosylation sites (Table 2) referred to as GP 1–4 corresponding to glycosylation at Asn-53, Asn-130, Asn-396 and Asn-580. In order to detect glycopeptides, a comparison was made of COX-2 either treated or untreated with endoglycosidase F to remove N-linked carbohydrate structures. When Lys-C peptide maps from native enzyme were compared to enzyme treated with endoglycosidase F (not shown) one broad peak (band G of Fig. 3) was found to shift to a faster running doublet. These peaks were confirmed both by mass spectral analysis and peptide sequencing as the F6 (sequence 107–135) and F6/7 (sequence 107–137) fragments, corresponding to deglycosylated glycopeptides from GP2. At present, we do not have conclusive evidence for or against glycosylation of GP1, 3 or 4 (see Section 4). Because glycopeptide bands tend to be heterogeneous, they may be too diffuse to resolve as clear peaks.

Similar peptide mapping strategies allowed the identification of the site of modification by [^{3}H]aspirin as Ser-516. COX-2 was treated either with aspirin alone or with aspirin + flurbiprofen. When enzyme samples were desalted and then passed through a C4 reverse-phase column to purify COX-2 (not shown), 30-fold more radioactivity was found in the aspirin-treated sample

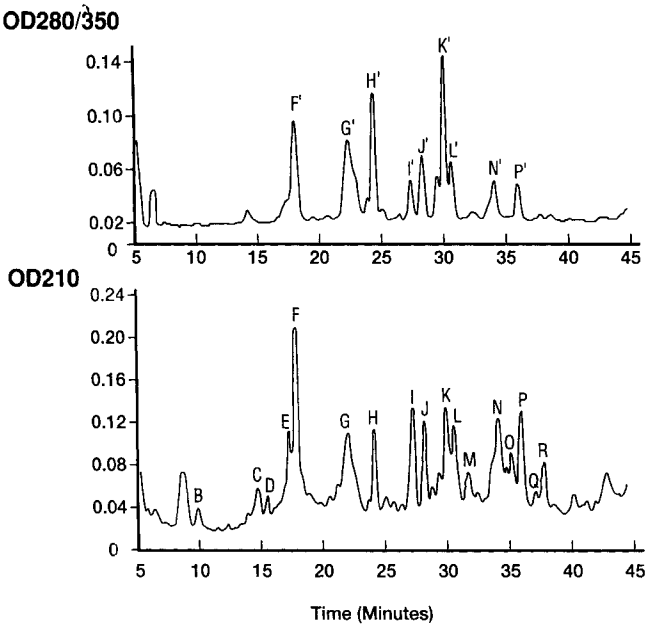

Fig. 3. Lys-C protease peptide map of COX-2. A peptide map of purified COX-2 was performed with Lys-C as described in Section 2. The peptides were separated by reverse-phase HPLC on an Altech C4 column. Absorption of the peptide bond was followed (bottom) by absorbence at 210 nm and by fluorescent (transfer from excited tryptophan and tyrosine) by monitoring at 350 nm (top). Peaks were collected, analysed and identified as described in Table 2.

compared to the aspirin + flubiprofen sample, indicating a high degree of specific labeling. Incorporation of specific label was estimated to occur at 70% of the enzyme monomers (average of three experiments 70% ± 4% SEM). The protein was further analysed by peptide mapping (Fig. 3) and individual peaks counted for radioactivity. The results are presented in Table 3. The Lys-Pro bond between F28 and F29 apparently resists protease cleavage because free F29 was never detected. Instead the longer F28/F29 peptide was found and this peptide, present as a series of related peaks, were found to be highly enriched in radioactivity. Since each of these peaks contain Ser-516, the amino acid analogous to the aspirin site of COX-1 and since the only other serine (F28/29, Ser-474) was not radioactive when sequenced (not shown), it is concluded that the analogous serine is acetylated in COX-2 as in COX-1. Comparing the labeling of F32 to F28/29, both of which contain two serine residues, the specificity of labeling into Ser-516 can be estimated to be >400 to 1. The molecular difference between the various Ser-516-containing peptides in Table 3 (peptides M, O and R) is not clear but may be due to variable posttranslational modification, such as phosphorylation or myristoylation.

Kinetic analysis of recombinant COX-2 was performed using both oxygen consumption and peroxidase assays with generally good agreement. As indicated in Table 4, recombinant COX-2 has kinetic properties similar to native COX-1 and COX-2. Extent assays using limiting substrate demonstrated the incorporation of two molecules of oxygen/molecule of arachidonic acid substrate (not shown). Various fatty acid substrates were utilized by COX-2 with different efficiencies and the order of substrate preference was close to that expected for COX-1 (manuscript, in prep.). The addition of heme is essential to attain COX-2 enzyme activity as measured either with cyclooxygenase or peroxidase assays.

4. Discussion

The development of isoform-specific inhibitors is theoretically possible and yet very few isoenzyme-specific inhibitors have been described. In contrast, numerous examples of specific inhibitors of receptor isotypes exist, such as agents which selectively inhibit the various adrenergic receptor subtypes. One reason for this contrast is that tools have not previously been available to pursue this goal. As one of the first cases where isoform-specific inhibitors are claimed, several recent reports [12,13,29–33] have documented selective COX-2 inhibitors. Presumably, other examples will follow as various targets are defined and evaluated at the molecular level.

The expression and purification of human COX-2 has allowed us to attempt scale-up purification and analysis of the recombinant COX-2 enzyme (see also [22,28,34]). COX-2 tends to aggregate; size-exclusion chromatography indicated the majority of the enzyme migrated as a protein dimer, however, even with BOG detergent in excess of its critical micellular concentration, some tendency for the enzyme to form larger aggregates was seen.

When dealing with novel expression systems, the potential for heterogeneity of expressed protein is enormous [35]. Prosite analysis of the primary sequence of human COX-2 predicts 4 potential N-linked glycosylation sites, 13 potential phosphorylation and 9 potential myristoylation sites. COX-1 has three N-linked glycosylation sites all of which are glycosylated [14,19,36] and each of these sites are conserved in COX-2. The fourth site of COX-2 Asn-580 was shown by mutation analysis [36] to be incompletely glycosylated in a murine recombinant COX-2 expressed in COS-1 cells. In the present study, Asn-130 was shown by HPLC analysis and peptide mapping to contain N-linked carbohydrate. Although it is uncertain from the present work whether Asn-53, Asn-396 and Asn-580 were glycosylated, from the changes in molecular weight after endoglycosidase treatment, we speculate that at least two sites are glycosyl-

Table 3
Selective labeling of Ser-516 by aspirin

Peak number	cpm/μg	Sequence	Assignment
A	546		F32
B	1088		
C	63		
D	2700		
E	0		
F	21		(F18)
G	0		F6 ~ GP2
H	0		
I	299		F22
J	366		F12
K	1260		F5 or F(18/19)
L	1831		
M	30440	PIDP + EMSAE	F10, F28/29
N	6979	GLMGN	F30/F(?)
O	103860	EMSAE	F28/29
P	7113		
Q			
R	35750	EMSAE	F28/29

The peaks shown in Fig. 3 were counted for radioactivity. Essentially, all the radioactivity was found in peaks which included the F29 peptide and, therefore, exclusively located in Ser-516 (see text).

Table 4
Comparison of kinetic data for COX isoforms

Isoform	Species	K_m (μM)	V_{max} (nmol/min)	Spec. activity (nmol/min/mg)
COX-1	ovine	6	100	30
COX-2	ovine	5	90	6
COX-2	human	3	80	25

The experiments were conducted at 37°C in 0.1 M Tris-HCl, 2 mM phenol, pH 7.7. The enzyme was added to start the reaction. The optimal velocity of oxygen was measured and used to determine the kinetic constants by Hanes's analysis.

ated in the major band (>90% of detergent extracted material). The inactive COX-2 68K band which is not extracted by BOG detergent may represent enzyme which was not properly folded because it did not enter the posttranslational modification pathway upon protein synthesis and, therefore, was not glycosylated and further modified.

Aspirin modified Ser-516 of COX-2, a residue which corresponds to Ser-530 of COX-1 [14,19]. The results support the concept that the two isoforms are structurally similar. A similar result was found when heme binding was investigated. Careful titration of the enzyme resulted in a one-to-one titration of enzyme monomers with added heme (ses also [26,37]). Cyclooxygenase and peroxidase activities of the recombinant enzyme were carefully evaluated after heme reconstitution. The enzyme showed similar kinetic properties to ovine COX-1 and the same substrate preference as COX-1. COX-2 oxygen consumption activity is inhibited by aspirin with a change in formation of 15-HETE as recently demonstrated [20,22].

With this body of evidence for similarity of structural and kinetic data between COX-1 and COX-2, coupled with the near identity of amino acids lining the substrate binding and catalytic site, it would appear to be a difficult task to exploit molecular differences between the two enzymes. Interestingly, proposed isoform-specific agents have revealed different kinetics of inhibition. This information may indicate that subtle amino acid differences of a more peripheral nature along the enzyme binding/reaction pathway may be exploited for specific inhibitor development. It is hoped that by developing a detailed understanding of this enzyme isoform pair, an era of isoenzyme-specific inhibitors may be forthcoming.

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