Acta Cryst. (1997). D**53**, 224–226

Characterization, crystallization and preliminary crystallographic analysis of human recombinant cyclooxygenase-2

STEFANIA DI MARCO,[a]* JOHN P. PRIESTLE,[a] MARKUS G. GRÜTTER,[a] LAWRENCE P. WENNOGLE[b] AND WILLIAM BOYAR[b] *at* [a]*Core Drug Discovery Technologies, Ciba-Geigy AG, CH-4002 Basel, Switzerland, and* [b]*Research Department, Ciba-Geigy AG, Pharmaceuticals Division, 556 Morris Avenue, Summit, NJ 07901, USA. E-mail: dimarco@fmi.ch*

(*Received 28 August 1996; accepted 13 December 1996*)

Abstract

Purified recombinant membrane apoprotein cyclooxygenase-2 (COX-2) has been reconstituted with heme and characterized. The holoprotein has been crystallized in complex with the selective inhibitor CGP 28238 [6-(2,4-difluorophenoxy)-5-methylsulfonylamino-1-indanone] by the sitting-drop method of vapor diffusion using polyethylene glycol 2000 monomethyl ether as precipitant, in the presence of the nonionic detergent β-octylglucoside. The crystals are orthorhombic, belonging to the space group $P2_12_12$ with cell dimensions $a = 209.56$, $b = 71.28$ and $c = 93.82$ Å, and diffract to 2.5 Å resolution. The asymmetric unit contains two COX-2 monomers, as confirmed by the molecular replacement solution and in agreement with the dimeric structure of the detergent-solubilized protein found with dynamic light scattering and size-exclusion chromatography. Structural work is in progress.

1. Introduction

Cyclooxygenase (COX), also called prostaglandin H_2 synthase, is a monotopic integral membrane glycoprotein (Picot, Loll & Garavito, 1994). This protein catalyzes the first step in the biosynthesis of prostaglandins, converting arachidonic acid to prostaglandin H_2, which is then rapidly converted into one of several prostanoids. Prostaglandin biosynthesis has been implicated in the pathophysiology of cardiovascular disease, cancer and inflammatory diseases (Marnett, 1990; Makheja, 1992; Abramson, 1991).

Cyclooxygenase exists in two isoforms, COX-1 and COX-2, which show 60% identity in their amino-acid sequences but differ in their regulation of expression and tissue distribution (DeWitt, 1991; Holtzman, Turk & Shornick, 1992; Meade, Smith & DeWitt, 1993; Jones, Carlton, McIntyre, Zimmerman & Prescott, 1993). A general model considers COX-1 to be a constitutive enzyme involved in cell–cell signalling and maintaining tissue homeostasis whereas COX-2 seems to be an inducible enzyme whose expression occurs in a limited number of cell types and is regulated by specific stimulatory events, leading to the hypothesis that this enzyme is responsible for the prostanoid biosynthesis involved in inflammation and mitogenesis (Pairet & Engelhardt, 1996). Several new drugs have reached the market based on COX-1 enzyme screens. These drugs all possess unwanted side effects resulting from inhibition of the constitutive enzyme, COX-1. Attention is now highly focused on selective inhibitors of the inducible COX-2. Here we present the characterization in solution of the reconstituted protein and crystals diffracting to 2.5 Å resolution of human recombinant COX-2 in complex with the potent and selective inhibitor CGP 28238 (Klein, Nüsing, Pfeilschifter & Ullrich, 1994). The structure is currently being solved by molecular replacement using the coordinates of the 3.5 Å X-ray structure of ovine COX-1 (Picot *et al.*, 1994). The three-dimensional structure of COX-2 will represent an indispensable contribution in the design of COX-2 specific drugs.

2. Methods

2.1. *Reconstitution and stabilization of the enzyme*

Pure recombinant human apoCOX-2 has been expressed in baculovirus and purified as described by Wennogle *et al.* (1995). The pure apoenzyme (1 mg ml^{-1}) first was dialyzed against 20 m*M* Tris–HCl, pH 7.5 in the presence of 100 m*M* NaCl and 1%(*w*/*v*) β-octylglucoside in order to remove EDTA. The enzyme then was reconstituted by adding a 1.5–2 fold molar excess of heme as a cofactor and a 20-fold molar excess of phenol as a stabilizer. The heme solution (10 m*M*) was freshly prepared from bovine hemin chloride in 100%(*v*/*v*) dimethyl sulfoxide (DMSO) and insoluble material was removed by filtration and centrifugation. The reconstituted holoenzyme was left at 277 K overnight. After reconstitution, excess heme was removed by dialysis. The protein was then concentrated using a Centricon-30 ultrafiltration device and immediately used in crystallization experiments. All steps were carried out at 277 K.

2.2. *Analytical methods*

Total iron content in the reconstituted and dialyzed protein was determined by flameless atomic absorption spectroscopy. Protein concentration was determined by the micro-biuret method (Goa, 1953) using horse IgG as a standard and confirmed with the Bio-Rad protein assay with bovine serum albumin as a standard. Automated Edman degradation for N-terminal sequence analysis was performed in a gas-phase sequencer (477A, Applied Biosystems). Phenylthiohydantoin (PTH)-amino acids were analyzed by gradient liquid chromatography on a PTH-amino acid analyzer (120A, Applied Biosystems). Matrix-assisted laser-desorption ionization/time-of-flight mass spectrometry (MALDI-TOF) was performed using sinapinic acid as matrix (Börnsen, Schär & Widmer, 1990).

2.3. *Dynamic light scattering*

Light scattering was performed with a Dynapro-801 Molecular Sizing Instrument (Protein Solutions, Inc, Charlottesville, VA, USA). Data analyses were performed on Protein Solutions' *Auto Pro Data* analysis software. The purified protein in solution was injected through a 0.02 µm syringe filter at room temperature into the Dynapro-801 detector, which uses a solid-state laser (20 mW power) to illuminate the sample with 780 nm light in a 7 µl quartz flow cell. The sample was measured at a protein concentration of

1 mg ml^{-1} in 20 m*M* Tris–HCl buffer, pH 7.5, containing 100 m*M* NaCl and 1%(*w*/*v*) *β*-octylglucoside.

2.4. *Analytical gel filtration*

High-pressure liquid chromatography (HPLC) gel filtration (Bio-Sil Sec-250, 7.8 × 300 mm) was performed at room temperature in 50 m*M* sodium phosphate buffer pH 6.6 containing 0.15 *M* NaCl and 1%(*w*/*v*) *β*-octylglucoside. A 20 µl aliquot of 10 mg ml^{-1} holoCOX-2 in 20 m*M* Tris–HCl buffer pH 7.5 containing 100 m*M* NaCl and 1%(*w*/*v*) *β*-octylglucoside was loaded onto the gel-filtration column, previously equilibrated with 50 m*M* sodium phosphate buffer pH 6.6 containing 150 m*M* NaCl and 1%(*w*/*v*) *β*-octylglucoside. The protein solution was eluted with the same buffer at a flow rate of 1 ml min^{-1}. The elution volume was compared against a standard curve of the column calibrated with molecular weight markers.

2.5. *Crystallization*

The crystallization screening employed methods such as the sparse-matrix method of trial conditions, which is biased towards known crystallization conditions for macromolecules (including those utilized for the few crystallized membrane proteins), in addition to a systematic crystallization approach (Riès-Kautt & Ducruix, 1992). Crystallization experiments of the reconstituted enzyme in complex with the inhibitor were performed at a protein concentration of 10 mg ml^{-1} incubated with fivefold molar excess of the inhibitor CGP 28238 for at least 10 min at 277 K. This solution was then mixed with an equal volume (2–5 µl) of the reservoir solution against which the drop containing the protein was equilibrated using the hanging- and sitting-drop methods of vapor diffusion at room temperature (McPherson, 1989).

2.6. *Data collection*

X-ray diffraction data were collected using a 30 cm MAR Research imaging-plate detector system. Graphite-monochromated Cu *Kα* radiation was provided by an Enraf–Nonius FR591 rotating-anode X-ray generator operated at 45 kV and 90 mA. The crystal-to-detector distance was set at 200 mm. Data were collected in steps of 0.5° frame^{-1} and an exposure time of 900 s frame^{-1}. Crystal orientation determination, data collection and on-line data evaluation were performed using the MAR Research imaging-plate software. To select strong spots from the collected images, the program *SPOTS* was used [adaptation of program *IMSTILLS* of the *CCP*4 package, Collaborative Computational Project, Number 4. (1994)]. Indexing was performed with the program *MARREFIX* (Kabsch, 1993). The measured intensity data were profile-fitted using the program *MARXDS* (Kabsch, 1988). Data scaling and merging were performed using the program *MARSCALE* (Kabsch, 1988).

2.7. *Molecular replacement*

Molecular replacement was carried out using the 3.5 Å structure of ovine COX-1 (Picot *et al.*, 1994) as the trial structure, truncated to reflect sequence differences between the two enzymes. Since the volume of the asymmetric unit indicated room for a dimer, the dimer structure of Picot *et al.* (1994) was used with the rotation-function program *ALMN* of the *CCP*4 program suite. Two clear peaks were found which were roughly 180° apart in rotation space and which were consistent over a wide range of resolution ranges and Patterson integration radii. The translation function was calculated for all possible primitive orthorhombic space groups and their various axes permutations with the program *AMoRe* (Navaza, 1994). Space group $P2_12_12$ gave the most convincing results and was verified by running the translation function in the monoclinic space group $P2_1$. Two dimers were found in the $P2_1$ asymmetric unit which indeed showed the packing to be consistent with $P2_12_12$. Partial refinement has been carried out using the program *X-PLOR* (Brünger, Kuriyan & Karplus, 1987). Although refinement is not complete, further evidence for the correctness of the structure has appeared. Strong electron density near the side-chain Asn130, one of the three putative glycosylation sites, could easily be fitted with an *N*-acetylglucosamine moiety and electron density representing the inhibitor has also been found in the active-site channel.

3. Results and discussion

Results obtained from 18 steps of N-terminal sequencing (ANPCCSHPCQNRGVCMSV), were consistent with the expected amino acids, based on the DNA sequence. The molecular mass, as determined by MALDI-TOF mass spectrometry, gave an approximate value of 71.5 kDa. It was not possible to determine the exact molecular mass because the peak was rather broad, likely to result from the presence of microheterogeneity of the carbohydrates on the protein. HoloCOX-2 in solution is a dimer with an approximate molecular weight of 150 kDa, as determined by dynamic light scattering and HPLC gel filtration. An amount varying from 3 to 10% of high molecular weight aggregates was observed. The reconstituted protein contained 1.12 Fe^{3+}/monomer as determined by atomic absorption spectroscopy. A monodisperse solution (less than 15% polydispersity) of detergent-solubilized and freshly reconstituted protein is the ideal starting point in order to obtain crystals. CGP 28238, a selective inhibitor of COX-2 (Klein *et al.*, 1994), was utilized for co-crystallization experiments. Brownish yellow rod-shaped crystals of dimensions 0.8 × 0.1 × 0.1 mm were obtained after one week and in 0.1 *M* MES–NaOH buffer using any of the following conditions: 20%(*w*/*v*) polyethylene glycol (PEG) 4000 at pH 5.2; 15%(*w*/*v*) PEG 6000 at pH 6.0 or 5.6; 30%(*w*/*v*) PEG 1000 at pH 6.0; or 20%(*w*/*v*) PEG 2000 monomethyl ether (MME) at pH 6.0 or 6.5, using the hanging-drop method. Brownish yellow rhombohedral-shaped crystals of dimensions around 0.1 × 0.15 × 0.05 mm were obtained after one week to two months with 30%(*v*/*v*) 2-methyl-2,4-pentanediol (MPD) in 0.1 *M* Tris–HCl at pH 8.5, or 0.1 *M* Bicine at pH 9.0 using the hanging-drop method. Crystals from PEG experiments were difficult to manipulate and were easily destroyed during mounting. They were stabilized by increasing the precipitant concentration by 10%(*w*/*v*) with no change in morphology or birefringence over several days. Few diffraction spots beyond 9 Å were observed. Crystals from MPD were easier to manipulate than the crystals obtained with PEG. One crystal, mounted directly from the drop, diffracted to around 8 Å resolution. Efforts to increase the size of both crystals resulted in lengths of up to 1 mm, but no corresponding increase in width and therefore, no increase in diffraction power.

Finally, two big brownish yellow layered-plate crystals were obtained using the sitting-drop method (Fig. 1). The crystallizing solution consisted of 20%(*w*/*v*) PEG 2000 MME and

Table. 1. *Data-collection parameters of the COX-2 crystal with the inhibitor CGP 28238*

R_{merge} is defined as $\sum(|\langle I\rangle - I_i|)/\sum I_i$, summed over all symmetry-related reflections.

Space group	$P2_12_12$
a (Å)	209.56
b (Å)	71.28
c (Å)	93.82
Unique reflections	46692
Average multiplicity	2.1
Data completeness (%)	89.0
Data completeness (%, last shell, 2.6–2.45 Å)	90.4
R_{merge} (%, 15–2.45 Å)	10.27
R_{merge} (%, last shell, 2.6–2.45 Å)	41.5

0.5%(*w*/*v*) β-octylglucoside in 0.1 *M* MES–NaOH buffer at pH 6.0. For data collection, one of these crystals was transferred from the crystallization drop to a stabilizing solution consisting of 30%(*w*/*v*) PEG 2000 MME and 0.5%(*w*/*v*) β-octylglucoside in 0.1 *M* MES–NaOH buffer at pH 6.0. Despite the poor morphology of these crystals, they diffracted to about 2.5 Å resolution and a native data set could be collected (Table 1). The asymmetric unit ($V_m = 2.65$ Å^3 Da^{-1}, solvent volume = 54%) suggests the presence of two COX-2 monomers, in agreement with the dimeric structure of the detergent-solubilized protein found with light scattering and size-exclusion chromatography. The molecular replacement solution confirmed that the asymmetric unit contains one dimer of COX-2, arranged together as found for COX-1 (Picot *et al.*, 1994). Although the refinement of the structure is incomplete, electron density for the inhibitor and one glycosylation site have been detected, confirming the choice of space group and the molecular replacement solution. Further refinement is needed to remove ambiguities about the conformation of the inhibitor CGP 28238 bound to COX-2. The results of such a study will allow the design of potent and specific inhibitors of COX-2 which could be used as new non-steroid anti-inflammatory drugs.

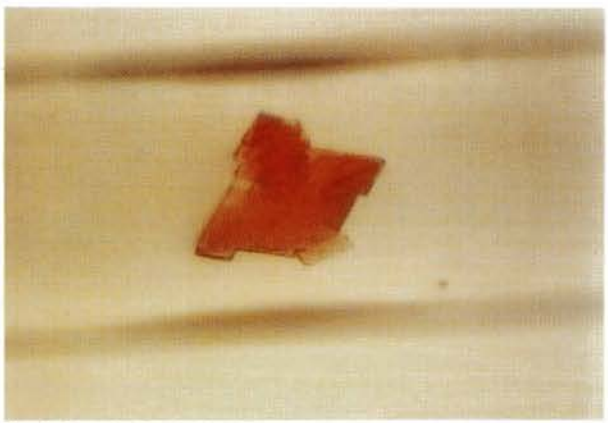

Fig. 1. Crystal of holoCOX-2 in complex with the inhibitor CGP 28238 which diffracted beyond 2.45 Å resolution. The crystal size was 0.45 × 0.25 × 0.15 mm.

Note added in proof: Shortly after submitting our manuscript and during the review process, two other research groups presented results on the structure of COX-2. Kurumbail *et al.* submitted an abstract for the XVII Congress of the International Union of Crystallography on the structure of mouse COX-2 in complex with a non-selective inhibitor of COX-2, flurbiprofen (Kurumbail *et al.*, 1996). The structure of human COX-2 in complex with different inhibitors has also been reported (Luong *et al.*, 1996).

References

Abramson, S. (1991). *Curr. Opin. Rheumatol.* **3**, 336–340.

Börnsen, O. K., Schär, M. & Widmer, H. M. (1990). *Chimia*, **44**, 412–416.

Brünger, A. T., Kuriyan, J. & Karplus, M. (1987). *Science*, **235**, 458–460.

Collaborative Computational Project, Number 4. (1994). *Acta Cryst.* D**50**, 760–763.

DeWitt, D. L. (1991). *Biochim. Biophys. Acta*, **1083**, 121–134.

Goa, J. (1953). *Scand. J. Clin. Lab. Invest.* **5**, 218–222.

Holtzman, M. J., Turk, J. & Shornick, L. P. (1992). *J. Biol. Chem.* **267**, 21438–21445.

Jones, D. A., Carlton, D. P., McIntyre, T. M., Zimmerman, G. A. & Prescott, S. M. (1993). *J. Biol. Chem.* **268**, 9049–9054.

Kabsch, W. (1988). *J. Appl. Cryst.* **21**, 67–71.

Kabsch, W. (1993). *J. Appl. Cryst.* **25**, 795–800.

Klein, T., Nüsing, R. M., Pfeilschifter, J. & Ullrich, V. (1994). *Biochem. Pharmacol.* **48**, 1605–1610.

Kurumbail, R. G., Stalling, W. C., Stevens, A. M., Pak, J., Gildehaus, D. A., Stegeman, R. Da., Gierse, J., Seibert, K. & Isakson, P. C. (1996). In *International Union of Crystallography, XVII Congress and General Assembly*, Seattle, Washington, USA, August 8–17, 1996. Abstract PS04.03b.12.

Luong, C., Miller, A., Barnett, J., Chow, J., Ramesha, C. & Browner, M. F. (1996). *Nature Struct. Biol.* **11**, 927–933.

McPherson, A. (1989). In *Preparation and Analysis of Protein Crystals*, edited by A. McPherson. Malabar, Florida: Krieger Publishing Co.

Makheja, A. N. (1992). *Mol. Cell. Biochem.* **111**, 137–142.

Marnett, L. J. (1990). *Environ. Hlth Perspect.* **88**, 5–12.

Meade, E. A., Smith, W. L. & DeWitt, D. L. (1993). *J. Biol. Chem.* **268**, 6610–6614.

Navaza, J. (1994) *Acta Cryst.* D**50**, 157–163.

Pairet, M. & Engelhardt, G. (1996). *Fundam. Clin. Pharmacol.* **10**, 1–15.

Picot, D., Loll, P. J. & Garavito, R. M. (1994). *Nature (London)*, **367**, 243–249.

Riès-Kautt, M. & Ducruix, A. (1992). In *Crystallization of Nucleic Acids and Proteins: a Practical Approach*, edited by A. Ducruix & R. Giegé, pp. 195–218. Oxford University Press.

Wennogle, L. P., Liang, H., Quintavalla, J. C., Bowen, B. R., Wasvary, J., Miller, D. B., Allentoff, A., Boyer, W., Kelly, M. & Marshall, P. (1995). *FEBS Lett.* **371**, 315–320.