Archives of Biochemistry and Biophysics 480 (2008) 41–50

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Large-scale expression, purification, and characterization of an engineered prostacyclin-synthesizing enzyme with therapeutic potential

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ARTICLE INFO

Article history:
Received 25 July 2008
and in revised form 4 September 2008
Available online 22 September 2008

Keywords:
Prostacyclin
Prostaglandin I2
Cyclooxygenase
Protein engineering
Prostacyclin synthase
Vascular protector
Anti-thrombosis

ABSTRACT

Recently, we reported that a novel hybrid enzyme (TriCat enzyme), engineered by linking human cyclooxygenase-2 (COX-2) with prostacyclin (PGI_2) synthase (PGIS) together through a transmembrane domain, was able to directly integrate the triple catalytic (TripCat) functions of COX-2 and PGIS and effectively convert arachidonic acid (AA) into the vascular protector, PGI_2 [K.H. Ruan, H. Deng, S.P. So, Biochemistry 45 (2006) 14003–14011]. In order to confirm the important biological activity and evaluate its therapeutic potential, it is critical to characterize the properties of the enzyme using the purified protein. The TriCat enzyme cDNA was subcloned into a baculovirus vector and its protein was expressed in Sf-9 cells in large-scale with a high-yield (~4% of the total membrane protein), as confirmed by Western blot and protein staining. The Sf-9 cells' membrane fraction, rich in TriCat enzyme, exhibited strong TriCat functions (K_m = 3 μM and K_{cat} = 100 molecules/min) for the TriCat enzyme and was 3-folds faster in converting AA to PGI_2 than the combination of the individual COX-2 and PGIS. Another superiority of the TriCat enzyme is its dual effect on platelet aggregation: it completely inhibited platelet aggregation at the low concentration of 2 μg/ml and then displayed the ability to reverse the initially aggregated platelets to their non-aggregated state. Furthermore, multiple substrate-binding sites were confirmed in the single protein by high-resolution NMR spectroscopy, using partially purified TriCat enzyme. These studies have clearly demonstrated that the isolated TriCat enzyme protein functions in the selective biosynthesis of the vascular protector, PGI_2, and revealed its potential for anti-thrombosis therapeutics.

The biosynthesis of prostanoids (prostaglandins and thromboxane) occurs via the COX[1] pathway from arachidonic acid (AA) in three/triple catalytic steps [1–5] by COX and its downstream synthases: AA, released from the membrane phosphoglycerides, is converted to the prostaglandin G_2 (PGG_2, catalytic step 1), and then to the prostaglandin endoperoxide (prostaglandin H_2 (PGH_2)) (catalytic step 2) by COX isoform-1 (COX-1) or COX-2. PGH_2 is further isomerized to the biologically active end-products, prostaglandin D_2 (PGD_2), E_2 (PGE_2), F_2 (PGF_2), and I_2 [PGI_2 (prostacyclin)] or thromboxane A_2 (TXA_2) by individual synthases (catalytic step 3) in tissue-specific processes. TXA_2, produced from PGH_2 by TXA_2 synthase (TXAS), has been implicated as a proaggregatory and vasoconstricting mediator which is one of the major factors causing stroke and heart attacks [6–7]. PGI_2 is the primary AA metabolite in vascular walls and has opposing biological properties to that of TXA_2 and therefore represents the most potent endogenous vascular protector by acting as an inhibitor of platelet aggregation [8] and a strong vasodilator on vascular beds [9–12]. A selective increase in the production of PGI_2 with a decrease in TXA_2 production is the ideal outcome for a model that prevents and protects against vascular diseases, including thrombosis, hypertension, strokes and heart attacks. From the century-old aspirin to the more recently developed COX-2 inhibitors, the drugs have not yet achieved this goal. Finding a way to specifically increase the production of the vascular protector, PGI_2, will be one of the most attractive therapeutic developments in pharmaceutical intervention. Recently, we have successfully engineered an innovative enzyme by linking COX-2 and PGIS together to form a single protein using a molecular cloning approach [13]. The engineered enzyme narrowly expressed in HEK293 and COS-7 cells could actively convert AA into PGI_2 through its triple catalytic activities. As a result, this engineered enzyme has been reported as a "Super Enzyme" with great potential for fighting heart disease by the American Chemical Society [14]. In this paper, the active Triple Catalytic (TriCat) enzyme was first successfully produced in large-scale using a baculovirus (BV) system, and its benefits for specifically up-regulating PGI_2 biosynthesis and its potent anti-thrombosis properties, which are superior to that of the individual COX-2 and

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[1] *Abbreviations used:* COX, cyclooxygenase; PGI_2, prostacyclin or prostaglandin I_2; PGIS, PGI_2 synthase; TXA_2, thromboxane A_2; TXAS, TXA_2 synthase; PCR, polymerase chain reaction; AA, arachidonic acid; HPLC, high-performance liquid chromatography; PRP, platelet rich plasma; BSA, bovine serum albumin; U46619, 9,11-Dideoxy-9alpha,11alpha-methanoepoxyprostaglandin F2alpha.

0003-9861/$ - see front matter © 2008 Published by Elsevier Inc.
doi:10.1016/j.abb.2008.09.010

PGIS, were confirmed and further characterized. The studies have revealed a great potential for developing the innovative protein into a new therapeutic intervention for vascular disease requiring increased levels of PGI_2.

Experimental procedures

Materials

COS-7 and HEK293 cell lines were purchased from ATCC (Manassas, VA). Medium for culturing the cell lines was purchased from Invitrogen. [^{14}C]-Arachidonic Acid (AA) was purchased from Amersham Biosciences. The non-labeled AA for the platelet aggregation studies was purchased from Chrono-Log Corp., (Havertown, PA) and Bio Data Corp., (Horsham, PA). The aspirin used in this experiment was purchased from Sigma.

Engineering of the TriCat enzyme cDNA containing the sequences of COX-2 and PGIS

The cDNA of the newly engineered TriCat enzyme (COX-2-10aa-PGIS) was previously described [13]. Briefly, COX-2 was linked to a 10 amino acid TM linker connected to PGIS. COX-2 was generated by a PCR approach and then subcloned into a pcDNA3.1 vector with a CMV promoter. The resultant pcDNA3.1-COX-2-10aa-PGIS was used for expressing the TriCat enzyme in HEK293 and COS-7 cells.

Subcloning of the COX-2-10aa-PGIS cDNA into the baculovirus (BV) vector

First, a BamHI insert was added to the front of the COX-2-10aa-PGIS cDNA's start codon, and then a 6His-tag sequence was inserted between N19 and P20 of the COX-2 sequence in the pcDNA3.1 vector using a PCR approach. The cDNA of the 6His-COX-2-10aa-PGIS (6His-TriCat enzyme) was cut from the pcDNA3.1 vector using EcoRI and BamHI restriction enzymes and then inserted into the BV-suitable vector, pVL1392, to obtain the final pVL1392-6His-TriCat enzyme construct.

Packing the BV for expression of the 6His-TriCat enzyme in Sf-9 cells and large-scale expression

The packing of the BV for high-yield expression of the TriCat enzyme in the Sf-9 insect cells was performed in two steps. (1) The Sf-9 cells were co-transfected with 2.0 μg of the pVL1392-6His-TriCat enzyme plasmid and 0.5 μg of Orbigen Sapphire DNA using a calcium phosphate precipitation method; (2) Six days after the co-transfection, the virus-containing supernatant was harvested and plaqued at dilutions of 10^{-5}, 10^{-6} and 10^{-7}. 1 week later, 10 plaques (A–J) were selected and each viral isolate was put into 1.0 ml of serum free Grace's media overnight. The following day, 0.5 mL of each viral plaque was added to an individual T25 flask of Sf-9 insect cells. After infection, the cells were harvested at different times to view the expression levels of the 6His-TriCat enzyme. The specific plaque (plaque H) of the transfected Sf-9 cells with high-yield expression of the TriCat enzyme was selected for further middle-scale (500 mL) and large-scale (5 L,) expression, both of which were performed in the Baculovirus/Monoclonal Antibody Facility, Baylor College of Medicine (Houston, TX).

Expression and co-expression of the individual COX-2 and PGIS in COS-7 or HEK293 cells

The recombinant synthases were expressed in COS-7 or HEK293 cells as described [15–16]. Briefly, the cells were cultured in a 100-mm cell culture dish with high glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% antibiotic and antimycotic, then grown at 37 °C in a humidified 5% CO_2 incubator. The cells were then transfected with a purified cDNA of the recombinant protein by the Lipofectamine 2000 method [17] following the manufacturer's instructions (Invitrogen). Approximately 48 h after transfection, the cells were harvested for further enzyme assay and a Western blot was performed. For the co-transfection, a 3:2 ratio of the cDNAs of COX-2 and PGIS were used.

Preparation of microsomal and membrane fractions of the Sf-9 cells

The general procedure for microsomal preparation was previously described [18]. The transfected or untransfected Sf-9 cells were harvested into ice-cold PBS buffer, pH 7.4, and collected by centrifugation. After the cells were washed three times, the pellet was resuspended in a small volume of the same buffer, sonicated briefly, and centrifuged at 10,000*g* for 10 min. The supernatant was collected and centrifuged at 200,000*g* for 40 min to pellet the microsomal and membrane fractions [18].

Electrophoresis and Western blot

Membrane proteins were separated by 7% (w/v) SDS–PAGE under denaturing conditions and then transferred to a nitrocellulose membrane. Bands recognized by specific primary antibodies against human PGIS or COX-2 were visualized with horseradish peroxidase-conjugated secondary antibody and chromogenic peroxidase substrates [13]. The intensities of the bands in the immunoblot were used to normalize the enzyme activities as well.

Enzyme activity determination for COX-2 and PGIS using the HPLC-scintillation analysis method [13]

To determine the activity of the enzyme that converted AA into PGI_2, the membrane protein was incubated with different concentrations of [^{14}C]-AA (0.3–60 μM) in a total reaction volume of 30–100 μL. After a 0.5–5 min incubation, the reaction was terminated by adding 200 μL of the solvent containing 0.1% acetic acid and 30% acetonitrile (solvent A). After centrifugation (12,000 rpm for 5 min), the supernatant was injected into a reverse phase C18 column (Varian Microsorb-MV 100-5, 4.5 × 250 mm) using the solvent A with a gradient from 35% to 100% of acetonitrile for 45 min at a flow-rate of 1.0 mL/min. The [^{14}C]-labeled AA metabolites, including [^{14}C]-6-keto-$PGF_{1\alpha}$ (degraded PGI_2) were monitored directly by a flow scintillation analyzer (Packard 150TR). The peak and the relative amount of [^{14}C]-6-keto-$PGF_{1\alpha}$ converted from [^{14}C]-AA was confirmed and calibrated by 6-keto-$PGF_{1\alpha}$ standards using Enzyme Immunoassays as described [13].

Anti-platelet aggregation assays

A sample of fresh blood was collected using a collection tube with 3.2% sodium citrate for anticoagulation. The platelet-rich plasma was separated from the blood by low speed centrifugation. A total of 0.5 mL of the platelet-rich plasma was incubated with either saline, membrane protein or cells (HEK293 or Sf-9) at 37 °C for 3 min. Sodium arachidonate (final concentration 5 mg/mL) was added to the platelet sample and the platelet aggregation was monitored by the anticoagulation analyzer (model 490, Chronolog Aggregometer with AggregoLink software; Havertown, Pennsylvania). Platelet aggregation was determined by conventional light transmittance aggregometry, in which aggregation was expressed as the maximal percent change in light transmittance from baseline, using the platelet-poor plasma as a reference.