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FEBS Letters 342 (1994) 33–37

FEBS 13821

FEBS LETTERS

Mutation of serine-516 in human prostaglandin G/H synthase-2 to methionine or aspirin acetylation of this residue stimulates 15-*R*-HETE synthesis

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Received 4 February 1994

Abstract

Prostaglandin G/H synthase (PGHS) is a key enzyme in cellular prostaglandin (PG) synthesis and is the target of non-steroidal anti-inflammatory agents. PGHS occurs in two isoforms, termed PGHS-1 and PGHS-2. These isoforms differ in several respects, including their enzymatic activity following acetylation by aspirin. While PG synthesis by both isoforms is inhibited by aspirin, 15-*R*-hydroxyeicosatetraenoic acid (15-*R*-HETE) synthesis by PGHS-2, but not PGHS-1, is stimulated by preincubation with aspirin. We have mutated the putative aspirin acetylation site of hPGHS-2, and expressed the mutants in COS-7 cells using recombinant vaccinia virus. Enzyme activity and inhibitor sensitivity studies provide evidence that Ser^{516} is the aspirin acetylation site of human PGHS-2 and that substitution of a methionine residue at this position can mimic the effects of aspirin acetylation on enzyme activity.

Key words: Prostaglandin G/H synthase-2; PGHS-2; Cyclooxygenase-2; COX-2; 15-HETE; Mutagenesis; Vaccinia virus

1. Introduction

The first two commited steps in the cellular synthesis of all prostaglandins (PGs), namely the oxygenation of arachidonic acid to prostaglandin G_2 (PGG_2) and the reduction of PGG_2 to prostaglandin H_2 (PGH_2) are catalyzed by the enzyme prostaglandin G/H synthase (PGHS) [1]. PGHS also represents the target of various non-steroidal anti-inflammatory drugs (NSAIDS), including aspirin and indomethacin [2,3].

A constitutively expressed form of PGHS, termed PGHS-1, has been detected in a variety of cell types from different species [4–6]. A second isoform (PGHS-2) is rapidly induced in response to growth factors, cytokines and endotoxin [3,7–11). This has lead to the suggestion that PGHS-1 may be responsible for PG synthesis under normal physiological conditions, with PGHS-2 being primarily responsible for PG synthesis during inflammatory responses [3,12,13]. PGHS-2 may therefore represent a novel target for NSAIDS. This has resulted in considerable interest in biochemical and pharmacological comparisons of PGHS-1 and PGHS-2 [12–14].

In addition to the synthesis of PGs following incubation with arachidonic acid, PGHS-1 and PGHS-2 both synthesize low levels of 15-HETE and 11-HETE [14–17]. Acetylation of PGHS-1 by aspirin results in an inhibition of PG synthesis, but does not alter 15-HETE synthesis [12–14]. This difference in activity suggests that the active sites of the two isoforms of PGHS have distinct structural features. The aspirin acetylation site of sheep PGHS-1 has been identified as Ser^{530} [18,19], and it has been proposed that the aspirin acetylation sites of human PGHS-1 (hPGHS-1) and human PGHS-2 (hPGHS-2) are Ser^{529} [20] and Ser^{516} [7], respectively. In an attempt to mimic the effects of aspirin acetylation on hPGHS-2 activity we mutated the putative aspirin acetylation site of the enzyme by site-directed mutagenesis and assessed the effects of amino acid substitutions on enzyme activity and the sensitivity of this activity to aspirin.

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Abbreviations: ASA, acetylsalicylic acid; DMSO, dimethyl sulphoxide; EDTA, ethylenediamine tetraacetic acid; 11-HETE, 11-hydroxyeicosatetraenoic acid; 15-HETE, 15-hydroxyeicosatetraenoic acid; NSAIDS, non-steroidal antiinflammatory drugs; PG, prostaglandin; PGHS, prostaglandin G/H synthase; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

2. Materials and methods

2.1. Chemicals and reagents

Nucleotides and dNTPs were obtained from Boehringer Mannheim (Montreal, Quebec, Canada). All other reagents were obtained from Pharmacia (Montreal, Quebec, Canada). ^{125}I-labeled protein A (10 mCi/mg) and [α-^{32}P]dCTP (3,000 Ci/mmol) were purchased from NEN-Dupont (Burlington, Ontario, Canada).

SSDI 0014-5793(94)00206-B

2.2. Cell culture

COS-7 and 143B TK$^-$ cell lines were obtained from the American Type Culture Collection and grown as monolayer cultures in a humidified environment of 6% CO_2 at 37°C in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum, 10 mM glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, and 100 μg/ml gentamicin (Gibco/BRL, Burlington, Ontario, Canada), 25 mM HEPES, pH 7.4. For 143B TK$^-$ cells, 25 μg/ml of 5-bromo-2′-deoxyuridine was also added to the culture medium.

2.3. Site-directed mutagenesis

All general recombinant DNA techniques and site-directed mutagenesis procedures were performed as described elsewhere [21]. Briefly, the *Pst*I fragment of hPGHS-2 was subcloned from pTM1-hPGHS-2-3′fl [14] into the multiple cloning site of Bluescript (Stratagene, LaJolla, CA) to yield pBS-Pst-hPGHS-2. The preparation of single stranded DNA from pBS-Pst-hPGHS-2 and the introduction of mutations into the cDNA of PGHS-2 was then performed using the Amersham oligonucleotide-directed mutagenesis kit (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. Single amino acid changes were introduced using appropriate oligonucleotides 33 nucleotides in length with 3 nucleotide mismatches. The *Pst*I fragments containing the mutated sequences were then subcloned back into the *Pst*I site of their plasmid of origin to yield the constructs pTM1-hPGHS-2(Ser^{516}-Met) and pTM1-hPGHS-2(Ser^{516}-Gln). The mutant proteins derived from these constructs are hPGHS-2(Ser^{516}-Met) and hPGHS-2(Ser^{516}-Gln), where the serine at residue 516 of hPGHS-2 is mutated to a methionine or glutamine residue, respectively. Oligonucleotides were synthesized using the 380B DNA synthesizer (Applied Biosystems, Mississauga, Ont., Canada). Introduction of the desired mutation was confirmed by dideoxy sequencing, using a T7 sequencing kit (Pharmacia).

2.4. Construction of recombinant vaccinia virus transfer vectors

The recombinant vaccinia virus used for the expression of human prostaglandin G/H synthase-2 (termed VV:hPGHS-2-3′fl) has previously been described [14]. All procedures for the generation of recombinant vaccinia viruses, infection of COS-7 cells with these viruses in the presence of the helper virus VV:TF7-3, and the preparation of microsomal protein fractions from infected cells have been described elsewhere [14].

2.5. Prostaglandin G/H synthase assays

Assays for the synthesis of PGE_2 and 15-HETE by microsome preparations from COS-7 cells have been described elsewhere [14]. Briefly each assay contained 50–125 μg of protein in a total volume of 200 μl containing 100 mM Tris-HCl, pH 7.4, and 10 mM EDTA. Samples were then incubated at room temperature for 30 min in the presence of aspirin (100 μM) or ethanol vehicle (2 μl), followed by incubation for 5 min in the presence of indomethacin (1 μl) or dimethyl sulphoxide vehicle. Reduced glutathione (2 μl), phenol (2 μl) and hematin (1 μl) were then added to final concentrations of 1 mM, 0.5 mM and 0.5 μM, respectively. Reactions were initiated by the addition of arachidonic acid (2 μl; final concentration 20 μM) followed by incubation for 30 min at room temperature. Reaction products were immediately extracted into chloroform and analyzed by reverse-phase HPLC using a solvent system of 75% methanol/0.01% acetic acid as previously described [14]. The identity of reaction products was verified by coinjection with authentic standards and mass spectrometry, and the amounts of reaction products were determined using a standard curve of authentic standards. Aliquots from these assays were also analyzed for PGE_2 concentrations using a radioimmunoassay (NEN-DuPont) as previously described [14].

3. Results

3.1. Expression of hPGHS-2 and hPGHS-2 mutants in COS-7 cells

By enzyme activity measurements and immunoblot analysis [14], PGHS expression is extremely low in microsomes from wild-type COS-7 cells. Immunoblot analysis (Fig. 1) demonstrates a high level expression of hPGHS-2, hPGHS-2(Ser^{516}-Met) or hPGHS-2(Ser^{516}-Gln) in 100,000 × *g* membrane preparations of COS-7 cells following infection with the appropriate recombi-

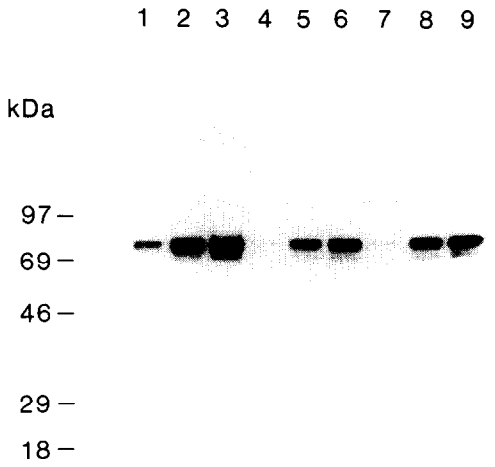

Fig. 1. Immunoblot analysis of vaccinia virus-directed expression of hPGHS-2 and hPGHS-2 mutants in COS-7 cells. COS-7 cells were infected with the indicated viruses, cultured for 26 h, harvested and then used for the preparation of microsomes. Microsomal proteins were subjected to SDS-PAGE on precast 10% Tris-glycine acrylamide gels (Novex, San Diego, CA) and electrophoretically transferred to nitrocellulose membranes. Immunoblot analysis was then performed using an anti-PGHS-2 antipeptide antiserum (Cayman, Ann Arbor, MI) and [^{125}I]protein A as previously described [21]. Expression of hPGHS-2 was assessed in microsomes prepared from cells co-infected with recombinant vaccinia virus for hPGHS-2 (lanes 1–3), hPGHS-2(Ser^{516}-Met) (lanes 4–6) or hPGHS-2(Ser^{516}-Gln) (lanes 7–9) and helper virus. The amount of protein loaded per lane was 1 μg (lanes 1, 4 and 7), 5 μg (lanes 2, 5 and 8) or 10 μg (lanes 3, 6 and 9). Blots were exposed to Kodak XAR-2 film at −80°C for 3 h. The migration positions of molecular weight markers are indicated.

Table 1
Prostaglandin and 15-HETE synthesis by hPGHS-2 and hPGHS-2(Ser^{516}-Met)

	Prostaglandin E_2 (ng)		15-HETE (ng)	
	−ASA	+ASA	−ASA	+ASA
T7 control	0.06	0.06	nd	nd
hPGHS-2	786	192	13	154
hPGHS-2(Ser^{516}-Met)	9	10	266	261

Microsome preparations from mock-infected COS-7 cells (T7 control) or COS-7 cells expressing hPGHS-2 or hPGHS-2(Ser^{516}-Met) were assayed in duplicate for enzyme activity as described in section 2. Results are shown as ng of product per assay, with the amount of protein for each assay being adjusted to give equivalent amounts of hPGHS-2 or mutant protein as determined by densitometric scanning of immunoblots (Fig. 1). Less than 10% variability was observed between duplicate samples. The amounts of protein per assay were 100 μg, 50 μg, and 125 μg for T7 control, hPGHS-2, and hPGHS-2(Ser^{516}-Met), respectively. nd = not detectable (< 1 ng).

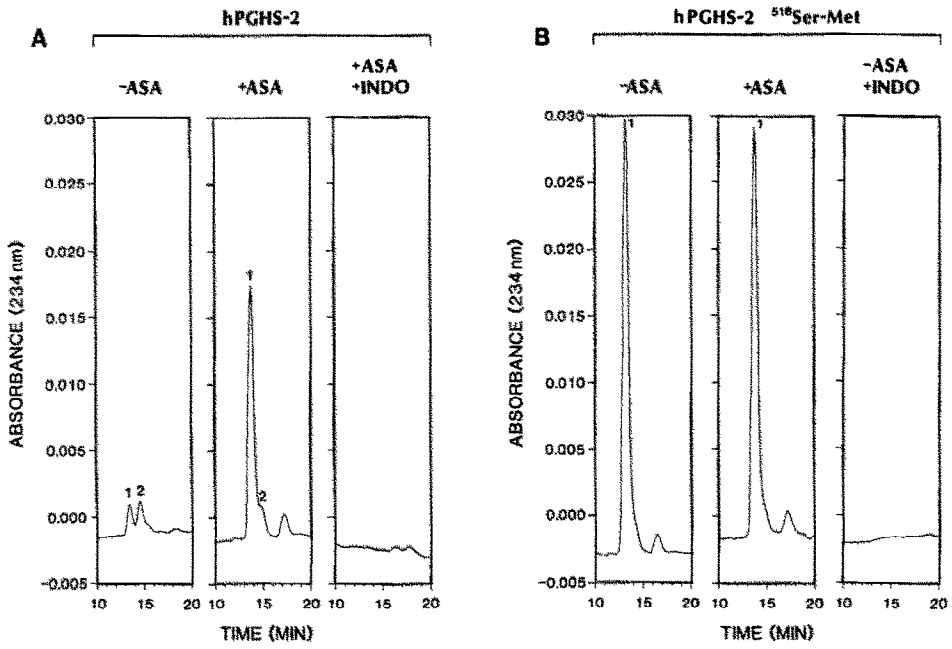

Fig. 2. 15-HETE synthesis by microsomes containing hPGHS-2 or hPGHS-2(Ser^{516}-Met). Microsomal proteins from COS-7 cells infected with recombinant vaccinia virus for hPGHS-2 (A) or hPGHS-2(Ser^{516}-Met) (B) were preincubated in the absence (−ASA) or presence (+ASA) of 100 μM aspirin for 30 min, followed by incubation with 20 μM arachidonic acid for 30 min. The indicated samples were incubated for 5 min with 100 μM indomethacin prior to the initiation of reactions. Reaction products were analyzed by reverse phase HPLC and the eluant monitored at 234 nm as previously described [14]. Peaks 1 and 2 correspond to 15-HETE and 11-HETE, respectively. The amounts of microsomal protein used in these assays was adjusted so that similar amounts of hPGHS-2 or hPGHS-2(Ser^{516}-Met) were present (Fig. 1 and Table 1).

nant vaccinia virus and helper virus for 26 h. In these experiments, expression of native hPGHS-2 was approximately 2-fold higher than that of the mutant proteins, as determined by scanning laser densitometry of the immunoblot (data not shown).

3.2. *Prostaglandin and 15-HETE synthesis by hPGHS-2 and hPGHS-2 mutants*

Consistent with our previous findings [14], PG synthesis in microsomes containing hPGHS-2 was inhibited by preincubation of the enzyme with aspirin (Table 1). In addition, hPGHS-2 expressed in this system synthesized low amounts of 15-HETE and 11-HETE (Table 1 and Fig. 2A), with 15-HETE levels being increased following preincubation with 100 μM aspirin. Fig. 2A also demonstrates that aspirin-stimulated 15-HETE synthesis by hPGHS-2 is completely inhibited by indomethacin, demonstrating that this NSAID can still bind to hPGHS-2 following aspirin acetylation of the enzyme.

Microsomes containing hPGHS-2(Ser^{516}-Met) synthesized approximately 90-fold lower concentrations of PGE_2 than the native protein (Table 1), with this activity not being altered by preincubation by aspirin. While PG synthesis by hPGHS-2(Ser^{516}-Met) was considerably lower than that observed with PGHS-2, the mutant protein synthesized approximately 20-fold higher concentrations of 15-HETE than the wild-type protein (Table 1 and Fig. 2B). Furthermore, 15-HETE and PG synthesis by hPGHS-2(Ser^{516}-Met) was unaltered by preincubation with 100 μM aspirin (Table 1 and Fig. 2B) or 100 μM salicylic acid (data not shown), but was completely inhibited by incubation with indomethacin (Fig. 2B). hPGHS- 2(Ser^{516}-Met) synthesizes higher concentrations of 15-HETE in the absence of aspirin treatment than does hPGHS-2 following aspirin treatment (Table 1).

The hPGHS-2 mutant in which Ser^{516} was replaced by glutamine residue synthesised approximately 70-fold lower concentrations of PGs than hPGHS-2(Ser^{516}-Met) and only 2-fold above control PG concentrations. However, this low level of synthesis was decreased to control values by preincubation with 100 μM indomethacin (data not shown). No detectable 15-HETE synthesis was observed with microsomes from mock-infected COS-7 cells or cells expressing hPGHS-2(Ser^{516}-Gln).

3.3. *15-R-HETE synthesis by hPGHS-2(Ser^{516}-Met)*

We have previously demonstrated that following preincubation with aspirin, hPGHS-2 synthesises the *R*-enantiomer of 15-HETE, with no detectable 15-*S*-HETE [14]. To confirm the stereoselectivity of 15-HETE synthesis by hPGHS-2(Ser^{516}-Met), reaction products were analyzed by chiral-phase HPLC (Fig. 3). This procedure confirmed that hPGHS-2(Ser^{516}-Met) synthesized 15-*R*-HETE, with no detectable 15-*S*-HETE.

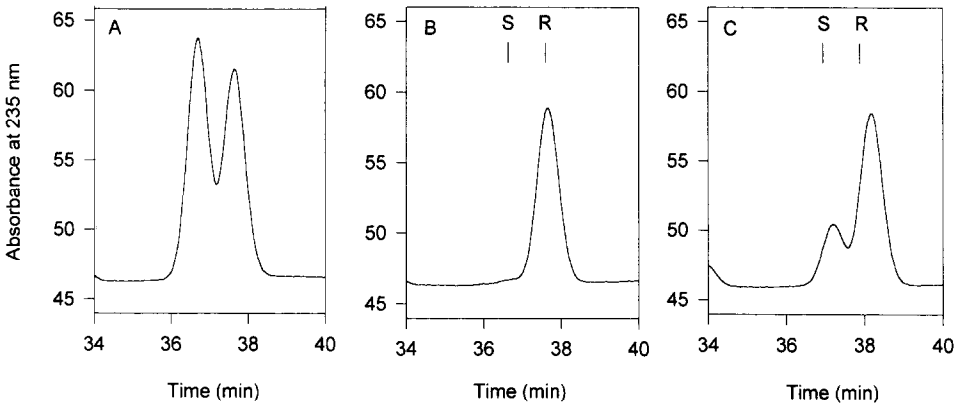

Fig. 3. Chiral phase HPLC analysis of 15-HETE synthesised by hPGHS-2(Ser^{516}-Met). The following samples were analyzed on a chiral phase column as previously described [14]. (A) A mixture containing 100 ng of each of the methyl esters of 15-*S*-HETE and 15-*R*-HETE. (B) 100 ng of the methyl ester of 15-HETE synthesized by microsomes from COS-7 cells containing hPGHS-2(Ser^{516}-Met). (C) Co-injection of 50 ng of the methyl esters of 15-*S*-HETE, 15-*R*-HETE and 15-HETE derived from hPGHS-2(Ser^{516}-Met). In B and C the retention times of 15-*S*-HETE and 15-*R*-HETE are indicated.

4. Discussion

In the present study, site-directed mutagenesis was used to mutate Ser^{516} of hPGHS-2, which is the putative aspirin acetylation site of the enzyme. Replacement of this residue with a methionine residue decreased PG synthesis by the enzyme dramatically, with the remaining activity being insensitive to treatment of the enzyme with aspirin. This is consistent with Ser^{516} being the aspirin acetylation site of hPGHS-2 and, similar to sheep PGHS-1 [18] and human PGHS-1 [20], that the serine residue at this site is not absolutely required for enzyme activity. These results also suggest that there is no other aspirin acetylation site in hPGHS-2 which affects PG production.

We have demonstrated that hPGHS-2(Ser^{516}-Met) can synthesize similar levels of 15-*R*-HETE from arachidonate to that observed following aspirin acetylation of hPGHS-2. This suggests that the side chain of methionine may sterically mimic an acetylated serine at the active site of hPGHS-2, and may provide a useful model system to study the effect of aspirin acetylation on the structure and activity of hPGHS-2. The demonstration that indomethacin can inhibit 15-HETE synthesis by hPGHS-2(Ser^{516}-Met) and aspirin-acetylated hPGHS-2 demonstrates that NSAIDS can still be accomodated at the active site of PGHS-2 following modification of Ser^{516}. When Ser^{516} was replaced with a glutamine residue, the resulting mutant could synthesize only extremely low levels of PGs and no detectable 15-HETE. This suggests that the side chain of glutamine, which is more polar and conformationally constrained than that of a methionine residue, prevents the productive binding of arachidonate to the enzyme.

The high level synthesis of 15-*R*-HETE by aspirin-acetylated hPGHS-2, which is mimicked by hPGHS-2(Ser^{516}-Met), is a feature which clearly distinguishes PGHS-2 from PGHS-1. A comparison of the structural features of PGHS-2 to those recently reported for PGHS-1 [22] may provide a greater understanding of the basis for this difference.

References

[1] Vane, J.R. and Botting, R. (1987) FASEB J. 1, 89–96.
[2] Smith, W.L. and Marnett, L.J. (1990) Biochim. Biophys. Acta 1083, 1–17.
[3] Xie, W., Robertson, D.L. and Simmons, D.L. (1992) Drug Dev. Res. 25, 249–265.
[4] DeWitt, D.L. and Smith, W.L. (1988) Proc. Natl. Acad. Sci. USA 85, 1212–1216.
[5] Merlie, J.P., Fagan, D., Mudd, J. and Needleman, P. (1988) J. Biol. Chem. 263, 3550–3553.
[6] Simmons, D.L., Xie, W., Chipman, J.G. and Evett, G.E. (1991) in: Prostaglandins, Leukotrienes, Lipoxins, and PAF (Bailey, J.M., ed.) pp. 67–78, Plenum Press, New York.
[7] Hla, T. and Neilson, K. (1992) Proc. Natl. Acad. Sci. USA 89, 7384–7388.
[8] O'Sullivan, G.M., Chilton, F.H., Huggins Jr., E.M. and McCall, C.E. (1992) J. Biol. Chem. 267, 14547–14550.
[9] O'Banion, M.K., Winn, V.D. and Young, D.A. (1992) Proc. Natl. Acad. Sci. USA 89, 4888–4892.
[10] Sirois, J., Simmons, D.L. and Richards, J.S. (1992) J. Biol. Chem. 267, 11586–11592.
[11] Pilbeam, C.C., Kawaguchi, H., Hakeda, Y., Voznesensky, O., Alander, C.B. and Raisz, L.G. (1993) J. Biol. Chem. 268, 25643–25649.
[12] Meade, E.A., Smith, W.L. and DeWitt, D.L. (1992) J. Biol. Chem. 268, 6610–6614.
[13] Mitchell, J.A., Akarasereenont, P., Thiemermann, C., Flower, R.J. and Vane, J.R. (1993) Proc. Natl. Acad. Sci. USA 90, 11693–11697.
[14] O'Neill, G.P., Mancini, J.A., Kargman, S., Yergey, J., Kwan, M.Y., Falgueyret, J.-P., Abramovitz, M., Kennedy, B.P., Ouellet, M., Cromlish, W., Culp, S., Evans, J.F., Ford-Hutchinson, A.W. and Vickers, P.J. (1994) Mol. Pharmacol. (in press).
[15] Bailey, J.M., Bryant, R.W., Whiting, J. and Salata, K. (1983) J. Lipid Res. 24, 1419–1428.

[16] Yanaja Setty, B.N., Stuart, M.J. and Walenga, R.W. (1985) Biochim. Biophys. Acta 833, 484–494.
[17] Hemler, M.E., Crawford, C.G. and Lands, W.E.M. (1978) Biochemistry 17, 1772–1779.
[18] DeWitt, D.L., El-Harith, E.A., Kraemer, S.A., Andrews, M.J., Yao, E.F., Armstrong, R.L. and Smith, W.L. (1990) J. Biol. Chem. 265, 5192–5198.
[19] Shimokawa, T. and Smith, W.L. (1992) J. Biol. Chem. 267, 12387–12392.
[20] Funk, C.D., Funk, L.B., Kennedy, M.E., Pong, A.S. and Fitzgerald, G.A. (1991) FASEB J. 5, 2304–2312.
[21] Vickers, P.J., Adam, M., Charleson, S., Coppolino, M.G., Evans, J.F. and Mancini, J.A. (1992) Mol. Pharmacol. 42, 94–102.
[22] Picot, D., Loll, P.J. and Garavito, M. (1994) Nature 367, 243–249.