Modeling Cyclooxygenase Inhibition. Implication of Active Site Hydration on the Selectivity of Ketoprofen Analogues

Albert Palomer,*,† Juan J. Pérez,‡ Susana Navea,† Oriol Llorens,‡ Jaume Pascual,† Lluïsa García,† and David Mauleón†

R & D Department, Laboratorios Menarini, Alfonso XII 587, 08918 Badalona, Spain, and Department d'Enginyeria Química, UPC, ETSEIB, Diagonal 647, 08028 Barcelona, Spain

Received December 20, 1999

Molecular modeling studies performed on the two cyclooxygenase isozymes (COXs) suggest that active site hydration is crucial for understanding inhibitor selectivity. In this work, models have been constructed considering some implicit water molecules, placed in the position suggested by GRID, that participate in the dynamic hydrogen-bonding network at the polar active site entrance together with protein residues 355, 524, 120, and 513. The selectivity observed for ketoprofen (**1**) and the structural analogues **2** and **3** may be rationalized in terms of such implicit hydration.

Introduction

Nonsteroidal antiinflammatory drugs (NSAIDs) are therapeutic agents useful in the treatment of inflammation, pain, and pyresis, although they exhibit an undesirable gastrotoxicity profile. Since NSAIDs directly target cyclooxygenases (COXs),[1] the discovery of the COX-2 isoform[2] has opened the possibility of developing COX-2 selective inhibitors to act as an effective NSAID without the gastrotoxic effects.[3] At present, two COX-2 selective inhibitors have successfully reached the market, rofecoxib[4] and celecoxib,[5] inducing a great interest in obtaining isozyme-specific drugs.[6]

The 3D structure of COX-1 is known from the first X-ray crystal structure described by Picot D. et al.[7] of the isoenzyme complexed with flurbiprofen, a nonselective NSAID. The structure of the human COX-2 enzyme, obtained from X-ray diffraction[8−10] or by homology modeling,[11] has also been published. The kinetic mechanism of COX inhibition has also been studied and it is now well-accepted that the different activity of a selective compound for each isoenzyme, cf. the selectivity, may arise from the difference in kinetics.[12,13] On the other hand, studies of the static 3D structure of the enzyme have been of great help to understand the enzyme inhibition mechanism and to design selective compounds, but it has been shown to be insufficient and some authors have suggested the importance of dynamic factors related to selective inhibition including enzyme flexibility[9] and rearrangement of hydrogen-bonding network at the entrance of the active site.[14] Moreover, dynamic models of COX−inhibitor complexes with SC-558,[11] NS-398,[15] indoprofen,[15] and flurbiprofen[16] have not only added a step further in understanding the mode of action of inhibitors but have also been used for the design of selective inhibitors based on the flurbiprofen structure.[16]

Water present in the ligand binding site of a protein has been recognized to play a major role in ligand−protein interactions.[17−19] To date, rational drug design techniques rarely incorporate the effect of these water molecules into the design strategies[18] and, to our knowledge, no study including COX enzyme hydration has been performed. Since water molecules are not covalently bound to the receptor, their effect depends largely on their variable dynamic position at the binding site.[18] In the case of COX inhibition, it has been hypothesized that the dynamic events associated with the molecular mechanism of time-dependent inhibition involve remodeling of the hydrogen-bonding network at the entrance of the active site;[14] therefore, hydration of this region may play a critical role in this dynamic process. Hydration effects may be considered by simply including some implicit water molecules in the calculations. Constitutive water molecules may be localized either from high-resolution X-ray diffraction studies or from calculations using the specific water probe with the GRID method.[20,21] A valuable starting point for this work was the presence of a constitutive water molecule in the structure of the COX-1/Br-aspirin complex[22] that was successfully localized using the GRID method (see Figure 1).

In the present work we describe the importance of studying the dynamic nature of the COX enzyme−inhibitor complexes considering some implicit water molecules near the active site entrance of the enzyme in order to rationalize the inhibition results obtained for the NSAID ketoprofen (**1**) and the structural analogues **2**[23] and **3**[23] (see Tables 1 and 2).

Results and Discussion

Present studies we carried out using the 3D structure of the flurbiprofen COX-2 and COX-1 complexes[11,24] once the ligand was removed. Initial structures of the COX complexes were generated manually by placing the inhibitors **1**−**3**, in a conformation similar to flurbiprofen, into the enzyme cavity using computer graphics by means of the InsightII program.[25] Manual docking, prior to complex minimization, was done by superimposition onto the original position of flurbiprofen with its carboxylate oriented toward residue Arg-120, the ben-

* To whom correspondence should be addressed. Tel: +34-3-4628800. Fax: +34-3-4628813. E-mail: apalomer@menarini.es.
† Laboratorios Menarini.
‡ ETSEIB.

10.1021/jm9906217 CCC: $19.00 © 2000 American Chemical Society
Published on Web 05/04/2000

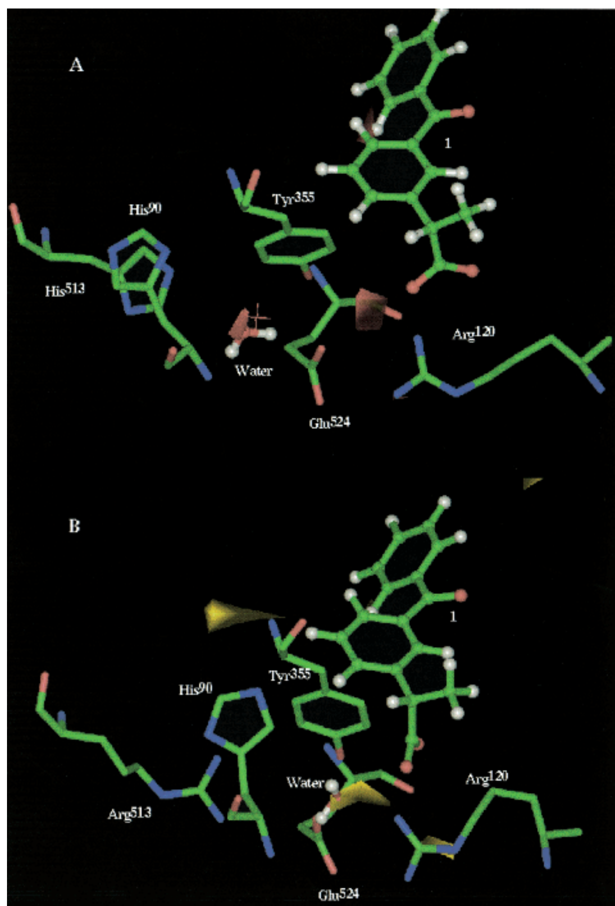

Figure 1. (A) Complex of **1** with COX-1: Interaction energy map contoured at −7 kcal/mol (red surface) obtained with the GRID water probe (OH2) in the absence of structural water molecules. The final water molecule is shown for reference together with the ligand and a selected subset of the COX-2 residues near the GRID water spots. The red cross indicates the position where a water molecule is located in the X-ray structure of the COX-1/Br-aspirin complex.[22] (B) Complex of **1** with COX-2: Interaction energy maps contoured at −7 kcal/mol (yellow surface) obtained with the GRID water probe (OH2) in the absence of structural water molecules. The final water molecule is shown for reference together with the ligand and a selected subset of the COX-2 residues near the GRID water spots.

zophenone carbonyl toward Ser-530, and placing the benzophenone group in the active site channel flanked by amino acids 530 (Ser), 385 (Tyr), and 523 (Ile in COX-1 and Val in COX-2).

The resulting complexes were then analyzed with the program GRID for the presence of putative water molecule binding sites. A valuable starting point for this work was the presence of a constitutive water molecule present in the structure of the COX-1/Br-aspirin complex[22] that was successfully localized using GRID (see Figure 1). Calculations were performed using the specific water probe (OH2) with the GRID method.[20] Particular attention was focused in the region where the constitutive water molecule is located in the crystal structure of the aspirin complex, indicated by a red cross in Figure 1. The intention was not to carry out a solvent simulation analysis but to assess whether the presence of a water molecule in the observed crystallographic position was energetically favorable and whether equivalent hydration positions could be predicted in the COX-1

Table 1. Compounds **1**−**3**

compd	R_1	R_2
1	(*S*)-CH_3[a]	H
2	(*rac*)-CH_3[b]	OH
3	(*rac*)-CH_3[b]	OCH_3

[a] **1** corresponds to the *S* enantiomer of ketoprofen (dexketoprofen). [b] Racemic **2** and **3** were obtained for activity measurement; meanwhile, the *S* enantiomers were used for docking experiments.

and COX-2 complexes of **1**−**3**. Clearly all the complexes presented a unique minima within 2.5 Å of the position of the crystallographic water using a cuttoff of −7 kcal/mol. Moreover, COX-1 complexed with **1**−**3** presented minima within 0.5 Å of the crystallographic water position (consistently detected in a COX-1 crystal); meanwhile, the COX-2 complexes presented minima within 2.5 Å of this location. For each complex, the corresponding water molecule was positioned and oriented following the GRID suggestions, and the resulting structures were further minimized. The resulting geometry-optimized complexes of both isozymes with compounds **1**−**3** were qualitatively similar to the one shown in Figure 1 for COX-2 complexed with **1**.

To assess the stability of the complexes we proceeded to perform the molecular dynamics (MD) simulations at a constant temperature of 300 K. After the initial 10 ps heating period and temperature stabilization, MD trajectories were run for 100 ps. During the MD simulations the ligand and water molecules together with the amino acids within 12 Å of this ligand-plus-water ensemble were allowed to relax, while the residues beyond this distance were held frozen. The distances between the inhibitor and the water molecule (see Figure 2) and between the latter and the amino acids 120 (Arg) and 355 (Tyr) (see Figure 3) were monitored along the complete 110 ps MD trajectory. MD simulation conditions were selected to properly emulate the molecular system at a reasonable computer cost. Computer limitations forced us to run the MD simulations with severe constrains, including a limited trajectory length (100 ps), the consideration of a limited subset of residues, and the complete absence of the cell membrane in the simulation environment.

From the results summarized in Table 2 and the MD trajectories shown in Figures 2 and 3 it can be deduced that COX-1 clearly produces stable complexes with the inhibitors studied, **1**−**3**. In contrast, COX-2 produces a stable complex only with **1**, while the water molecule in the complexes with **2** and **3** do not remain stable along the trajectory and evaporate after 26 and 58 ps, respectively. In the COX-1 complexes with **1** and **3**, the water molecule remains stable after a shift from the original position within the initial 17 ps. Similarly, the water molecule in the COX-2 complex with **1** remains stable along the 110 ps MD trajectory, even after a temporary migration to a position 6.5 Å away from the equilibrium. In addition, similar results were obtained for the latter COX-2/**1** complex when the simulation was exceptionally run during 200 ps to ensure the stability of the complex (results not shown).

Table 2. COX Inhibition Activities and Stability Profile of the Hydrated Enzyme−Inhibitor Complexes of Compounds **1**−**3**

	IC_{50} (μM)		
compd	COX-2[a,c]	COX-1[b,c]	stability of the enzyme−H_2O−inhibitor complexes[d] along the 110 ps molecular dynamics simulations
1	0.027	0.0019	the COX-1 and COX-2[e] complexes remain stable
2	0.30	0.051	the COX-1 complex remains stable; meanwhile, the H_2O molecule evaporates from the COX-2 complex after 26 ps
3	2.8	0.042	the COX-1 complex remains stable; meanwhile, the H_2O molecule evaporates from the COX-2 complex after 58 ps

[a] PGE_2 generation by LPS-stimulated monocytes isolated from human blood.[27] [b] TxB_2 generation in the presence of 1 μM arachidonic acid by platelets isolated from human blood.[27] [c] Values are mean of at least two independent determinations. [d] Molecular dynamics (MD) was runned for 100 ps MD at 300 K after heating and temperature stabilization during 10 ps. [e] Exceptionally, MD for the COX-2/**1** complex was runned during 200 ps (after 20 ps heating and temperature stabilization) to ensure the complex stability.

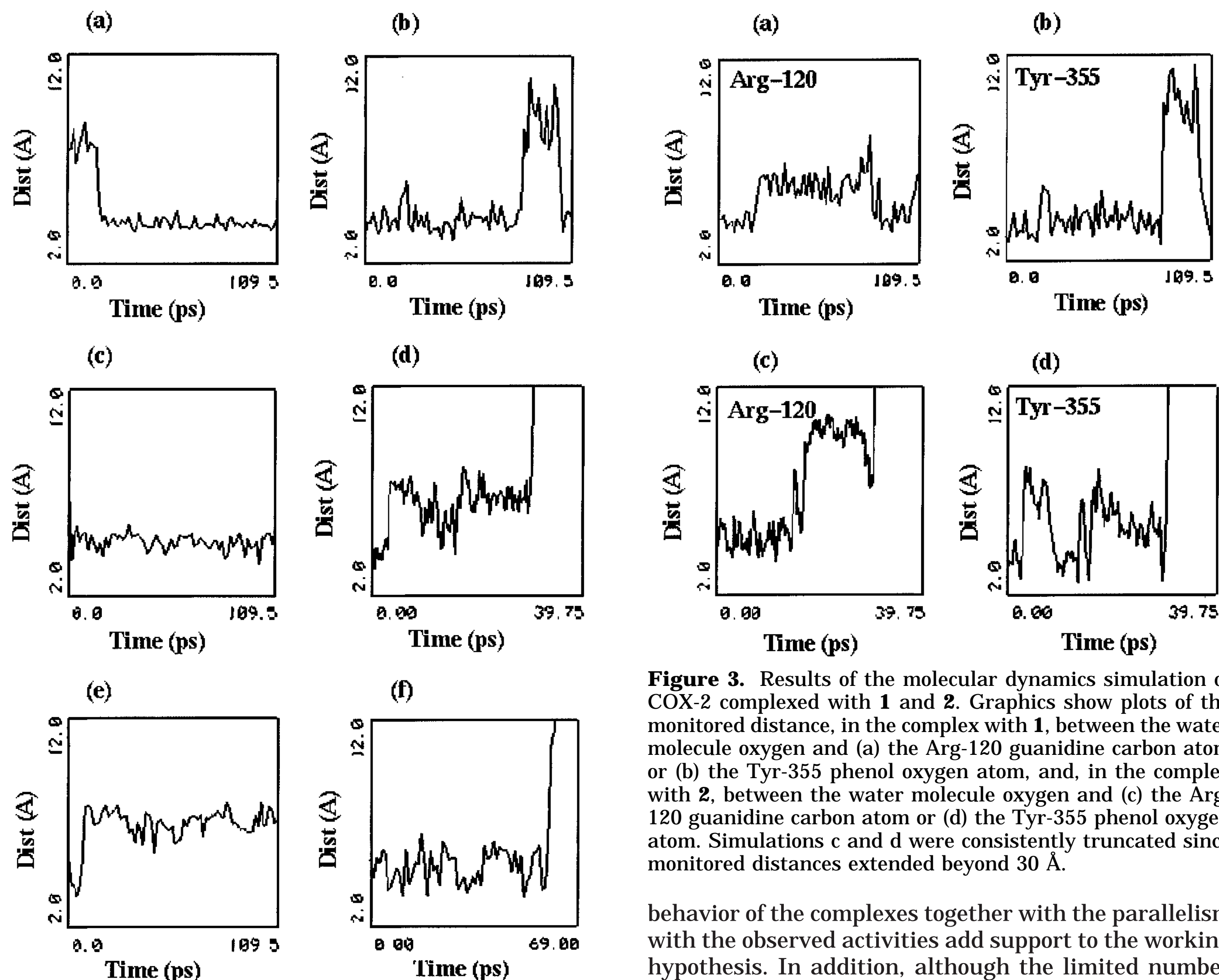

Figure 2. Results of the molecular dynamics simulation of COX-1 and COX-2 complexed with **1**−**3**. Graphics show plots of the monitored distance between the water molecule oxygen atom and the ligand carboxylate carbon atom in the complex of (a) COX-1 with **1**, (b) COX-2 with **1**, (c) COX-1 with **2**, (d) COX-2 with **2**, (e) COX-1 with **3**, and (f) COX-2 with **3**. Simulations d and f were consistently truncated since the monitored distances extended beyond 30 Å.

Figure 3. Results of the molecular dynamics simulation of COX-2 complexed with **1** and **2**. Graphics show plots of the monitored distance, in the complex with **1**, between the water molecule oxygen and (a) the Arg-120 guanidine carbon atom or (b) the Tyr-355 phenol oxygen atom, and, in the complex with **2**, between the water molecule oxygen and (c) the Arg-120 guanidine carbon atom or (d) the Tyr-355 phenol oxygen atom. Simulations c and d were consistently truncated since monitored distances extended beyond 30 Å.

Finally, the above-discussed results on the dynamic stability of the COX complexes significantly parallel the activity observed for the ketoprofen structural analogues **1**−**3** (see Table 2), therefore giving a rationale for the activity of the cyclooxygenase inhibitors **1**−**3** in terms of the dynamic stability of their hydrated enzyme−ligand complexes. Moreover, while the results deduced from the short simulations may be considered as preliminary, the observed differences in the dynamic behavior of the complexes together with the parallelism with the observed activities add support to the working hypothesis. In addition, although the limited number of compounds present in this study is insufficient to venture some mechanistic implications, the results may help us to understand the intricate kinetics observed for the inhibition of cyclooxygenases by NSAIDs.

Conclusion

In the present work we have established the importance of considering implicit water molecules near the active site entrance of the cyclooxygenases to model the dynamic nature of the enzyme−inhibitor complexes and to further rationalize the inhibition results obtained for compounds **1**−**3**, presumably interacting with this region. Molecular models have been constructed by considering such implicit water molecules that participate in the dynamic hydrogen-bonding network at the polar active site entrance together with residues 355, 524, 120, and 513. The water molecules have been positioned on the basis of crystallographic data together with the

suggestions by GRID. The inhibitory activity observed for ketoprofen (**1**) and the structural analogues **2** and **3** have been rationalized in terms of the dynamic stability of such hydrated complexes.

Experimental Section

Molecular Modeling. All molecular modeling studies were performed on a Silicon Graphics O_2 computer running MSI[25,26] and GRID software.[20] The basic modeling methodologies leading to the energy-minimized complexes and to molecular dynamic trajectories (MD) were performed using the cvff molecular mechanics force field implemented in Discover with a dielectric constant of 4*r* and a 12 Å cutoff to compute the nonbonded interactions. Energy minimization was performed using the standard steepest descent and conjugate gradients minimization algorithms implemented in the program leaving the complete structure free to relax. For MD, the ligand and water molecules together with the amino acids within 12 Å of this ligand-plus-water ensemble were allowed relaxation, meanwhile, the residues beyond this distance were held frozen. The assembly of amino acid residues allowed to relax was dynamically recalculated during the MD trajectory. After an initial 10 ps period of heating and temperature stabilization, MD was performed at 300 K with an integration step of 1 fs. Although MD simulations in the present study suffer from severe constrains (i.e. 100 ps trajectory, consideration of a limited subset of residues, and complete absence of the cell membrane) these conditions may properly emulate the molecular system at a reasonable computer cost. Exceptionally, MD for the COX-2 complexed with **1** was run during 200 ps after 20 ps heating and temperature stabilization to ensure the complex stability.

GRID Calculations. GRID was used to assess the presence of water molecules. Calculations focused on a region including the ligand binding site of COX and the position where the water molecule is located in the aspirin complex. Calculations were carried out in a standard lattice corresponding to a 4 Å extension of the ligand-plus-water ensemble (34 × 28 × 26 Å) with a 1.0 Å grid spacing and using the GRID force field with the standard water probe (OH2).[21] In the next step, the program MINIM, included in the GRID package,[20] was used to find favorable minima inside the grid box. As expected, using a cuttoff of −7 kcal/mol, the calculations showed several minima for each of the complexes but clearly all presented a unique minima within 2.5 Å of the position of the crystallographic water. In each complex, a water molecule was located in the position and the orientation resulting from the GRID analysis.

Acknowledgment. This work was financed in part by a grant from the Spanish Ministry of Education (CDTI) Nr. 98-0413. The authors thank F. López, J. Martí, and B. Ballart for the synthesis of compounds **2** and **3** and F. Cabré and A. Carabaza for the biological testing of products and for helpful discussions and assistance in reviewing the manuscript.

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JM9906217