Mechanistic insights into cyclooxygenase irreversible inactivation byaspirin

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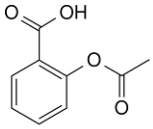
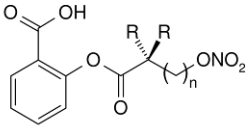
Paolo Tosco,*[a] Loretta Lazzarato[a]

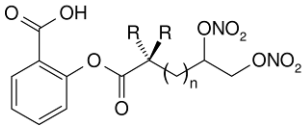
Aspirin (acetylsalicylic acid, Figure 1) belongs to the broad class of non-steroidal anti-inflammatory drugs (NSAIDs), whose therapeutic effects are largely amenable to inhibition of prostaglandin synthesis by cyclooxygenase (COX). Aspirin is unique among NSAIDs since it covalently modifies both isoforms of COX enzymes, thus inactivating them irreversibly. Its major drawback is a significant gastrotoxicity; symptoms may range from gastritis to peptic ulcer and severe gastrointestinal haemorrhage.[1,2] Our research group has recently realized a series of nitric oxide (NO)-releasing Aspirin-like molecules aimed at reducing the gastrotoxicity of the parent drug (Figure 1).[3] These hybrid compounds, which combine the anti-inflammatory and anti-aggregatory properties of the lead with NO-mediated gastroprotective effects, have proven to be irreversible inactivators of both COX isoforms: after in vitro incubation with the inhibitor, the enzymes do not recover their catalytic activity upon washing.[4] The molecular basis of COX irreversible inhibition by Aspirin relies in the ability of the latter to selectively transfer its acetyl group to Ser530. While this residue is not directly involved in the catalytic function of the enzyme, the increased bulk of the acylated side chain hinders proper binding of arachidonic acid in the cyclooxygenase channel.[5,6] Since our hybrids are nitrooxy-substituted higher homologues of Aspirin, it seems reasonable to hypothesize that they might act as COX acylating agents like the parent drug. However, since other NSAIDs (*e.g.*, indomethacin) behave as time-dependent, irreversible inhibitors of COX without forming any covalent bond within the active site,[7] we decided to undertake a theoretical investigation to clarify whether our compounds are actually covalent inactivators or tight-binding inhibitors. While site-directed mutagenesis experiments and the x-ray structure of COX-2 inactivated by bromoacetylsalicylic acid[6] leave no doubt about how Aspirin inhibits COX, they fail to elucidate the mechanism of acyl transfer at a molecular level. Therefore, before studying our hybrids, we needed a proof of concept on Aspirin itself. Herein, we present our preliminary results and propose a putative mechanism of Ser530 transesterification by acetylsalicylic acid.



Aspirin



R = H n = 1-5
R = CH_3 n = 1



R = H n = 0-3
R = CH_3 n = 2

Despite Aspirin's low affinity for COX (K_i = 20 mM), acetylation of Ser530 takes place very efficiently once Aspirin has bound in the active site, as indicated by the high values of k_{inact}.[8] Both the selectivity and the efficiency of Aspirin are quite surprising, since no neighbouring groups are present in vicinity of Ser530 to enhance its nucleophilicity, such as histidine and glutamate in serine proteases.[9] Site-directed mutagenesis experiments have underlined the importance of Arg120 and Tyr385 for Aspirin's activity. Based on its reduced potency on Arg120Gln mutants and its loss of activity on Arg120Ala mutants, it has been suggested that acetylsalicylate initially forms a charge-enhanced hydrogen bond with Arg120 through its carboxylate moiety, which puts the molecule in the correct orientation to subsequently acetylate Ser530.[6,10] The abolishment of activity in Tyr385Phe mutants has suggested a crucial role of the hydroxyl group of Tyr385 as a hydrogen bond donor in orienting and polarizing the acetyl group of Aspirin, thus increasing its reactivity towards transesterification by Ser530. Tyr385 might then be able to stabilize the incipient negative charge of a putative tetrahedral intermediate, mimicking the oxyanion hole of serine proteases.[10] This hypothesis is complemented by the finding that redox cycling of the peroxidase, which involves formation of a radical on Tyr385, and consequent loss of hydrogen bond-donating capabilities, inhibits COX acetylation by Aspirin.[11] The salicylate structure itself appears to selectively target Ser530; in fact, unlike other acylating agents such as *N*-acetylimidazole, no other residues are acetylated.[12] The stability of the acetylated serine to hydrolysis has been ascribed to the low probability of interaction with bulk water due to the hydrophobic nature of the COX channel.[6] The carboxylate-binding region represented by Arg120 and the target residue Ser530 are often described as very close in space, and ideally located to promote inactivation of the enzyme by Aspirin.[8,9] Actually, as Loll and co-workers pointed out,[6] acetylsalicylic acid, after its likely initial ion-pairing to Arg120, needs to diffuse 5 Å upwards in the COX channel to reach a position from which Ser530 acetylation can take place (Figure 1).

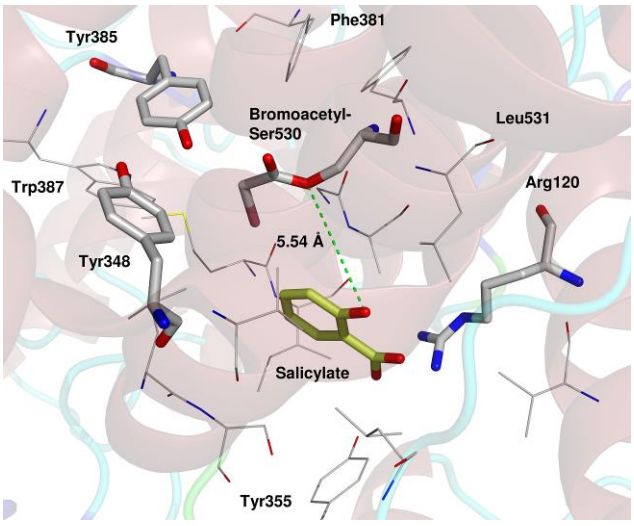


Figure 1. The crystal structure of COX-2 active site after inactivation by bromoacetylsalicylic acid as obtained by Loll and co-workers.[6] All hydrogens were missing in the original coordinate file. The distance between the phenolic group of salicylate and the oxygen atom belonging to Ser530 is represented as a green dotted line.

Unfortunately, the precise sequence of events can only be inferred, since the x-ray structure shows the inhibitor-enzyme complex after acylation has already occurred. With this observation in our mind, we first attempted to dock Aspirin in the active site of both COX-1 and COX-2 isozymes, to see how intact acetylsalicylate would bind. Among the structures available in the Protein Data Bank,[13] the best resolved ones were chosen, namely 1Q4G for COX-1[14] and 1PXX for COX-2.[15] The co-crystallized ligands were removed and flexible docking of acetylsalicylate was accomplished with AutoDock 4.0,[16] keeping the protein structure rigid. On both isozymes a single cluster of binding poses, almost identical to each other, was obtained, largely reminiscent of the one experimentally found for salicylate by Loll and co-workers. While this outcome was largely expected on the basis of mutagenesis data pinpointing the key role of Arg120, in these poses the acetyl carbon lies 5 Å away from the Ser530 OH group, and therefore acylation cannot be expected to occur. In order to find a reasonable starting conformation for a quantum-mechanical/ molecular-mechanical (QM/MM) study, we needed to simulate the outcome of the upward diffusion following the initial binding as postulated by Loll and co-workers.[6] For this purpose, a biased conformational sampling was accomplished by molecular dynamics (MD) in CHARMM.[17] The two isozymes were solvated in a octahedral box of explicit water under periodic boundary conditions (PBC), then 10 complexes were generated with Aspirin assuming random orientations in the active site of each isoform, setting as a constraint that the distance between the acetyl carbon and the Ser530 OH group should be below 3.5 Å. After preliminary minimization and equilibration at 300 K, a simulated annealing (SA) procedure by MD was carried out using a dual heat-bath thermostat, which allowed heating the ligand up to 1000 K and then slowly cooling it to 300 K, while the enzyme and bulk water were kept constantly at 300 K. This procedure allowed thorough sampling of Aspirin conformations inside the cavity, while preventing any distortion of the protein structure; additionally, harmonic constraints of appropriate weight were set on backbone and side-chain atoms. The whole SA cycle was repeated 5 times, removing the 3.5 Å constraint on the acetyl carbon-Ser530 OH distance after the first run. This precaution was taken since our goal was to set an initial bias in order to sample only relevant conformations, avoiding to waste CPU time on enzyme-inhibitor complexes which would never be able to give rise to Ser530 acylation. However, we wanted to be sure not to force the protocol to find unreasonable, high-energy conformations just because of the presence of a tight constraint throughout all SA cycles. Performing 4 unconstrained SA runs warrants that fake minima due to the existence of an artificial biasing potential cannot be found. The 8 initial starting conformations converged onto only 3 different final enzyme-inhibitor complexes, labelled *a*, *b* and *c*, very similar on either isoform; in Figure 2 those obtained on COX-1 are shown. While complexes *b* and *c* appear compatible with the hypothesis of an initial ion-pairing with Arg120 through the carboxylate group, followed by upward diffusion towards Ser530, complex *a* differs quite markedly from the x-ray structure pictured in Figure 1, since the carboxylate group is on the opposite side, facing Ile523 instead of Arg120. Once obtained the initial inhibitor-enzyme complexes, we switched from a pure MM potential to a hybrid QM/MM Hamiltonian, using the semiempirical SCC-DFTB level of theory as implemented in CHARMM[18] for the QM calculations. Since our interest was focussed on the region were acylation should take place, all molecules which potentially might be involved in the acylation mechanism, namely Aspirin, the residues Tyr348, Tyr385, Ser530, and nearby water molecules, were treated by QM, while the rest of the protein, as well as bulk water, were treated by traditional molecular mechanics. The resulting Hamiltonian can be expressed as

$$\boldsymbol{H} = \boldsymbol{H}_{QM} + \boldsymbol{H}_{MM} + \boldsymbol{H}_{QM/MM}$$

were $\boldsymbol{H}_{QM}$ is the Hamiltonian describing atoms treated by QM, $\boldsymbol{H}_{MM}$ the Hamiltonian of atoms treated by classic MM, and $\boldsymbol{H}_{QM/MM}$ represents the interactions between the MM and the QM regions.[19] To further reduce the computational burden, we decided to switch from PBC to stochastic boundary conditions (SBC), cutting a 45-Å-radius water droplet containing the whole enzyme-inhibitor complex out of the periodic system. A 16-Å-radius sphere centred on the acetyl carbon of Aspirin was defined as the region of interest, and left completely unconstrained. Instead, to get rid of possible distortions taking place at the periphery of the system under SBC, harmonic constraints derived from experimental B-factors[20] were applied on the residues lying in a 4-Å-wide boundary region around the region of interest, while all atoms beyond this region were held fixed. The QM/MM approach, which takes into account the electronic structure of molecules enclosed in the QM region, allows for bonds to be formed and broken in this region if the appropriate conditions are met, making it possible to study the feasibility of acetylation of Ser530 by Aspirin under the conditions of simulation. As soon as a preliminary minimization of complex *a* was carried out on both COX-1 and COX-2 using the hybrid QM/MM potential, migration of the acetyl moiety from the phenol to the carboxyl group was observed, giving rise to the corresponding anhydride (complex ***a'***, Figure 3). We were quite surprised by this outcome: while the formation of an anhydride might be a reasonable explanation for the unusual reactivity of Aspirin in the COX active site, if actually Aspirin behaved as a mechanism-based inhibitor, this should result in peculiar inhibition kinetics,[21] which have never been reported. In the literature, there are some early reports about the “acid anhydride” character of Aspirin,[22] confirmed by other authors who claimed the anhydride intermediate to be involved in the mechanism of hydrolysis of Aspirin to acetic acid and salicylate.[23] However, later independent works by Fersht,[24] Jencks[25] and Kemp[26] demonstrated through elegant experiments that, while Aspirin can actually exist under the anhydride form, the latter is not involved in the mechanism of solvolysis (either by water or alcohols), which instead proceeds under intramolecular general base catalysis provided by the vicinal carboxylate moiety (Scheme 1). The reason why the anhydride intermediate does not play a role in solvolysis of Aspirin was found to rely in the greater nucleophilicity of the phenoxide anion with respect to solvent, which favours the intramolecular reaction reforming Aspirin.[24]

Figure 2. The three complexes *a*, *b*, *c* between Aspirin and COX-1 obtained by simulated annealing. Non-polar hydrogens have been omitted for clarity.

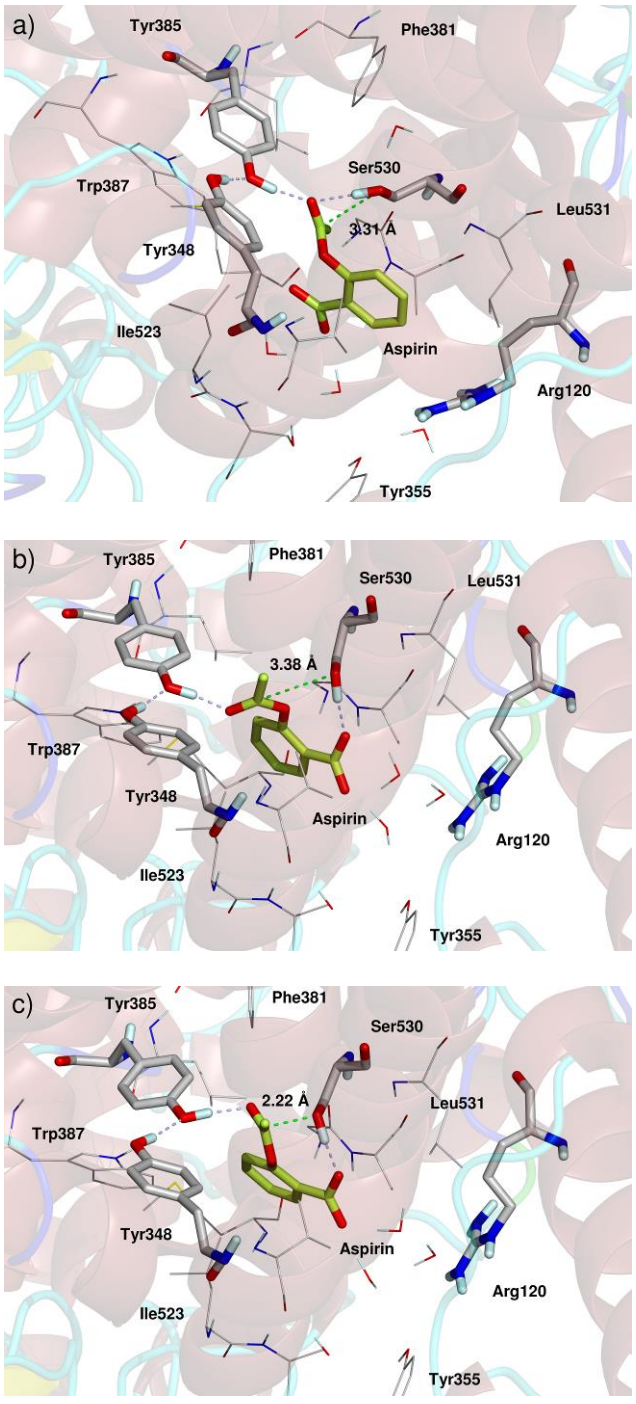
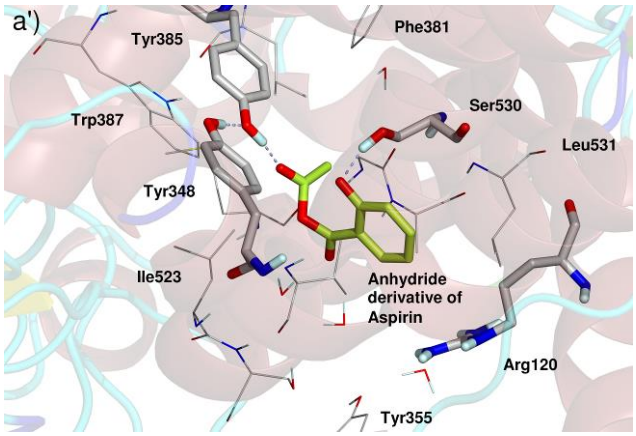
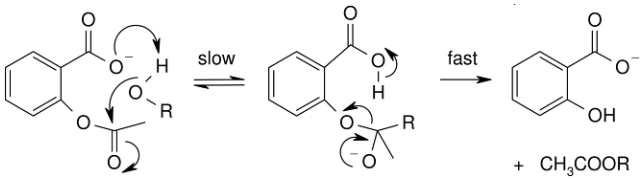


Figure 3. Complex *a'*, originated by acetyl migration following a simple energy minimization using the hybrid QM/MM potential.



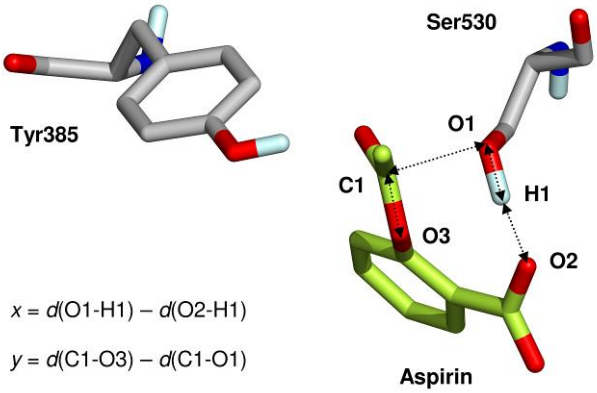
Scheme 1. Solvolysis of Aspirin under general base catalysis conditions (R = H, alkyl).



Indeed, taking a closer look at complex *a'* (Figure 3), it is evident that the phenoxide anion is in a much more favourable position than the hydroxyl group of Ser530 to react with the acetyl carbon to reform Aspirin. To verify that this is actually the case, 1-ns QM/MM MD simulations on complex *a'* were accomplished at constant *V*,*T*. No acetylation occurred on either isozyme; rather, the acetyl group was observed to migrate smoothly from the phenol to the carboxyl group and back, consistently with the experimental findings by the previously cited authors. Complex *a* has therefore to be regarded as a pose from which Aspirin is not able to trigger acetylation of Ser530. Moreover, as already noticed, the collocation of the carboxyl group on the opposite side with respect to Arg120 probably makes this binding mode rather unlikely. Complexes *b* and *c*, instead, appear to have the correct geometry to undergo the general base catalysis mechanism outlined in Scheme 1, since the hydroxyl proton of Ser530 faces the carboxylate moiety of Aspirin, while the oxygen atom is not far away from the acetyl carbon. For both complexes 1-ns QM/MM MD simulations were carried out. We were pleased to observe that after a few picoseconds these two starting conformations were able to trigger acetylation of Ser530 both on COX-1 and on COX-2. In particular, complex *b* first evolves to complex *c*, which appears to be a very effective pro-acylating conformation; the first 250 ps of the MD trajectories of complex *b* on both isozymes are available in Supporting information. Careful examination of MD trajectories shows that acetylation needs the formation of a stable hydrogen bond between the hydroxyl group of Tyr385 (donor) and the oxygen atom of the acetyl moiety on Aspirin (acceptor). Once this hydrogen bond has been established, as soon as the Ser530 O-H bond and the C^{ar}-O(CO) bonds become parallel, a proton migrates from the Ser530 OH group to Aspirin's carboxylate, and immediately afterwards the acetyl moiety migrates from Aspirin to Ser530, passing through a tetrahedral intermediate. Very interestingly, after the transesterification has occurred it appears that the salicylate leaving group has no tendency to accomplish the reverse reaction on the acetylated Ser530, which is consistent with the experimental observation that COX inhibition is irreversible. The role of Aspirin's carboxylate in providing intramolecular general base catalysis also gives a sound explanation to the stability of acetylated Ser530 to hydrolysis. Even if probably a much longer MD simulation would be necessary to observe the salicylate leaving group moving away from the reaction site, it is evident from the experimental x-ray structure[6] (Figure 1) that after Aspirin has acetylated Ser530, salicylate undergoes electrostatic attraction by Arg120. Therefore, once the only basic moiety has left the reaction site, in the absence of a specialized proton relay system such as in esterases, water *per se* is not nucleophilic enough to regenerate the active enzyme. Apart from the short simulation time, there is another reason why the 5-Å backward diffusion towards Arg120 is not observed in the trajectory. In fact, after the acetyl group has been transferred from Aspirin's phenol group to COX, the proton abstracted from Ser530's side chain would be expected to migrate from the carboxylate group to the phenoxide anion, on the basis of the higher basicity of the latter. However, this transfer does not take place in the time span covered by the simulation. While this might well be considered as an artefact due to the semiempirical SCC-DFTB QM method, it is worth mentioning that the energy difference between the two tautomers has been demonstrated to be quite small, both experimentally and by ab initio QM.[27] Actually, after carrying out a QM minimization on the two isolated salicylate tautomers with a DFT RB3LYP/6-31G+(d) method, the phenoxide tautomer resulted 0.76 kcal mol^{-1} more stable than the carboxylate one. When the same minimization was accomplished with the SCC-DFTB method, the carboxylate tautomer underwent spontaneous conversion to the phenoxide tautomer, probably due to underestimation of the interconversion energy barrier. The fact that during our MD simulations the negative charge remains on the phenoxide anion probably

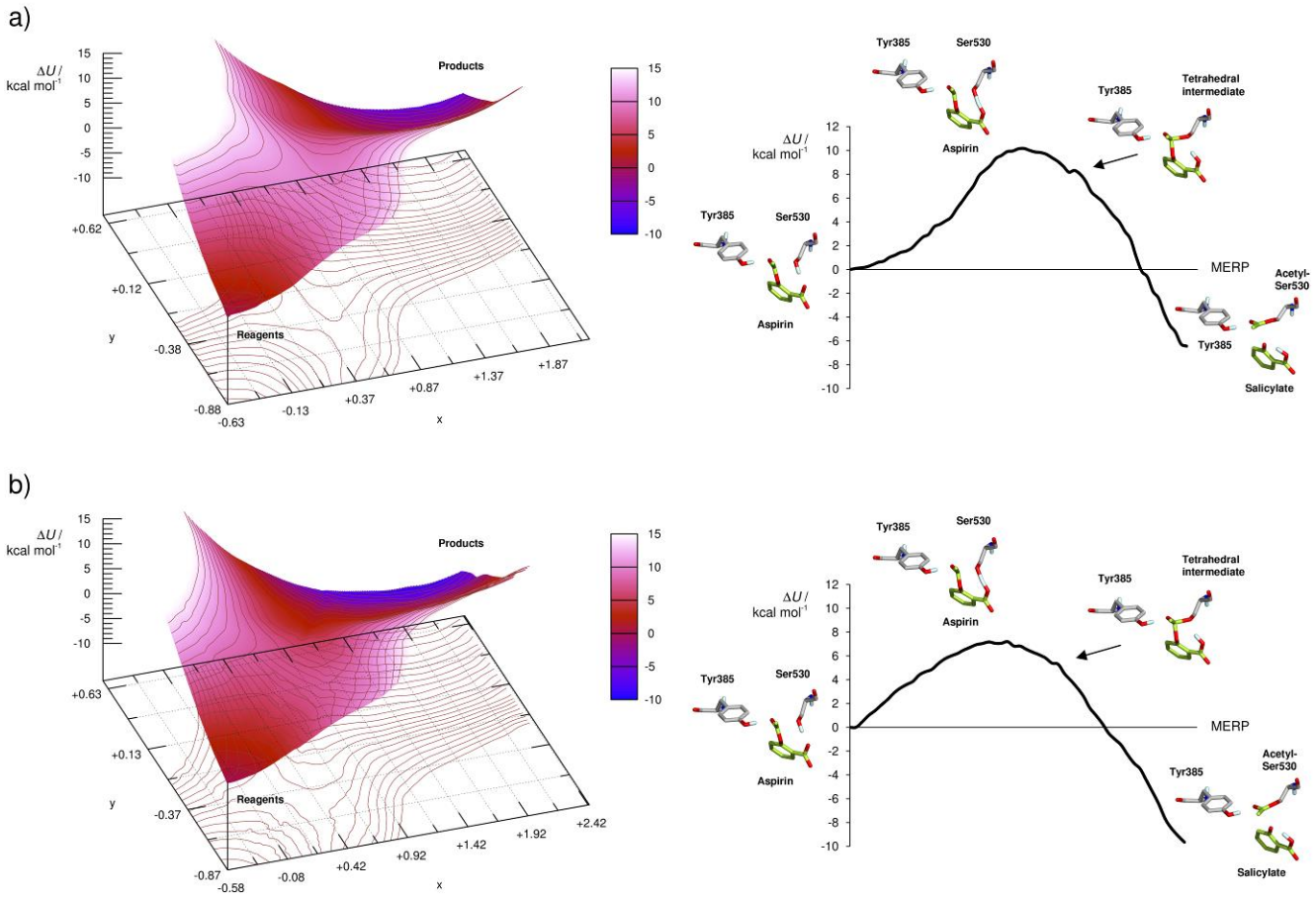
prevents salicylate from ion-pairing with Arg120, since a charge-enhanced hydrogen bond between the phenoxide anion and Tyr385 OH group prevails. Nonetheless, even if salicylate does not move very far away from the reaction site, acetyl-Ser530 appears absolutely stable. Using the MD trajectories as a guide, an adiabatic potential energy surface (PES) describing the transesterification reaction was built. Starting from the 3D coordinates of complex *c*, two reaction coordinates *x* and *y* were defined as linear combinations of distances between atoms involved in breaking and formation of covalent bonds (Figure 4).

Figure 4. Definition of reaction coordinates *x* and *y*.



A series of sequential QM/MM energy minimizations were carried out, one for each pair of *x*,*y* values, using the same conditions as for MD (SBC, harmonically-constrained boundary region, fixed outer region). The region of interest was left completely free to relax, except for the two reaction coordinates which were restrained with a high force constant (1000 kcal mol^{-1} $Å^{-2}$), in order to drive the reaction from the initial to the final state along the path outlined by MD. The minimized geometries of the residues making up the QM region were isolated, and two single-point energy calculations were carried out, one with the semi-empirical SCC-DFTB method, the other with a DFT method at the RB3LYP/6-31G(d) level. Diffuse sp functions were not included in the basis set since, when applied on a small test region of the PES, they proved not to affect significantly calculated energies, while inducing a 16-fold increase of CPU time. The DFT correction to the total QM/MM energy was accomplished as previously described,[28] subtracting the SCC-DFTB contribution for the QM region and adding the RB3LYP energy. The obtained RB3LYP/6-31G(d)//SCC-DFTB-CHARMM22 PESs for acetylation of COX-1 and COX-2 are shown in Figure 5a, 5b respectively, together with the plots of the potential energy and the structure of the relevant intermediates along the minimum energy reaction path (MERP). A word of caution is needed when considering these data: firstly, they represent simple potential energies, and not free energies; secondly, they have been obtained by sampling single snapshots along the MERP rather than an ensemble of conformations. For these reasons, it is probably safer to assume that they provide only a rough, qualitative estimate of the energetic profile of the transesterification process in the COX active site. Analysis of the PESs obtained on the two isozymes explains why the acetylation of Ser530 takes place so smoothly during MD. In fact, the activation barrier of the transesterification is quite low, while the products are definitely more stable than the reagents, which justifies the fact that the backward reaction does not take place. Interestingly, the first, well-shaped saddle point corresponds to the structure of a complex where the tetrahedral intermediate has not formed yet, while a proton has migrated half-way between Ser530's OH and Aspirin's carboxylate. The involvement of such an intermediate in intramolecular general-base-catalyzed solvolysis of Aspirin has been previously proposed by other investigators on the basis of experimental evidence.[29] The unusual stability of this transition state has been attributed to the formation of a particularly strong intramolecular hydrogen bond, termed "one-proton solvation bridge".[29] The fact that our QM/MM simulation is able to reproduce this low-energy transition state gives robustness to our results. A second, more ill-defined saddle point is present on the way to the final products, corresponding to the tetrahedral intermediate, which forms after the proton transfer from Ser530 to Aspirin has already completed. The structure of this tetrahedral intermediate is consistent with the role played by Tyr385 in stabilizing the negative charge as proposed by Hochgesang and co-workers.[10] While the SCC-DFTB method appears to underestimate the energy of the transition states by about 5-7 kcal mol^{-1}, the overall shape of the PES is quite similar to the one obtained applying the DFT correction; the pure SCC-DFTB-CHARMM22 PESs and MERPs are reported in the Supporting information. To further challenge our mechanistic hypothesis, we performed a Tyr385Phe *in silico* mutation on both COX isoforms, in order to verify the actual importance of this residue in the transesterification mechanism. It is immediately evident from the examination of the QM/MM MD trajectories (see Supporting information) that in the absence of Tyr385's OH group Aspirin is no more univocally oriented as in the wild-type enzymes, but it wanders in the active site without being able to assume a pro-acylating conformation. It is worthwhile to notice that migration of the acetyl from the phenol to the carboxylate group occurs; however, as already mentioned, the anhydride form is unable to trigger acylation of Ser530.

Figure 5. RB3LYP/6-31G(d)//SCC-DFTB-CHARMM22 Potential energy surfaces for the transesterification of Ser530 by Aspirin according to the proposed mechanism on COX-1 (*a*) and COX-2 (*b*), respectively. The potential energy of the system with respect to the initial state (ΔU) along the minimum energy reaction path (MERP) is also reported, together with the 3D structures of relevant intermediates.



In conclusion, our theoretical study has led to a reasonable mechanistic hypothesis for acetylation of cyclooxygenase by Aspirin, which is consistent with previous experimental findings by other investigators. In particular, Ser530 appears to be acetylated under intramolecular general base catalysis provided by Aspirin's vicinal carboxylate group; it is widely accepted that solvolysis of acetylsalicylate both by water and alcohols occurs by the same mechanism.[24-26] Moreover, a transition state with the same geometry proposed by Minor and Schowen[29] was found, and the key role of Tyr385 individuated by site-directed mutagenesis[10] has been confirmed. Future challenges include: a more precise determination of the reaction free energy profile by extensive conformational sampling by QM/MM MD, possibly using a higher level of theory for the QM part; an estimate of the energetic cost of the 5-Å upward diffusion after the initial ion-pairing to Arg120 to assume the pro-acylating starting conformation found by simulated annealing; the assessment of the adaptability of the protocol herein described to other irreversible COX inhibitors.

Computational Methods

All molecular models were built using standard bond lengths and angles with the MOE software package.[30] MM and QM/MM computatations were performed with CHARMM (version c33b1)[17] using the CHARMM22 force field. Parameters for Aspirin necessary for pure MM calculations were derived from the GAFF force field[31] using the CHARMMGEN facility included in the AMBER 10 suite.[32] Electrostatic charges were fitted through the RESP approach[33] to the ab initio HF/6-31G(d) electrostatic potential computed with GAMESS-US.[34] 3D Coordinates for COX-1 and COX-2 were retrieved from the Protein Data Bank[13] (PDB codes 1Q4G and 1PXX, respectively); hydrogen atoms were added in standard positions and then minimized in CHARMM. All geometry optimizations were carried out using a termination criterion based on gradient (< 0.01 kcal mol^{-1}). Docking of Aspirin in the active site was accomplished with AutoDock 4.0[16] using the Lamarckian genetic algorithm (default parameters, 200 runs per isoform). Random enzyme-Aspirin complexes were generated with an in-house SVL script running in MOE, imposing that the distance between the acetyl carbon and the Ser530 OH group should be below 3.5 Å. The complexes were solvated in a octahedral periodic box under PBC, and a preliminary minimization with harmonic constraints on backbone (10 kcal mol^{-1} Å^{-2}) and side chain atoms (2.5 kcal mol^{-1} Å^{-2}) was carried out, while leaving Aspirin and hydrogen atoms unconstrained. Equilibration of water was then accomplished, first with self-guided Langevin dynamics, then at constant *p*,*T* (300 K) in order to achieve the correct density in the solvated system, finally at constant *V*,*T* using a Nosè-Hoover thermostat as implemented in CHARMM. A SA protocol was designed were the ligand was heated to 1000 K and then slowly cooled to 300 K in 100-K steps, allowing 5-ps sampling in each temperature window, while protein and bulk water were constantly kept at

300 K. At the end of each SA run an energy minimization was carried out, and the resulting geometry was used for the following SA cycle. QM/MM calculations were accomplished under SBC at the SCC-DFTB level of theory as implemented in CHARMM;[18] link atoms were used to fill empty valences at the boundary between the MM and the QM regions. 1-ns QM/MM MD were accomplished at constant *V*,*T* (300 K) with a Nosè-Hoover thermostat. The PESs were computed setting sequential constraints on two reaction coordinates while allowing the rest of the system free to relax. The DFT corrections to the potential energy were calculated using PC-GAMESS.[35]

Acknowledgements

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Keywords: Aspirin · cyclooxygenase · acylation · enzyme catalysis · QM/MM

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