Computational molecular biology approaches to ligand-target interactions

Paola Lupieri,¹ Chuong Ha Hung Nguyen,¹ Zhaleh Ghaemi Bafghi,¹ Alejandro Giorgetti,² and Paolo Carloni^{1,3}

¹SISSA, International School for Advanced Studies, via Beirut 2-4, I-34014 Trieste, Italy

²University of Verona, Department of Biotechnology, Ca' Vignal 1 Strada Le Grazie 15, I-37134 Verona, Italy

³DEMOCRITOS Modeling Center for Research in Atomistic Simulation and Italian Institute of Technology—SISSA Unit, via Beirut 2-4, I-34014 Trieste, Italy

(Received 5 December 2008; accepted 11 February 2009; published online 10 March 2009)

Binding of small molecules to their targets triggers complex pathways. Computational approaches are keys for predictions of the molecular events involved in such cascades. Here we review current efforts at characterizing the molecular determinants in the largest membrane-bound receptor family, the G-protein-coupled receptors (GPCRs). We focus on odorant receptors, which constitute more than half GPCRs. The work presented in this review uncovers structural and energetic aspects of components of the cellular cascade. Finally, a computational approach in the context of radioactive boron-based antitumoral therapies is briefly described. [DOI: 10.2976/1.3092784]

CORRESPONDENCE

Paolo Carloni: carloni@sisa.it

INTRODUCTION

Small molecules, such as metabolites and second messengers, control a myriad of cellular functions by binding to their target macromolecules. By encountering their cellular partners, ligands govern processes such as growth, programmed cell death, sensing, and metabolism. This key event triggers complex cellular pathways characterized by reactions, environmental changes, intermolecular interactions, and allosteric modifications.

All of these processes involve molecular recognition; i.e., the process by which two or more molecules interact to form a specific complex. Although far from being understood (Whitesides *et al.*, 2008), the process is surely dominated by short-range, often transient, interactions at the contact surface of the molecules. Even conformational changes and assemblies of very large macromolecular aggregates, which can be propagated through long distances (tens of angstroms), are the effect of local interactions between small mol-

ecules (like messengers) or macromolecules with their cellular targets.

Ultimately, therefore, understanding the molecular basis of ligand-target interactions requires the integration of biological complexes into cellular pathways (the so-called “systems biology”). Nonmolecular modeling, which is advancing tremendously our understanding (see, e.g., Segre *et al.*, 2002; Alon, 2006), needs to be paralleled by a quantitative molecular description of pathways, so far mostly lacking. This will impact strongly on pharmaceutical sciences and toxicology, as drugs target (and mutations affect) pathways, rather than a single biomolecule. It is also crucial in nanobiotechnology, e.g., to design artificial sensing devices, which in Nature involve cascade of events and not a single protein.

Computational molecular biology (CMB) or structural biology (Schwede and Peitsch, 2008) approaches are keys to face these challenges. Very broadly speaking, there are two major strategies for computational molecular biology.

The first strategy, the so-called protein bioinformatics, is aimed at the development of computational tools that enable to decipher the information encoded in the protein sequences, thus enabling the prediction of structure and function. Among the many ways to obtain three-dimensional (3D) structural models, here we mention the comparative modeling approach (Chothia and Lesk, 1986), based on the fact that proteins diverging from a common ancestor have similar 3D structures. Therefore, the structure of a protein of interest (target), can be modeled based on the three-dimensional coordinates extracted from an evolutionary correlated protein of known structure (template) (see, e.g., Chothia and Lesk, 1986; Tramontano, 2006).

The second strategy is based on the laws of physics. One of the most important methods here is molecular dynamics (MD) (Frenkel and Smit, 2002; Berendsen, 2007), which predicts structural, dynamical, and energetic (bio)molecular properties based on Newton laws of motion. For a system of N atoms with mass m_i and position $\vec{r}_i$, the force acting on each atom is given by:

$$\vec{F}_i = - \frac{dE(\vec{r}_1, \dots, \vec{r}_N)}{d\vec{r}_i} = m_i \ddot{\vec{r}}_i \quad (i = 1, \dots, N). \quad (1)$$

A key ingredient of the method is of course the force field E , which is usually constructed so as to reproduce experimental quantities and/or quantum chemical calculations (Leach, 2001; Schwede and Peitsch, 2008). The quality of the force fields is continuously improving, in parallel with the availability of more powerful computational resources.

This allows one nowadays to produce stable trajectories covering a time scale of tens of nanoseconds. Several schemes have been developed to control the temperature and the pressure of the simulation, a feature that allows one to reproduce the physiological conditions under which processes take place or experiments are performed (Berendsen *et al.*, 1984; Schneider and Stoll, 1978; Quigley and Probert, 2004). In this way, MD enables the exploration of the conformational energy landscape accessible to biomolecules. Thus, it provides a connection between the structure and the dynamics of biomolecules.

Combining different CMB approaches such as those outlined here is very powerful, as the data are often complementary one to the other (Schwede and Peitsch, 2008).

To illustrate how CMB approaches work in practice for a cellular pathway, we review here an example of current effort at characterizing the olfactory signaling pathway at the molecular level, through the analysis at different computational levels of each of the participating proteins. We close the review by presenting a ligand-target interaction study for anti-tumoral research. For this project, which we have just started, we provide a perspective from the approach outlined here. The connecting element between this and the previous topic is the characterization of signaling pathways triggered by ligand-target interactions. In addition, a discussion of the last

topic gives the opportunity to present the use of other fundamental CMB approaches for ligand-target interactions.

The first is molecular docking, which is used to predict poses of ligands onto their targets (Kitchen *et al.*, 2004). Such poses are obtained by minimizing a scoring function (Eldridge *et al.*, 1997). This function evaluates, in a simplified and computationally efficient way, the interaction between the ligand and the target, as well as the entropic contributions associated with the formation of the ligand-target complex. Molecular docking is routinely used in drug screening from large databases (Moitessier *et al.*, 2008) as well as in the design of new ligands (Kitchen *et al.*, 2004).

The second is the so-called quantum mechanics/molecular mechanics (QM/MM) method, which is of increasing importance in drug design. In this approach, invented by Warshel and Levitt (1976), one small part of the system (such as an enzymatic active site or a receptor binding site) is treated at the quantum-mechanical level (either with semi-empirical method or by first principles), while the rest (i.e., the protein frame and the solvent) is described with the force field E of Eq. (1). In the QM/MM approach, one can treat transition metal ion-containing (bio)molecules, which are very difficult to describe by using effective potentials. This is achieved by including the metal site in the QM part. Examples include Pt-based drugs, which target DNA (Magistrato *et al.*, 2006). The distortion imposed on DNA by these drugs is dictated by electronic effects, which are incorporated in the QM/MM approach (Dal Peraro *et al.*, 2007). In addition, the QM/MM method can be used to investigate the action of alkylating agents, as covalent bonding is not included in scoring functions. Examples include the formation of a covalent bond between duocarmycins, a potent class of anticancer agents, and the adenine nucleobases of DNA (Spiegel *et al.*, 2006). Instead, with the QM/MM approach, bond-forming and bond-breaking phenomena can be described (Rothlisberger and Carloni, 2007; Senn and Thiel, 2008). Next, it can be used to predict spectroscopic properties (such as NMR; Piana *et al.*, 2001) and vibrational properties (Miani *et al.*, 2007) of drugs bound to their targets. Finally, it can be used to study reactions of enzyme targets (Dal Peraro *et al.*, 2007).

OLFACTIVE SIGNALING PATHWAY

A molecular view on odorant receptor signaling

G-protein-coupled receptors (GPCRs) are the largest membrane-bound receptor family expressed by mammals (encompassing more than 1% of the genome). They are involved in an enormous variety of intra- and extracellular signaling, including detection of light, sense of smell, neurotransmission, inflammation, and cardiac and smooth muscle contractility (Kroeze *et al.*, 2003; Sakmar, 2002). Ligand (or photon) binding to GPCRs activates a cascade of events, producing an electrical signal as output. They are of

utmost pharmaceutical relevance, being the targets of almost 30% of all marketed drugs (Landry and Gies, 2008).

All of the members of the GPCR superfamily share a similar seven transmembrane (TM) helices domain connected by alternating extracellular and intracellular loops, an extracellular N-terminus, and an intracellular C-terminus (Strader *et al.*, 1994). They may be assembled as dimers or form oligomeric complexes within the membrane (Filizola *et al.*, 2005; Pin *et al.*, 2007). Despite sharing a common structure, they show considerable diverse primary structures. Based upon evolutionary studies, GPCRs can be divided into six main classes, the so-called A–F classification system (Kolakowski, 1994). In particular, the Class A or the rhodopsin receptor family is the largest and most studied.

More than half GPCRs (about 900) are olfactory receptors (ORs) (Buck and Axel, 1991; Takeda *et al.*, 2002). This clearly underlies the crucial role of the sense of smell during evolution. ORs possess high affinity for thousands of volatile molecules associated with odor. With such a large number of different ORs, the olfactory system is capable to discriminate between $\sim 10\,000$ different odors: one odorant can activate numerous types of ORs, while a single OR can be activated by several different odorants (Malnic *et al.*, 1999).

ORs belong to the Class A of GPCRs and in turn can be divided into two main classes. Class 1 ORs, the so-called fish-like ORs, share a rather high sequence identity (SI). Class 2 ORs, the mammalian-like ORs, comprise all the others. Class 1 ORs bind structurally similar ligands such as,

among others, aliphatic acids, alcohols, and aldehydes. Class 2 ORs instead bind structurally divergent odorants (Khafizov *et al.*, 2007).

The initial part of the ORs cascade is the same across the GPCR family (Fig. 1). In the cilia of olfactory sensory neurons, odorant molecules binding to ORs cause conformation changes, with the consequence of activating its cognate G-protein. The activation of the G-protein is associated with a decrease of affinity for GDP and an increase of affinity for GTP. This, in turn, stimulates the adenylate cyclase (AC) type III enzyme (Bakalyar and Reed, 1990), which converts ATP into cyclic AMP (cAMP). cAMP binds to and opens the cytoplasmic domains of the olfactory cyclic nucleotide gated (CNG) ion channels. This allows Na^+ and Ca^{2+} cations to flow along their electrochemical gradients from the extracellular to the intracellular side of the membrane (Kaupp and Seifert, 2002; Pifferi *et al.*, 2006a). The increased Ca^{2+} concentration in the cilia causes the opening of Ca^{2+} -activated Cl^- channels and the subsequent Cl^- efflux, which further depolarizes the cell (Kleene, 1993; Lowe and Gold, 1993; Frings *et al.*, 2000). Thus, the chemical interactions of ORs with volatile molecules lead ultimately to the production of action potentials that will carry information about the external world to the brain (Menini, 1999; Firestein, 2001). The axons of the olfactory sensory neurons from the nasal cavity send information to second-order neurons in the olfactory bulb, which in turn project to the olfactory cortex and then to other brain areas. The increase of Ca^{2+} concentration has an inhibitory effect, which eventually terminates the signal, as

Figure 1. Schematic view of the odorant receptor cascade. (Adapted from Pifferi *et al.*, 2006a). Reprinted with permission from the Federation of The European Biochemical Societies.

evidently required for the function of this apparatus. This is achieved by Ca^{2+} binding to calmodulin (CaM), which also stimulates the activity of a phosphodiesterase (PDE). Ca^{2+} is then extruded by a $\text{Na}^+/\text{Ca}^{2+}$ exchanger. The lack of experimental structural information for most components of the pathway calls upon CMB modeling at different levels, possibly with inclusion of experiments performed at the same time. The homology modeling techniques that can be used to supply for the lack of the experimental information can in general be decomposed into four principal steps:

1. Template search. In this part of the work, programs such as BLAST (Altschul *et al.*, 1990) or FASTA (Lipman and Pearson, 1985) are used for the search of evolutionary correlated proteins with known structure.
2. Sequence alignment of the target and templates. This step is the most critical one for the modeling. Most of the time, the use of pairwise sequence alignments may produce low quality models, in particular when dealing with low sequence identities. In the latter cases, more sophisticated and recent developed techniques, such as the family characterization using Profiles and/or Hidden Markov Models algorithms, are used in order to automatically optimize the alignments (Krogh *et al.*, 1994). The optimization takes into account features conserved all along the family of proteins, allowing a better definition of the aligned columns.
3. Transfer of the coordinates from the template to the target, followed by an energy optimization of the structure. This step involves the modeling of conserved regions (core of the protein); modeling of the less conserved regions, in particular regions in which gaps are present; and side chain modeling.
4. Quality assessment of the model using visual inspection, software validation tools, and validation with biological knowledge coming from experiments. The validation against experiments is fundamental and makes the homology modeling an iterative process. In this case, models are refined with the introduction of restraints extracted not only from the sequence alignments but also from experiments; the refined models are then used for the design of new experiments, which will be used for a subsequent validation, until arriving at convergence.

The components of the signaling pathway for which structural information is available, are instead amenable for MD. In particular, one can use techniques to explore the free energy landscapes of molecular processes. Here we will review an application of the metadynamics method (Laio and Parrinello, 2002). It is based on the introduction of few collective coordinates, which are functions of the coordinates of the system and which describe the process under observation. The free energy is then evaluated as a function of these coordinates. An artificial dynamics in the space of these col-

lective variables is defined by making use of a history-dependent potential. This is constructed as a sum of Gaussians centered along the trajectory of the collective variables. In the course of the simulation the potential gradually fills the minima of the free energy surface. In the end, the sum of the Gaussians will thus reproduce, modulus a constant and with the opposite sign, the free energy profile dependent on the chosen collective variables. Of crucial importance is the choice of the collective coordinates, which should be significative for the process to monitor and which are normally difficult to sample with the standard dynamics because of the high energy barriers that separate the free energy minima.

Here we report a brief survey of our contribution OR cascades. Many studies by other groups have been performed, which cannot be all reported here because of limitations of space (Gao *et al.*, 2006; Hunter *et al.*, 2008; Goddard and Abrol, 2007). Those required for this review can be found in the [Supplementary Materials \(SI\)](#).

Structural prediction and MD studies

ORs. Experimental structural information at atomic level of these receptors is currently lacking. Structural predictions of the ORs were carried out by several groups (Singer, 2000; Floriano *et al.*, 2004; Khafizov *et al.*, 2007; Goddard and Abrol, 2007). A nontemplate-based modeling approach was used to predict, by means of molecular simulation, the structures of several members of the following families: the β -adrenergic receptors, olfactory receptors, and taste receptors.

The observation that structural features are well conserved across all Class A GPCRs (which include ORs and rhodopsin; Kolakowski, 1994), along with the determination of the structure of other GPCRs [such as the human β_2 adrenergic receptor (Cherezov *et al.*, 2007), the turkey β_1 adrenergic receptor (Warne *et al.*, 2008) and the human A2A adenosine receptor (Jaakola *et al.*, 2008)], has led to the conclusion that template-based structural predictions, together with the use of restraints extracted from point mutagenesis experiments, may offer reliable models. This is confirmed by recent studies (Jørgensen *et al.*, 2007; Costanzi, 2008), which show that GPCR x-ray structures exhibit a large predictability and can thus be used as templates for structural models of GPCRs sharing similar sequences.

The identification of nine crucial amino acids involved in ligand binding and selectivity on the helices TM3, TM5, and TM6 of ORs set the stage for such modeling (Khafizov *et al.*, 2007). The structural predictions of the ORs for which ligand binding data are available (overall 29) were validated against experimental information regarding one of the modeled receptors (MOR174-9; Katada *et al.*, 2005). They were then used to predict which amino acids could be involved in the ligand binding.

The obtained results suggested that: (i) in Class 1 ORs, the residues in positions 3.40, 5.45, 5.46, 5.50, 5.51, 6.44,

6.47, 6.48, and 6.51 in Fig. 2 might form electrostatic and van der Waals interactions for most ligands; and (ii) in Class 2 ORs, the positions 3.40, 5.45, and 6.51 are occupied mostly by apolar groups, and the positions 5.50 and 6.47 by polar groups. In addition, the nature of the residues in positions 5.46, 5.51, 6.44, and 6.48 is similar to that of the corresponding residues in Class 1 ORs; (iii) 14 positions (indicated as 3.29, 3.32, 3.33, 3.36, 3.37, 4.56–4.58, 5.41, 5.42, 5.54, 6.43, 6.50, and 6.52 in Fig. 2), besides the already cited ones, may play an important role for ligand binding.

Performing further mutagenesis experiments will dramatically improve the general comprehension of the odorant recognition process and refine the model. Only one subunit is considered here; multimeric structures could be modeled in the future (George *et al.*, 2002; Reggio, 2006).

Very recently, the first structure of a GPCR in an empty state (opsin) has been reported (Scheerer *et al.*, 2008). The structural information might now be used to perform comparative studies of ligand-bound and ligand-free ORs.

G-proteins, adenylyl cyclases, and their complex in signaling. In their inactive state, G-proteins exist as heterotrimers, formed by the so-called α -subunit ($G\alpha$) in complex with GDP and a dimer formed by the tightly bound β - and γ -subunits ($G\beta$ and $G\gamma$; Schmidt *et al.*, 1992) (Fig. SI_1 in

the Supplementary Materials). $G\alpha$ is formed by a helical domain composed of six helices and by a catalytic domain (also referred as RAS-like or GTPase domain). The two domains are connected by two flexible linkers (linkers I and II) and their interface hosts the nucleotide binding pocket. $G\beta$ consists of an N-terminal α -helix followed by a β -propeller domain, formed by seven “WD” repeat motifs, each made by approximately 43 amino acids. Its overall fold is completed by the interactions of strands from WD1 and WD7. The N-terminal helix of $G\alpha$ interacts with WD1 of $G\beta$, while the N-terminus of β 2 strand, α 2 helix and β 3/ α 2 loop of $G\alpha$ interact with six out of seven WD repeats of $G\beta$ (WD1–WD5, WD7). $G\gamma$, which is smaller than the other two subunits, consists of two helices connected by a loop. The N-terminal helix of $G\gamma$ forms a tight coiled-coil interaction with the N-terminal helix of $G\beta$.

Upon ligand binding, the active GPCR-bound G-protein undergoes a structural rearrangement, including a movement of the α 5 helix (Oldham *et al.*, 2006; Galés *et al.*, 2006). These rearrangements lead to GDP release, with formation of the empty state. NMR spectroscopy suggests that such empty state is transient and conformationally dynamic (Abdulaev *et al.*, 2006).

Figure 2. Side (a) and top (b) views of a representative structural model of ORs (the traces of $C\alpha$ carbons in TM helices are only shown). The positions of the residues that may be involved in ligand binding are indicated following the numbering of (Ballesteros and Weinstein, 1995). Nine of them, indicated with 3.40, 5.45, 5.46, 5.50, 5.51, 6.44, 6.47, 6.48, and 6.51, coincide with those previously obtained for MOR174-9 receptor (Katada *et al.*, 2005).

GTP then replaces GDP and a new structural change occurs, which causes the detachment of one subunit (the so-called $G\alpha$ subunit) from the other two. The $G\alpha$ subunit binds and activates various enzymes and effectors (Kristiansen, 2004; Oldham and Hamm, 2006; Johnston and Siderovski, 2007). In the case of ORs, it activates AC type III.

By using MD simulations, it has been shown that large scale motions of the inactive state involve the relative movement of the helical and catalytic domains (Khafizov *et al.*, 2007). This is consistent with previous observations (Ceruso *et al.*, 2004; Mello *et al.*, 1998), indicating that this motion may be important for GDP release (Galés *et al.*, 2006). As expected (Wall *et al.*, 1998), almost all of the residues relevant for the $G\alpha i1/G\beta 1$ interface (identified by computational alanine scanning) are conserved or conservatively mutated. Instead, when the G-proteins are found in their empty state, GDP removal causes instabilities in the $\beta 6$ - $\alpha 5$ region, consistently with the suggestion that $\alpha 5$ helix might be displaced in this process (Ceruso *et al.*, 2004; Oldham *et al.*, 2006). The latter rearrangement is assisted by the conformational flexibility of a region spanned by two residues (Gly202-Gly203). This is consistent with the observation that the G203A mutation locks the trimer in its inactive state, inhibiting $G\beta 1$ $\gamma 2$ release (Miller *et al.*, 1988; Lee *et al.*, 1992). In addition, the calculations suggest that the empty state may structurally pre-adopt the active one.

ACs are the most abundant enzymes catalyzing the synthesis of the universal second messenger cAMP from ATP. They can be activated or inhibited by binding with GTP-bound α -subunit of specific G-proteins. cAMP, synthesized

by the enzyme, activates target proteins such as protein kinases, ion channels, and transcription factors, finally resulting in a cellular response to the primary stimulus.

These proteins contain two TM domains (M1 and M2), each crossing the membrane six times. The main functional parts are located in the cytoplasm and can be subdivided into the N-terminus, C1a, C1b, C2a, and C2b. The C1 region exists between TM helices 6 and 7 and the C2 region follows TM helix 12. The C1a and C2a domains form a catalytic dimer where ATP binds and is converted to cAMP.

By comparative modeling, it was possible to predict the structural determinants of the cytoplasmic domain of an enzyme involved in ORs signaling (AC III) in complex with its cognate $G\alpha$ -subunit (Golf), in the presence of the essential Mg^{2+} ion and forskolin (MPFsk). The template is the highly similar MPFsk-bound AC III/ G_{sa} · Mg^{2+} ·2'-deoxy-3'-adenosine monophosphate complex (SI > 50%). This model (Fig. 3) suggests that the active site residues binding to MPFsk (Tyr443, Thr512, Lys516, Glu518, Ile940, Gly941, and Ser942) are the same as in the template (Tesmer *et al.*, 1997). MD simulations could now be performed to relax the structure and investigate the hydration of the active site cavity.

CNG channels. CNG ion channels are tetrameric proteins gated by cGMP and cAMP second messengers. They produce the electrical signal in response not only to odor stimulation but also to light in the vision process. CNG channels belong to the superfamily of tetrameric voltage-gated ion channels (Biel and Michalakakis, 2007; Anselmi *et al.*, 2007).

Figure 3. Structural model of the cytoplasmic domain of human AC type III in complex with its cognate $G\alpha$ -subunit. The C1 domain, C2 domain (of AC III), and the G_{sa} are in blue, red, and gray, respectively. Forskolin (MPFsk) binding site in C1-C2 heterodimer (of AC III) is showed in the close-up view on the right.

They consist of: (i) a transmembrane domain formed by six transmembrane helices (S1-S6) and a pore helix (P-helix); and (ii) a cytoplasmic domain formed by the cyclic nucleotide binding domain, which is linked to the transmembrane domain through the so-called C-linker region.

To relate functional differences of CNG channels with other channels of the superfamily, their sequence was compared with that of the voltage-gated K^+ (Kv) channels (Anselmi *et al.*, 2007). It was found that CNG channels are less voltage dependent than Kv channels because of several factors, including: (i) the lower charge of their voltage sensor (the S4 helix) relatively to that of the Kv channels; and (ii) the presence of a conserved proline in the S4-S5 linker region, which is likely to reduce the mechanical coupling between S4, S5, and S6 helices.

The pore region was next modeled on the template of a CNG channel from bovine rod. The latter exhibits a very high SI with the ORs' CNG channels (58%). Therefore, the two channels are expected to have extremely similar structural determinants. The sequence alignment, along with experimental constraints, was then used to provide a structural basis of these channels (Giorgetti *et al.*, 2005).

The experimental constraints were obtained by cysteine scanning mutagenesis of residues present principally along the channel axis. Mutated channels were then studied by measuring the differences of current blockage upon the introduction of metals, such as Cd^{2+} , and agents capable of interacting with cysteines in the solution (Nair *et al.*, 2006). New constraints have been recently included in homology modeling of CNG channel for residues Asp413 and Tyr418 in both open and closed states. In the open state, the distances between them should be of about 11–13 Å, since the mutant channels D413C and Y418C are inhibited by Cd^{2+} , while in the closed state they are not blocked, so these distances should be larger than 14 Å (Nair *et al.*, in preparation) (Fig. 4).

Figure 4. Structural models of the P-helix (in blue), S6 (in yellow), and the C-linker (in red) of CNG channel in the closed and open state. For clarity, only two subunits are shown. Insets: top view of the N-term@C-linker (in red).

On the basis of the combination of experimental and theoretical studies, it has been proposed that a rotational movement begins in the C-linker region. This rotational movement is then transmitted upwards, making the upper part of S6 rotate anticlockwise. Due to the direct interaction of S6 with the P-helix, this motion is transmitted to the latter, which rearranges itself so that its terminal Thr360 residues and, therefore, the lower part of the pore wall, lead to the opening of the pore lumen. Thus, the initial event of cyclic nucleotide binding is transmitted to the pore walls by a remarkable and sophisticated coupling of conformational changes spanning throughout the entire cytoplasmic and transmembrane domains of the channel.

Chloride channels. Members of the bestrophin proteins are among the Ca^{2+} -activated Cl^- channels playing a role for the olfactory transduction (Pifferi *et al.*, 2006b; Boccaccio and Menini, 2007; Kleene, 1993). According to two topological models (Jentsch *et al.*, 2002; Loewen and Forsyth, 2005), the N- and C-terminal domains of bestrophins would be located at the intracellular side of the membrane and would be connected to four or five hydrophobic domains forming the channel.

CMB techniques are currently in use to identify aspartate and glutamate residues binding Ca^{2+} and to predict the effects of their mutations to alanine. Selected mutations are investigated by electrophysiological experiments (Xiao *et al.*, 2008; Kranjc *et al.*, in preparation).

Calmodulin. Calmodulin is a calcium-modulated protein found ubiquitously in eukaryotes. It is capable of regulating biological activities of various calcium-sensitive enzymes, ion channels, and other proteins by changing the conformation upon binding to calcium, which then enables the binding to specific proteins for a specific response. In particular, they regulate the activities of the CNG channels in the olfactory signaling pathway by: (i) binding the channel subunits CNGA4 and CNGB1b, thus decreasing the probability of opening CNG channels; (ii) binding and activating the CaM-dependent phosphodiesterase (PDE1C2) and therefore transforming cAMP in AMP; and (iii) phosphorylating the Ser1076 residue of AC III, inhibiting it. This is achieved by activating CaM-dependent protein kinase II (Fiorin *et al.*, 2006).

Calmodulin is an acidic protein composed of two globular domains connected together by a flexible linker. Each end contains two EF-hand motifs, each of which can bind a calcium ion (see Fig. SI_2 in the Supplementary Materials). Despite a large number of experimental and theoretical studies, the detailed mechanisms which allow CaM to recognize its target peptide segment are not fully understood. Metadynamics-based free energy simulations (Laio and Parrinello, 2002) were used to investigate the final steps of CaM-peptide complex formation. Structural information for CaM in complex with the olfactory CNG channel target segment is currently not available. Therefore, the complex be-

tween CaM and M13, a peptide which is part of the skeletal muscle myosin light chain kinase (skMLCK), was considered. This complex is experimentally well studied and involves an important biological CaM partner in the muscle tissue. The accuracy of calculations was established with a comparison between calculated and NMR-derived structural and dynamical properties. The results of the calculations (Fiorin *et al.*, 2006) provide novel insights into the mechanism of protein/peptide recognition: it was shown that the process is associated with a free energy gain similar to that experimentally measured for the CaM complex with the homologous smooth muscle MLCK peptide (Ehrhardt *et al.*, 1995). It was suggested that binding is dominated by entropic effects, in agreement with previous proposals. Furthermore, it was demonstrated that the large flexibility of the conserved methionines side chains plays a key role in the binding mechanism. Finally, a rationale is provided for the experimental observation that in all CaM complexes the C-terminal domain seems to be hierarchically more important in establishing the interaction.

The metadynamics simulation in this work has provided a first step toward predicting the complete energetics of the molecular recognition of calmodulin and CNG channels.

All the material presented here has been reported elsewhere, except for the modeling of the AC III/Golf complex, which was performed for this study here.

3CTAs LIGANDS IN BORON NEUTRON CAPTURE ANTICANCER THERAPY

Boron neutron capture therapy (BNCT) combines chemotherapy and radiotherapy approaches for antitumoral intervention. It is based on the nuclear capture and fission reactions of ^{10}B interacting with thermal neutrons (Soloway *et al.*, 1998). These yield destructive effects that are limited to boron-containing cells. BNCT may selectively destroy malignant cells and spare normal cells, provided that the boron carrier adopted is able to concentrate much more ^{10}B in the tumoral cells than in the surrounding normal ones.

Recently designed boron-cage containing derivatives of deoxythymidine (dT) [3CTAs in Fig. 5(a)] might satisfy these requirements by concentrating large quantities of boron specifically in the cell nuclei (Al-Madhoun *et al.*, 2004; Narayanasamy *et al.*, 2006). In fact, they can be incorporated in new filaments of DNA through the salvage pathway of nucleoside phosphorylation.

The first and most important step of this mechanism is the enzymatic phosphorylation of dT or its boronated analog by the dimeric enzyme human thymidine kinase 1 (TK1) (Eriksson *et al.*, 2002):

The design of ligands with enzymatic efficiency larger than current ones (their $k_{\text{cat}}/K_{\text{M}}$ values are 60% of that of wild-type or lower) may improve the potency of this BNCT strat-

egy (Al-Madhoun *et al.*, 2004; Narayanasamy *et al.*, 2006). Thus, dissecting facets of the reaction mechanism may help this design. As a first step towards this goal, we have predicted the structural determinants of the complex between the human enzyme and its natural substrate as well as the 3CTAs for which *in vitro* thermodynamic and kinetic evaluations have been performed [Fig. 5(a)].

STRUCTURAL PREDICTIONS

Michaelis complex. A key issue in drug design of ligands targeting thymidine kinases is the structural prediction of the Mg^{2+} ion's coordination polyhedron in the active site [Fig. 5(b)], which is highly nontrivial: in fact, this has been achieved by MD simulations with *ad hoc* constraints imposed (Cavalli *et al.*, 2001). This can be done only if the interactions of the ion with the rest of the protein are known (i.e. the calculations have little predictive power). Recent MD simulations on one subunit of TK1, carried out for this Perspective (see Supplementary Materials), provided the first insights on the Michaelis complex of the enzyme. The Mg^{2+} ion turned out to be coordinated by six oxygen atoms from three water molecules, the second and third phosphate groups of ATP, and a serine (see Supplementary Materials). The octahedral geometry of Mg^{2+} coordination was well maintained during the course of MD simulation (see Supplementary Materials, Figs. SI_5b and SI_5c). The binding pose of ATP is stabilized mainly by hydrogen bonds and by electrostatic interactions with the protein atoms. The dT moiety interacts with the protein as in the x-ray structure of TK1 (Welin *et al.*, 2004; Birringer *et al.*, 2005; Segura-Peña *et al.*, 2007). Work is in progress to test our computational protocol for the dimeric structure.

3CTAs-TK1 complexes. Molecular docking on the x-ray structure of TK1 shows that the binding poses of the ligands are fairly similar to those of dT (see Supplementary Materials). However, the methylene atoms and the carborane cage (which is located outside the protein) cause some steric hindrance with the residues in the active site cavity [Fig 5(c)]. Consequently, the interactions between thymine and the protein are expected to be weaker.

PERSPECTIVES

A key development of this project is represented by QM/MM calculations of the reaction mechanism of the enzyme (see, e.g., Dal Peraro *et al.*, 2007) in complex with the substrate and with 3CTAs. These calculations may provide a rationale for the lower $k_{\text{cat}}/K_{\text{M}}$ of boronated drugs relative to that of dT (Al-Madhoun *et al.*, 2004; Narayanasamy *et al.*, 2006). Based on this information, more efficient prodrugs than those developed so far might also be designed.

In the perspective highlighted in this paper, however, an exciting advance is the investigation of *all* the steps involved in the fate of the drugs, from the administration in the ill tissues to tumoral DNA.

Figure 5. (a) A group of 3CTAs for which the k_{cat}/K_M and Michaelis constants have been measured (Al-Madhoun *et al.*, 2004; Narayanasamy *et al.*, 2006). A methylene linker, at times with hydroxyl substituents, connects the nucleobase to an icosahedral cage, whose vertexes are occupied by ten boron atoms and two carbon atoms. The latter are either in position ortho (*Group 1* 3CTAs, as in this picture) or para (*Group 2* 3CTAs; see [Supplementary Materials](#)). Polyols attached to the cage may be present. (b) MD structure of the Michaelis complex after 6 ns. (c) Docking pose of compound no. 10 in (a) (balls and sticks), in comparison to that of dT in the x-ray structure (Welin *et al.*, 2004; thick line). This is the most promising dT derivative because it shows one of the highest k_{cat}/K_M (~40% of that of dT) among the 3CTAs and hydrophobic properties due to which it should be able to cross membranes by passive diffusion (Al-Madhoun *et al.*, 2004; Byun *et al.*, 2006).

The first step is the simulation of the 3CTAs crossing of the membrane, which occurs by passive diffusion (Barth *et al.*, 2004; Byun *et al.*, 2005). Free energy approaches such as metadynamics (Laio and Parrinello, 2002) could be used. Next, there are the chemical modifications of the derivatives, occurring in the phosphorylation salvage pathway of dT in the cytoplasm (Eriksson *et al.*, 2002; van Rompay *et al.*, 2003). This involves three successive phosphorylation reactions, catalyzed respectively by TK1, thymidilate kinase, and a nucleoside diphosphate kinase:

The boronated derivatives should then cross the nuclear membrane by passive diffusion through the membrane pores (van Rompay *et al.*, 2003). This process could also be simulated by MD. Finally, they should interact with DNA polymerase to be incorporated into a new DNA strand. The process might be simulated with QM/MM methods.

The information obtained by these CMB studies might then be used to develop prodrugs with improved efficiency and selectivity.

CONCLUDING REMARKS

In this review, some aspects of the modeling of OR cascade of events were presented. CMB studies may provide struc-

tural determinants of most of the key components, and, in one case, they provide a molecular (quantitative) view on their mutual interactions.

The approach can be extended to other signaling pathways. One possible extension has been outlined here in the context of anticancer therapy.

Challenges for the near future include the development and application of methods that permit the full description at the molecular level of large protein complexes, most of all including membrane proteins with unknown structure. In addition, a mapping between the detailed level of CMB and very coarse (yet very effective) systems biology is required.

Advancement in experimental structural biology (Markwick *et al.*, 2008; Neylon, 2008; Riekel *et al.*, 2005), along with algorithms for free energy calculations (Ensing *et al.*, 2006), multiscale modeling (Sherwood *et al.*, 2008), and protein-protein docking (Lensink *et al.*, 2007) make us confident that such challenges can be undertaken in short time and that these approaches may provide a great improvement to our understanding of cell and molecular biology events.

ACKNOWLEDGMENTS

We acknowledge all of the coauthors discussed in the present review, as well as Professor Staffan Eriksson (University of Uppsala, Sweden), Professor Gianrossano Giannini (University of Trieste, Italy), Professor Anna Tramontano (University of La Sapienza, Roma, Italy), and Dr. Gianluca Lattanzi (University of Bari, Italy) for many stimulating discussions. This work was in part supported by Illy Caffè (Trieste), Glaxo (Stevenage, UK), Human Frontier Science Program, INFM, CINECA.

Paola Lupieri, Chuong Ha Hung Nguyen and Zhaleh Ghaemi Bafghi contributed equally to this work.

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