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Molecular modeling and crystal structure of ERK2–hypothemycin complexes

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ABSTRACT

Resorcylic acid lactones containing a *cis*-enone—such as hypothemycin—are susceptible to Michael addition reactions and are potent and specific inhibitors of about 45 of the known Ser/Thr/Tyr protein kinases. These inhibitors bind reversibly, and then form a covalent adduct with a completely conserved cysteine in the ATP binding site of their target kinases. As a paradigm for the structures of the *cis*-enone resorcylic acid lactone complexes with this subset of kinases, we have modeled the structure of ERK2–hypothemycin reversible and covalent complexes using docking and extended molecular dynamics simulations. Subsequently, we determined the 2.5 Å resolution crystal structure of the complex that was in excellent accord with the modeled structure. The results were used to discuss structure–activity relationships, and provide a structural template for the development of irreversible inhibitors that complement the ATP binding site of kinases.

1. Introduction

Resorcylic acid lactones (RALs) are polyketide natural products with a large macrocyclic ring fused used to resorcylic acid. Some RALs that contain an α,β-unsaturated ketone in the macrocycle, as exemplified by the *cis*-enone RALs hypothemycin, 5Z-7-oxozeaenol and L-783,277, are potent inhibitors of certain protein kinases (Williams et al., 1998; Zhao et al., 1999; Ninomiya-Tsuji et al., 2003). Recently, it been reported that hypothemycin covalently binds to a subset of ~45 known Ser/Thr/Tyr protein kinases that contain a Cys (Cys-164 in rat and 166 in human ERK2) adjacent to the completely conserved Asp that is involved in binding the Mg complexed to ATP; kinases that do not have this Cys residue are not inhibited (Schirmer et al., 2006). The subset of kinases inhibited include popular therapeutic targets such as VEGFR, PDGFR, MEK, ERK, TAK1 and others (Williams et al., 1998; Zhao et al., 1999; Ninomiya-Tsuji et al., 2003; Schirmer et al., 2006). Notably, the targets include four enzymes in the ERK proliferation pathway—MEK1/2, ERK1/2—so these inhibitors provide an extremely effective blockade of the cell proliferation pathway (Murphy and Blenis, 2006; Rubinfeld and Seger, 2005; MacCorkle and Tan, 2005). Hypothemycin and related RALs possess potent antiproliferative activities (Schirmer et al., 2006; Hearn et al., 2007). Indeed, they are extremely efficient at inhibiting cells harboring the ERK-dependent B-RAF V600E mutation commonly associated with melanoma. Importantly, the fact that only the kinases possessing the target Cys are irreversibly inhibited has clear advantages for controlling and enhancing selectivity (Garber and Arbor, 2006; Bridges, 2001; Dancey and Sausville, 2003).

As a paradigm for the structures of RAL complexes with their target protein kinases, we determined the structure of hypothemycin bound to ERK2 with molecular modeling and crystal structure analyses. Docking and extended molecular dynamics (MD) simulations in explicit water solvent were performed on the reversible and covalent hypothemycin–ERK2 complexes. These simulations were performed in order to predict the binding mode of the inhibitor and to investigate dynamic features that may be relevant for inhibitor binding. We found that hypothemycin binds the ATP binding site by establishing a number of hydrophobic interactions as well as hydrogen bonds with active site residues and water. Interestingly, while some of these interactions are conserved for the whole duration (28 ns) of the MD simulations, others showed time-dependent behavior. Finally, the 2.5 Å resolution crystal structure of ERK2 covalently bound to hypothemycin was determined and found to be in excellent agreement with the modeled structures. The results were used to infer the structural features most relevant for ERK2 covalent inhibition by resorcylic acid lactone *cis*-enones and to provide a basis for future inhibitor design.

Abbreviations: ERK, extracellular regulated kinase; RAL, resorcylic acid lactone; MM, molecular mechanics; MD, molecular dynamics.

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2. Materials and methods

2.1. Docking and molecular dynamics simulations

The starting protein structure used in our investigation is the crystal structure of unphosphorylated rat ERK2 (PDB code 1ERK) (Zhang et al., 1994). Rat and human ERK2 are highly conserved, differing for two additional aminoacids at the N-terminus of the human sequence and for one mutation (L44 V) in a loop located in the exterior of the protein. The crystal structure of isolated hypothemycin (Agatsuma et al., 1993) was used as starting point for docking and MD simulations.

The initial orientation of hypothemycin in the ATP binding site of ERK2 was obtained with the automated docking program AutoDock3.0.5 (Morris et al., 1998). The Lamarckian genetic algorithm (LGA) was applied to model the interaction of the ligand with the protein. The docking area of ERK2 was defined using the AutoDock module ADT. The grid site was constrained to a 15 Å cubic space centered on the middle of the ATP binding site. Default parameters were used for docking, and a hundred independent runs were performed with LGA. The docked orientations were clustered and sorted according to their predicted docked energies estimated with the AutoDock empirical scoring function, and then visually inspected with MidasPlus (Ferrin et al., 1988).

The most favorable docking solution was used as starting point for MD simulations of the ERK2–hypothemycin complex in water. MD simulations were performed with the *sander* module of AMBER 9 (Case et al., 2006) and the ff03 (Duan et al., 2003) force-field. Hydrogens were added to the protein using the internal coordinates of the AMBER all-atom data base. All Lys and Arg residues were positively charged and Glu and Asp residues negatively charged.

The atomic charges of hypothemycin were obtained from an electrostatic potential (ESP) fit to the 6-31G* ab initio wave function, using Gaussian98, followed by standard RESP (Bayly et al., 1993) fit using the antechamber module of Amber. Amber atom types consistent with the ff03 (Duan et al., 2003) force-field were assigned to hypothemycin. Missing force-field parameters were assigned based on similarities with existing parameters and then tested to make sure that the minimized structure of the isolated ligand was in agreement with the crystal structure of hypothemycin (Agatsuma et al., 1993). Force-field parameters of hypothemycin covalently bound to the cysteine were obtained as described below. MD simulations of the reversible complex clearly suggested that the cysteine approaches the 7′,8′ double bond from the *re*-face of the double bond (which is also more solvent exposed), resulting in a Michael adduct with the *R*-stereochemistry (see Section 3 and Fig. 1). Therefore, a truncated model of the Michael adduct was generated by modeling the cysteine attached to carbon 8′ as a *S*-methyl group in the *R* configuration. Then, the resulting structure of the adduct was energy-minimized with AM1 and atomic charges were derived using the same procedure already described for hypothemycin. Atom types were re-assigned to reflect saturation of the double bond. Finally, a new residue topology containing an entire cysteine residue linked with hypothemycin was defined by merging the Amber cysteine topology and the truncated Michael adduct topology described above. Only marginal adjustments of atomic charges of Sγ and Cβ atoms were required to ensure that the total charge of the modified residue was neutral.

The ERK2–hypothemycin complexes were solvated in an octahedral box with water molecules extending 8 Å outside the protein on all sides, resulting in more than ten thousand waters. The electrostatics were treated with the particle-mesh Ewald method (Darden et al., 1993) with a grid size of 80^3 Å, a fourth-order B-spline interpolation and a tolerance of 10^{-5}. The simulations employed a residue-based cutoff of 8 Å, and bond lengths involving hydrogens were constrained using the SHAKE (Ryckaert et al., 1977) algorithm and a time step of 2 fs. The nonbonded pair list was updated every 25 fs. The solvated complexes were minimized with 10,000 steps of conjugate gradient minimization and then equilibrated with MD at 300 K as follows: first, 20 ps MD under constant volume were performed to equilibrate the temperature around 300 K with 1 kcal/mol restraint on the protein and ligand heavy atoms to avoid undesirable drifts of the structure; then, MD was continued under constant pressure conditions (1 atm) for 50 ps with 1 kcal/mol restraint on heavy atoms, followed by additional 20 ps MD with reduced (0.5 kcal/mol) restraints, after which a short equilibration of 200 ps without restraints was performed. At the end, additional 10,000 steps of conjugate gradient minimization were performed.

To investigate the dynamic features of the complexes, significantly more extended MD simulations were performed starting from the equilibrated structures described above, using the same parameters. Production runs consisting in 28 ns constant pressure MD without restraints were performed on the reversible and covalent complexes. Coordinates were collected every 0.5 ps, resulting in a total of 58.000 snapshots collected and then analyzed with the *ptraj* module of amber for measuring distances between selected atoms and performing hydrogen bond analyses. Moreover, the coordinates were averaged every 20 ps for visual inspection with MidasPlus.

In order to mimic the early steps of covalent bond formation, five independent 1 ns MD simulations with gradually increasing restraints on the shortening of the Sγ–C8′ distance were also performed. These simulations were started from five different ERK2–hypothemycin geometries sampled during the 28 ns reversible complex simulation. At this aim, the distance was gradually shortened from 4.0 to 2.5 Å by imposing a strong (100 kcal mol^{-1} $Å^{-2}$) restraint coupled to this distance (*i.e.* a restraint that increases gradually from 0 to 100 kcal mol^{-1} $Å^{-2}$ as long as the distance decreases from 4.0 to 2.5 Å).

2.2. Crystal structure determination

The rat ERK2 kinase domain (residues 1–368) was purified according to previous methods (Khokhlatchev et al., 1997). Hypothemycin was dissolved in DMSO at a concentration of 100 mM. The covalent complex was created by adding Hypothemycin to dilute ERK protein (0.5 mg/ml in 20 mM Tris 7.5,

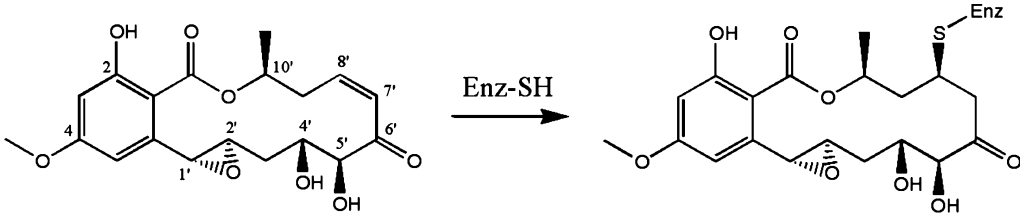

Fig. 1. Structure of hypothemycin and its Michael adduct formed with the cysteine of target protein kinases.