

Validated ligand mapping of ACE active site

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Summary

Crystal structures of angiotensin-converting enzyme (ACE) complexed with three inhibitors (lisinopril, captopril, enalapril) provided experimental data for testing the validity of a prior active site model predicting the bound conformation of the inhibitors. The ACE active site model – predicted over 18 years ago using a series of potent ACE inhibitors of diverse chemical structure – was recreated using published data and commercial software. Comparison between the predicted structures of the three inhibitors bound to the active site of ACE and those determined experimentally yielded root mean square deviation (RMSD) values of 0.43–0.81 Å, among the distances defining the active site map. The bound conformations of the chemically relevant atoms were accurately deduced from the geometry of ligands, applying the assumption that the geometry of the active site groups responsible for binding and catalysis of amide hydrolysis was constrained. The mapping of bound inhibitors at the ACE active site was validated for known experimental compounds, so that the constrained conformational search methodology may be applied with confidence when no experimentally determined structure of the enzyme yet exists, but potent, diverse inhibitors are available.

Introduction

Current paradigms of structure-based drug design presuppose detailed knowledge of the structure of the therapeutic target and/or complex to design potential inhibitors. Yet, despite the increasing number of high-resolution 3D structures publicly available, a distinct knowledge gap remains between the identification of potential disease targets, and the generation of accurate structure-based models to use in drug discovery. Methodologies like the active-analog approach aim to bridge this gap, by taking advantage of

three-dimensional chemical information implicitly stored in a series of active drug molecules.

The active-analog approach was used to predict the active conformations of angiotensin-converting enzyme (ACE) inhibitors bound at the (C-terminal) active site of ACE in 1987 by Mayer et al. [1] This and previous schematic-type models [2] were the basis for discovery efforts that produced the large family of ACE inhibitors employed as safe, effective, orally available, anti-hypertensive drugs throughout the last two decades. Since the prediction of the ACE active site in 1987, new inhibitors have been developed for therapeutic application. Computational power has increased dramatically as well. These two parallel developments enabled refinement of the original

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ACE active site model – the inclusion of structural information from new inhibitors added further constraints to the system, and greater computational power allowed better sampling of conformational space. Likewise, advances in protein engineering and structural determination enabled Natesh et al. to solve high-resolution, X-ray crystallography structures of human testicular ACE [3] (EC 3.4.15.1), and ACE complexed with lisinopril [3], captopril [4] and enalapril [4] (potent ACE inhibitors of known therapeutic relevance).

The active site model proposed by Mayer et al. [1] was evaluated with respect to the three crystal structures and found to be accurate within 0.37–0.77 Å root mean square deviation (RMSD) across the model distances. Using published information on new ACE inhibitors, the commercial software package Sybyl 6.9.1 and a single-processor workstation, an active site model of higher resolution was developed. This model has been shown to be accurate with respect to the three published X-ray crystallography structures of ACE-inhibitor complexes, within 0.43–0.81 Å RMSD. These observations represent the achievement of a significant milestone in the fields of protein science and drug design – this is the earliest known *in silico* prediction shown to be highly accurate with respect to high-resolution experimental data. Further, the methodology for achieving these results is shown to be accessible to anyone with a modern workstation and the appropriate software.

Such results corroborate the feasibility of using the implicit three-dimensional information in chemically diverse inhibitors to accurately predict the bound conformations of ligands at enzyme active sites; in this case, the C-terminal active site of ACE (different binding and biological responses result from N-terminal site binding [5–7]). This study provides the proof of concept that the active-analog approach may be applied for active site mapping with enzymes where no high-resolution structures of the enzyme or enzyme-inhibitor complexes exist.

Results and discussion

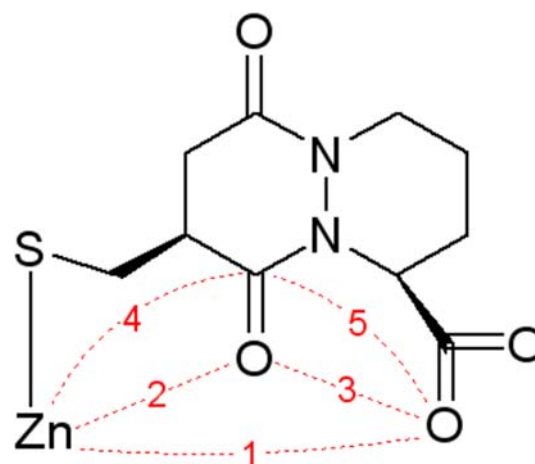
The distance maps reveal that prediction matches experimental evidence

The three X-ray crystal structures with bound inhibitor (lisinopril, enalapril or captopril) were

analyzed in the framework of the predicted active site using distance maps. Figure 1 establishes that the distance maps derived from the three experimentally determined structures fit precisely within the active site prediction, allowing for some delocalization (less than 1.1 Å) of the zinc atom. The crystal structures show evidence that the coordinated zinc may not have a precisely localized position, when bound to different inhibitors.

The bound zinc atom is delocalized among the crystal structures

Figure 2 displays the inherent deviation among crystal structures as a superposition. The root mean square (RMS) deviation among active site anchor points in lisinopril and enalapril, which share a carboxylate zinc-binding moiety, is notably small, 0.040 Å. But the RMS deviation between active site anchor points in enalapril and captopril (that employs a sulfhydryl zinc-binding group), is higher, 0.198 Å. Lisinopril, enalapril and captopril



	<u>Predicted model</u>	<u>Crystal structures</u>	<u>Deviation</u>
1:	7.188 - 7.812	8.485 - 8.673	~1.1
2:	4.812 - 5.312	5.718 - 5.981	~0.6
3:	3.562	3.532 - 3.628	~0.0
4:	4.812 - 5.062	4.876 - 5.181	~0.1
5:	3.938	3.989 - 4.047	~0.0

Figure 1. All molecules in the ACE inhibitor series have common structural features as deduced by structure–activity studies. A distance map (DMAP) was defined to evaluate the geometrical arrangement of these features across the whole series. The first molecule (most constrained) examined in the series is shown, with the five characteristic distances numbered and indicated by broken red lines. All values in angstroms.

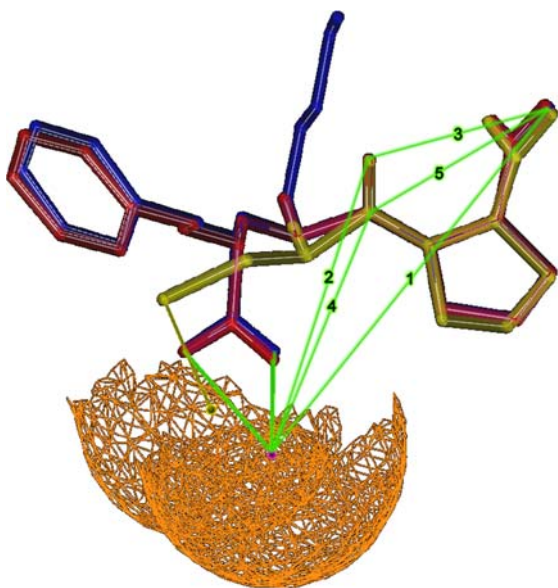


Figure 2. The 3D crystal structures of lisinopril (blue), enalapril (red) and captopril (yellow), the three ACE inhibitors structurally determined in complex with ACE. Electron density contours around the bound zinc atoms are shown as orange meshes. Lisinopril and enalapril employ a carboxylate zinc-binding moiety while captopril utilizes a sulfur; the two distinct zinc loci are evidence of zinc delocalization in the crystal structures. The superposition of lisinopril and enalapril is very tight, and the common geometry appears almost as one. Numbered distance map dimensions are shown for reference (green lines).

are all nanomolar inhibitors of ACE activity, so an absolute overlap of the active site groups in relation to the zinc atom is not necessary to obtain nanomolar inhibition. The enzyme/inhibitor complex optimizes the binding interaction with each ligand while maintaining the common binding mode deduced.

The predicted model implies the accurate active conformations

Figure 3 shows a superposition of the predicted lisinopril conformations, and the lisinopril active conformation from the crystal structure. The constrained portions of the predicted conformations overlap almost exactly with the crystal structure. Vectors are shown to indicate where unconstrained regions connect to the constrained region. The relatively unconstrained pendant phenyl ring and lysine-like chain conformations include the conformation show in the crystal structure (data not shown).

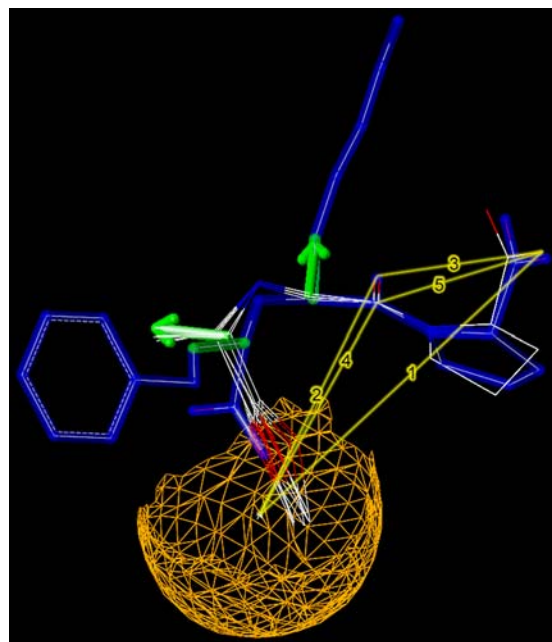


Figure 3. Five predicted lisinopril geometries derived from the constrained search model (shown as line models), compared with the experimentally determined lisinopril structure (shown as blue tubes). The superposition is tight, allowing for some delocalization around the zinc atom. Only the constrained portions of the lisinopril model are shown. Vectors to connect the unconstrained portions are shown as green arrows; the corresponding bonds on the lisinopril crystal structure are highlighted as green tubes. Yellow lines indicate the distance map for the lisinopril crystal structure.

The predicted pharmacophore is visually placed in the context of the ACE active site in Figure 4, using ramipril as an example (no experimental data is available for ACE complexed with ramipril). About 1275 conformations of ramipril, which all tightly fit the predicted model constraints, are shown at the active site of ACE. It is clear that the bulk of the inhibitor is tightly constrained, with some delocalization relative to the zinc atom. Figure 4 also shows that all three inhibitors from the crystal structures bind in the same identical active site of ACE, in the same orientation. Further, the molecular volumes shown by color in Figure 4 illustrate how the active site anchor atoms clearly occupy a common volume, and each inhibitor also fixes additional atoms to occupy unique volumes. The characteristic distance map (among pharmacophoric anchor points) is consistent for the crystal structures and the predicted ramipril structures.

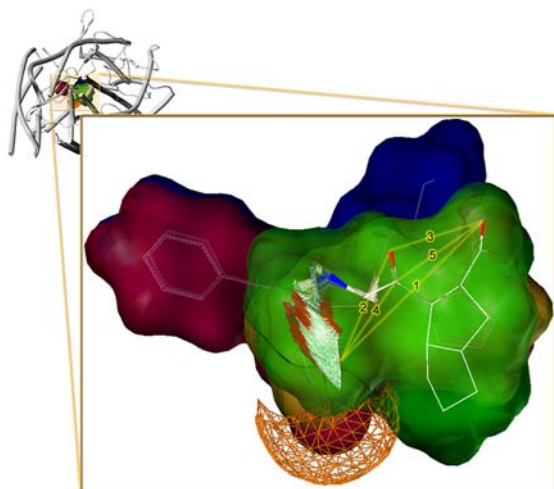


Figure 4. One thousand two hundred and seventy five conformations of the inhibitor ramipril (Altace[®]) predicted at the active site of ACE, compared with the experimental structures of lisinopril, enalapril and captopril. The Connolly molecular surfaces of lisinopril (blue), enalapril (red) and captopril (yellow) are shown to overlap significantly with the predicted ramipril structures (green). All three inhibitors bind the same identical active site on ACE; the active site anchor points coincide as predicted. The predicted ramipril conformations fit within the common volume of the experimentally determined inhibitors. The orange mesh represents an electron density contour encapsulating all zinc loci observed in the crystal structures. Only the constrained portion of ramipril is shown.

Figure 1 illustrates a trend in the deviation across the active site anchor points – the terminal carboxyl and amide carbonyl anchor points (and therefore distances 3 and 5) have much smaller deviation from the crystal structures than those distances involving the zinc (distances 1, 2, 4). This is likely due to two contributing causes. First, applying molecular-level modeling assumptions to a zinc atom that requires quantum-level coordination of its *d*-electrons may have introduced some inaccuracy. The zinc was modelled in a likely coordination geometry (T_5 ; trigonal bipyramidal), but with idealized bond lengths, angles, and plane distortions using molecular mechanics. Clearly, when bound in the active site of ACE, the zinc ligand is coordinated among several atoms and the net result is not necessarily ideal equilibrium geometry with respect to a single oxygen/sulfur coordinating group from the inhibitor. Adjusting the Tripos zinc parameters to appropriate values for zinc ligands in the PDB did not significantly change the predicted distance maps – accurately

predicting metal-coordination geometries will require consideration of the quantum *d*-orbitals.

Secondly, the difference in zinc position among crystal structures suggests that the zinc has no absolute locus, which allows each moiety to coordinate zinc with a slightly different geometry – this is consistent with both the predicted active site model and the crystal structures. The ACE inhibitors include the carboxyl (i.e., lisinopril, enalapril) and sulfhydryl (i.e., captopril) groups, plus hydroxamic acid and phosphate functionalities. The addition of other zinc-binding moieties in the series adds constraints to the analysis and enables a more precise active site prediction. The precision of the comparison with the three crystal structures argues that the constraints from the structures of the inhibitors alone were sufficient to converge upon an accurate, useful prediction of essential active site geometry. Careful addition of constraints at other points could refine the conformational prediction further, perhaps to specify the location of the common pendant phenyl ring, for example.

Several of the new inhibitors included in the study are notable, beyond the three with determined structures. For examples, Omapatril (Vanlev[®]), molecule 29, is known to be a potent inhibitor of both ACE and neutral endopeptidase (NEP). It is shown to fit the predicted ACE active site model, which may provide useful insight for mapping ligands bound at the NEP active site.

These results suggest that a molecule must satisfy the constrained geometry in the active site model to be a potent ACE inhibitor. It is not rigorously established whether these conditions are sufficient, though analysis of molecules 1 and 2 may suggest a minimal molecule.

Increased computational power allowed better sampling of conformational space, relative to the 1987 prediction of Mayer et al. [1] This refined active site model was rebuilt starting with original literature information on ACE inhibitors. Three major differences exist in the current model: (i) All therapeutically relevant drugs are included; (ii) zinc was modelled as a zinc atom with a particular coordination geometry and characteristic parameters, versus a dummy atom; (iii) better sampling of conformational space enabled the use of a higher resolution grid (0.125 Å), resulting in the higher accuracy in prediction of non-zinc anchor points. Though the overall RMSD (relative to

crystal structures) for the 1987 prediction is slightly lower than that for the current model, the deviation in the original prediction is systematic, where the deviation in this model is attributable only to the difficulty of modelling zinc coordination geometries with molecular mechanics.

Many ACE inhibitors of diverse chemical structure existed with sufficient inherent three-dimensional information to determine their bound structures. The increasing prevalence of combinatorial chemical methods is rapidly accelerating the rate at which such inhibitors can be detected. To conduct a similar study on a different disease target-inhibitor system, a researcher need not have access to 20 years of published data – high-throughput methods could be used to generate a series of inhibitors, ideally including inhibitors with both high affinity and low affinity. Such inhibitors could form the basis of a constrained conformational search, from which a set of constraining distance maps would result. These distance maps could be a focus for rational design aimed at generating more potent inhibitors for a series, etc. Holding the active site anchors constant and intelligently substituting (by applying exhaustive or stochastic computational methods) could yield novel inhibitors of high potency for any system utilizing such a high quality active site model.

A predicted active site model of ACE-bound inhibitors [1] was validated against experimentally determined structural data on three inhibitor/ACE complexes and found to be accurate to within 0.43–0.81 Å RMSD. This validates the basic assumptions that active sites of enzymes are very geometrically constrained for substrate recognition and catalysis, so that the three-dimensional geometry of the active site can be determined by analysis of a sufficiently chemically diverse set of inhibitors. The active-analog methodology used to generate the active site model of ACE is generalizable, and reproducible with modest computational resources, commercial software and published data on inhibitors. The proposed active site model is sufficiently accurate to be useful for design of novel inhibitors in the absence of structural information on the therapeutic target itself. These results should encourage the prediction of similar active site models of many therapeutic enzymatic targets

where no high-resolution structure of the enzyme yet exists.

Procedures

Generating a series of potent inhibitors and 3D structures

A series of thirty potent ACE inhibitors with published IC_{50} values below 50 nM was constructed (Figure 5) from published data. [1] As of submission, all major commercialized ACE inhibitors were included, to demonstrate the clinical relevance of the active site model. These included: captopril (Capoten[®]), enalapril (Vasotec[®]), benazepril (Lotensin[®]), quinapril (Accupril[®]), ramipril (Altace[®]), trandolapril (Mavik[®]), fosinopril (Monopril[®]), cilazapril (Inhibace[®]), perindopril (Aceon[®]), lisinopril (Prinivil[®]) and omapatril (Vanlev[®]). (Supplementary Material includes 3D coordinates of the full series.)

All three-dimensional structures in this work were generated using Sybyl 6.9.1, by reference to a published two-dimensional representation. Simulated annealing/gradient minimization was found to outperform CONCORD [8] in predicting high-quality initial conformations. This performance was evaluated by RMSD from small molecule crystal structures in the Cambridge Structural Database [9] for representative inhibitors in the series. The zinc atom type was defined by the supplemental Tripos metals parameter set included with Sybyl; this corresponds to the T_5 trigonal bipyramidal coordination geometry found to be most common for zinc ligands in the RCSB Protein Data Bank (PDB) [10]. Charges were not calculated, so potential energy evaluation was conducted strictly in the context of fundamental bond angle/length, torsion and van der Waals potentials without consideration of electrostatics.

Defining a distance map

Numerous structure–activity studies have been performed on the ACE inhibitor system [1]. The fundamental structural requirements for ACE inhibition include: (a) a terminal carboxyl group to satisfy ionic interactions with a positively charged residue assumed in the ACE active site; (b) a carbonyl group to participate in assumed

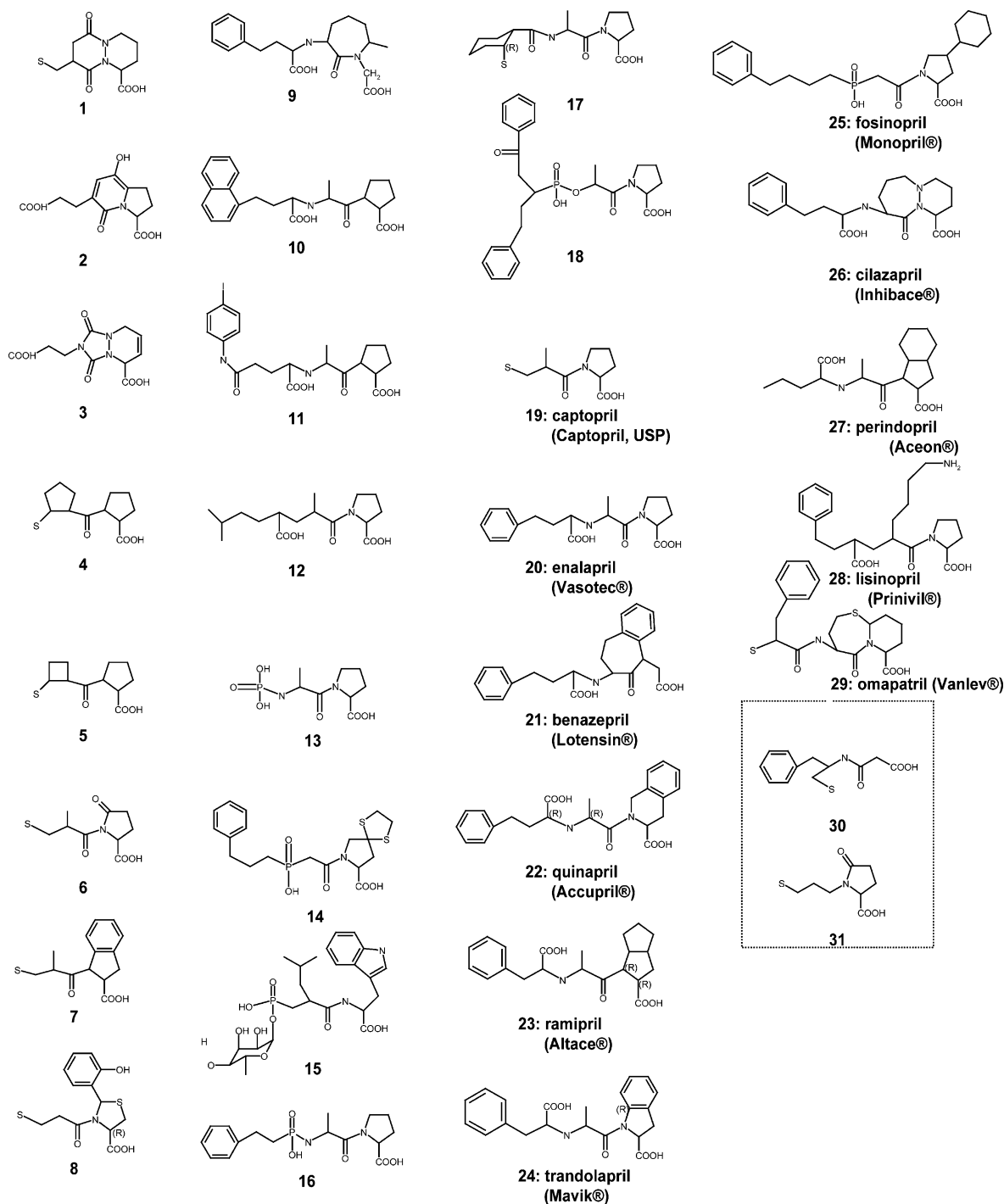


Figure 5. 2D chemical sketches of potent ACE inhibitors (#1–29) and two inactive compounds used as negative controls (#30–31). All chiral centers are in the *S* configuration unless explicitly noted here as *R* configuration. The corresponding database of 3D molecular coordinates is available online as Supplementary Material.

hydrogen bonding with the ACE active site; (c) a functional group assumed to coordinate a zinc atom in the ACE active site. These rules define

pharmacophoric/active site anchor points, among which the distance map is defined, as shown in Figure 1. Figure 1 also defines a set of five

distances [11–15], which captures the relative orientation among the four pharmacophoric anchors to generate an active site model including the zinc atom. To establish a means of evaluating whether or not two conformers are equivalent in distance map space, the distance maps are evaluated on a multidimensional grid. The spacing of evaluation points along each grid axis determines the resolution of the distance maps. Several possible grid resolutions, from 0.500 to 0.100 Å were iteratively evaluated – a 0.125 Å grid resolution was optimal.

Scanning conformational space

For the systematic search, van der Waals radii were scaled according to the parameters calibrated by Iijima et al. on the set of twenty naturally occurring amino acids [12]. All rotatable bonds local to the active site anchors were scanned at 2° increments; bonds far from the active site anchors were scanned at 2–90° increments. Unconstrained results (not shown) indicated that the 2° angle increment was more than adequate for sampling conformational space.

Alignment of 3D structures

In order to preserve the orientation of the ligands in the active site as observed in the crystal structures, the program POSSE [13] was used to align whole ACE proteins by superimposing secondary structural elements, which superimposed the active sites, and therefore superimposed the bound inhibitors. This method gives a physically meaningful superposition, not merely a ‘best fit’ presentation. When superimposing predicted inhibitor models (with no corresponding protein) upon crystal structures, a least-squares fit was used to minimize the distance among atoms in the common amide bond.

Coordinates for the three ACE X-ray crystal structures with bound inhibitor (lisinopril, 1O86.pdb [3]; captopril, 1UZf.pdb [4]; enalapril, 1UZE.pdb [4]), and for ACE without inhibitor (1O8A.pdb [3]) were downloaded from the RCSB PDB [14].

Supplemental Material [1, 15, 16], including full 3D coordinates of all inhibitors used in this series, are available online in Sybyl MOL2 format.

The authors have no competing financial interests.

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