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Mapping of the Active Site of Alcohol Dehydrogenase with Low-Molecular Ligands

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Abstract—In search of an active alcohol dehydrogenase inhibitor, the structure of which may serve as the basis for a potential drug design, the active site of alcohol dehydrogenase containing NAD and Zn^{2+} ions was mapped using the method of molecular mechanics. Molecular docking was performed using a number of ligands containing characteristic functional groups: formate ion, ammonia, ammonium ion, methanol, and methylamine. Sites of preferable binding were revealed for each ligand and arranged in order of decreasing energy of binding to the enzyme. A comparison of the predicted ligand-binding sites and the experimental data on the location of water and inhibitor binding sites in the known structures of corresponding alcohol dehydrogenase complexes indicated a coincidence of the complex formation sites, which confirms the validity of the method and provides the requirements for a highly effective inhibitor (the pharmacophore model).

Key words: alcohol dehydrogenase; proteins, structure, ligands; docking; molecular mechanics

INTRODUCTION

Alcohol dehydrogenase (EC 1.1.1.1) is one of the most important enzymes in the group of NAD-dependent dehydrogenases. It participates in the cellular metabolism of low-molecular mass alcohols catalyzing the NAD-dependent *in vivo* oxidation of primary and secondary alcohols (in particular, methanol, propanol, butanol, and ethylene glycol) to aldehydes or ketones.[2]

The problem of poisoning by methanol and ethylene glycol currently has great relevance [1–4]. Inhibition of alcohol dehydrogenase is also one of the most promising ways of bringing the organism out of acute intoxication with ethanol and its analogues [5]. The main pathogenetic mechanism of these forms of intoxication is a critical increase in the level of aldehydes in blood, which can result in death. Inhibition of the activity of alcohol dehydrogenase can prevent the formation of critical quantities of aldehydes, and alcohol dehydrogenase inhibitors may thus serve as effective antidotes.

For example, this effect is exerted by 4-methylpyrazole [6], which is an active component of Fomepizole, a drug permitted for clinical use in the United States. However, it has an adverse side effect, the impairment of the extrapyramidal system responsible for the involuntary regulation of complex motor acts. Various mechanisms of inhibitor binding to alcohol dehydrogenase are known [6], and the search among compounds of other chemical classes for new inhibitors of enzyme devoid of extrapyramidal impairments seems to be promising.

A great number of chemical compounds have been tested for their ability to inhibit the activity of alcohol dehydrogenase. Among the known inhibitors of the enzyme are nirvanol, tisercin, methiaden, acluracil, metronidazol, iotalamic acid, and others [5]. Thus, the availability of various classes of chemical compounds generally enables the selection of ligands for the catalytic rather than for the coenzyme site and, ultimately, the prediction of the basic structure of a highly specific drug with minimal side effects. Note that the known alcohol dehydrogenase inhibitors cannot be used as antidotes due to their high toxicity and limited accessibility [5].

Alcohol dehydrogenases have been thoroughly studied in many respects. In particular, more than 200 alcohol dehydrogenases from various organisms have been sequenced, and their crystal structures have been determined (at a resolution of up to 1.66 Å) and published for 32 of them (see database of the Brookhaven Protein Data Bank (PDB): http://www.rcsb.org/pdb/). Human and horse alcohol dehydrogenases have been subjected to especially detailed studies. The structures of a number of relative enzymes (lactate, malate, and glyceraldehyde-3-phosphate dehydrogenases) have also been determined. All this provides a basis for the structural analysis of the active site of human alcohol dehydrogenase in order to construct its highly specific inhibitors.

To achieve this goal, we applied a well-proven method for mapping the enzyme active sites using a set

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[2] Abbreviations: AS, active site.

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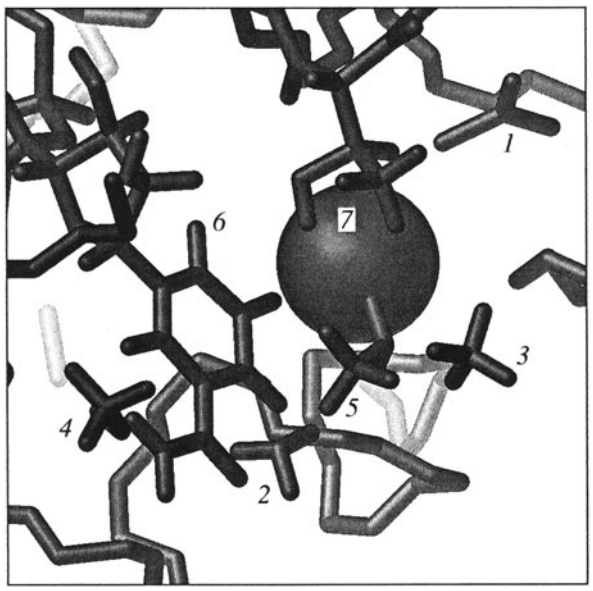

Fig. 1. Mapping of the active site of alcohol dehydrogenase. Superposition of ligands in complexes. Numbers indicate: *1*, formate ion (complex 1.6, see table); *2*, ammonia (complex 2.4); *3*, ammonium ion (complex 3.3), *4*, methanol (complex 4.2); *5*, methylamine (complex 5.1); *6*, cofactor NAD; *7*, Zn atom.

of functional groups (ligands) [7–9] (see the Experimental section). The term ligands is used here in a broad sense and includes functional groups that may be of interest for the further construction of more complex inhibitor molecules.

RESULTS AND DISCUSSION

Ligand Docking at the Active Site of Alcohol Dehydrogenase

The alcohol dehydrogenase AS was studied by the method of simulation of the Brownian motion of small molecules (ligands) in the AS cavity. As a result, several sites of preferable binding were revealed for each ligand corresponding to the minimal interaction energy with the alcohol dehydrogenase macromolecule. Binding sites were then sorted and selected based on the geometric as well as energetic criteria in order to use the coordinates of these minima for the subsequent construction of a highly specific inhibitor. The quantitative characteristics of the binding sites for each ligand are given in the table, and the orientations of ligands in the most interesting binding sites are shown in Fig. 1. For the AS mapping, we used five ligands of various natures that reflect a diversity of functional groups potentially capable of specific interaction with the enzyme.

Two positions of each ligand were considered distinct if the average distance between them was more than 2.5 Å, which is slightly less than the average size of a ligand itself. The maximum number of the energetically most favorable ligand-protein complexes was originally anticipated to be less than ten. Indeed, the search for the energetically most favorable conformations and their analysis revealed that this threshold exceeds the number of binding sites for each ligand tested (see table). This fact indicates that our procedure was comprehensive. The greatest number of complexes (seven) was revealed for the formate ion, and three to four complexes were found for the other ligands. In most cases, the binding sites of each compound to be analyzed did not coincide with those found for other ligands: they were ≥3 Å apart from each other. Conformation 2 of ammonia (complex 2.1.) and conformation 1 of methylamine (complex 5.1.) were the exceptions, and their binding sites were less than 2 Å apart.

Binding sites that can form one or more specific hydrogen bonds in a close proximity to the enzyme catalytic site are especially interesting for the detection of highly specific interaction between alcohol dehydrogenase and its potential inhibitors. The characteristics of most strong hydrogen bonds for the ligands in this position are given in the table. These can be useful for the subsequent design of promising chemical substances.

In essence, this complete search for all possible conformational ligand states by the Monte Carlo method, the diversity of compounds and their fragments used as ligands in this test, and the wide range of binding sites (with respect to both their binding energy values and their position in the enzyme AS) provide evidence that we revealed all the potential ligand-binding sites in the AS field by using this model.

A Comparison of Binding Sites Found with the Experimental Data on Inhibitor and Water Binding

A combination of most useful sites of complex formation enables the design of pharmacophore models that give an idea of the requirements for an effective inhibitor of the enzyme. An independent assessment of the model ligand-binding sites was obtained from their comparison with water- and inhibitor-binding sites in the structures determined experimentally. The comparison with water seems to be necessary, because it is natural to assume that water molecules are capable of occupying the majority of potential binding sites in the crystal structure of an enzyme. Note that water was not initially included in our model system (the list of ligands) because (1) the water molecule is not an adequate pharmacophore fragment and (2) this would require calculating the ensemble of clusters of water molecules (a whole network) for the strict modeling of water-binding sites; however, in this case, direct comparison with ligand-binding sites would be impeded. Thus, we focused on the experimental data, especially as they offer rather extensive statistics.

The analysis of all possible water-binding sites in the alcohol dehydrogenase AS revealed 22 sites of their

Ligand-protein complexes at the active site of alcohol dehydrogenase

Number	Ligand	Complex number	Interaction energy,* kcal/mol				Hydrogen bonds**				Ligand environment***		
			E_{total}	E_{vw}	E_{el}	$E_{hb\Sigma}$	ligand (donor/acceptor)	residue	bond length, Å	E_{hb}, kcal/mol	residues in AS	distance (Å) from	
												NAD	Zn
1	Formate ion	1.1.	–32.34	–4.91	–26.89	–0.54	–	–	–	–	Val318	4.9	3.7
		1.2.	–30.92	–5.48	–24.92	–0.52	–	–	–	–	Val294	3.3	3.3
		1.3.	–30.70	–6.90	–23.25	–0.55	–	–	–	–	Phe93	3.4	3.4
											Cys174		
		1.4.	–30.59	–4.76	–24.20	–1.63	a	Thr48	1.9	–1.36	Thr48	3.5	3.4
		1.5.	–26.94	–2.87	–22.82	–1.24	a	NAD	2.3	–0.70		7.4	6.6
		1.6.	–7.57	–2.19	0.21	–5.59	d	Asp49	1.8	–2.63	Asp49	9.5	6.8
							a	Cys46	2.1	–0.99	Cys46		
							a	His67	1.8	–1.20	His67		
											Gly44		
											Glu68		
		1.7.	–6.24	–2.83	0.43	–3.84	d	His67	2.1	–1.17	His67	8.7	8.7
							a	Tyr319	2.0	–1.31	Tyr319		
							a	Phe93	2.1	–0.93	Phe93		
											Pro91		
											Gly173		
2	Ammonia	2.1.	–4.50	–3.57	–0.08	–0.86	d	NAD	2.3	–0.86		3.8	4.4
		2.2.	–4.43	–3.67	–0.06	–0.11	–	–	–	–	Phe93	4.0	3.4
		2.3.	–4.27	–2.64	–0.12	–1.51	d	Ala317	2.0	–1.46	Ala317	5.7	10.0
											Thr178		
											Ser182		
											Gly320		
		2.4.	2.34	5.49	–0.33	–2.82	d	Thr178	1.8	–2.73	Thr178	3.0	5.2
											Phe93		
											Cys174		
											Tyr319		
3	Ammonium ion	3.1.	–7.43	–2.16	–0.94	–4.34	d	Phe93	2.0	–1.52	Phe93	8.1	9.7
							d	Thr94	1.8	–2.63	Thr94		
											Asn114		
		3.2.	–5.15	5.31	–3.50	–6.96	d	Val151	1.8	–1.54	Val151	17.6	9.5
							d	Thr143	2.0	–1.34	Thr143		
							d	Thr150	1.8	–2.69	Thr150		
							d	His139	2.4	–0.58	His139		
											Gly142		
		3.3.	0.12	–2.84	2.96	0	–	–	–	–	Leu57	5.8	8.0
											Leu116		
		3.4.	0.13	–2.88	3.01	0	–	–	–	–	Phe93	5.6	7.2
											Leu141		

Table. (Contd.)

Number	Ligand	Complex number	Interaction energy,* kcal/mol				Hydrogen bonds**				Ligand environment***		
			E_{total}	E_{vw}	E_{el}	$E_{hb\Sigma}$	ligand (donor/acceptor)	residue	bond length, Å	E_{hb}, kcal/mol	residues in AS	distance (Å) from	
												NAD	Zn
4	Methanol	4.1.	−12.10	−3.30	−3.97	−4.84	d	Pro296	2.1	−1.29	Pro296	15.7	2.2
							d	Gln299	2.0	−1.54	Gln299		
											Pro295		
											Ser298		
		4.2.	−8.40	−4.89	−0.32	−3.18	d	Val292	1.9	−1.64	Val292	6.5	11.4
							d	Gly293	2.0	−1.44	Gly293		
											Val294		
		4.3.	−7.21	−3.58	−1.36	−2.27	d	Pro296	2.1	−1.30	Pro296	14.6	3.6
							a	Ser298	2.1	−0.98	Ser298		
											Pro295		
		4.4.	−6.55	−3.56	−0.41	−2.59	d	Ile291	1.9	−1.62	Ile291	8.1	11.4
							a	Ala317	2.1	−0.97	Ala317		
5	Methylamine	5.1.	−6.58	−5.64	0	−0.95	d	NAD	2.2	−0.97	Phe93	3.5	3.6
		5.2.	−6.03	−4.66	0	−1.38	d	NAD	2.1	−1.38	Phe93	3.8	5.7
		5.3.	−5.89	−4.68	0	−1.21	d	NAD	2.2	−1.21	Phe93	4.6	6.6

*E_{total}, Energy of interaction of a ligand in a complex with the enzyme, cofactor, and Zn atoms and its constituents: E_{vw}, energy of van der Waals interactions; E_{el}, energy of electrostatic interactions; and $E_{hb\Sigma}$, energy of hydrogen bonds.

**For hydrogen bonds, there are indicated residues that interact with ligand [ligand serves as (d) a donor and (a) an acceptor], lengths and energy, E_{hb}, of bonds. Hydrogen bonds whose energy does not exceed the absolute value of 0.5 kcal/mol are not indicated. A dash indicates the absence of hydrogen bond.

***The amino acid residues whose atoms are located from the ligand at a distance no more than 3 Å are indicated. Distances between the most negatively charged atom of each ligand and the reactive carbon atom C4 of NAD and Zn atom are shown.

preferable binding. These water-binding sites were then compared with the calculated ligand-binding sites in the model structure and with inhibitor binding sites that were experimentally determined for various alcohol dehydrogenases. Binding sites for the majority of ligands turned out to coincide with the sites of preferable water binding. This fact indicates that the water-binding sites may serve as the basis for a comparison with the binding of inhibitors and ligands, because they adequately reflect the energy requirements for binding sites.

Six and four sites that correspond to preferable water binding were revealed when comparing with ligands and inhibitors, respectively. Among them, three water-binding sites coincided and might be considered to be the most preferable sites of ligand, inhibitor, and water binding in the alcohol dehydrogenase AS. Although the number of coincident binding sites may appear to be insignificant, we should note that the majority of 22 water-binding sites are likely to be responsible for only a weak interaction with the protein, while the ligand-binding sites are energetically most favorable.

As an additional test, we compared the inhibitor-binding sites that were identified experimentally and the ligand-binding sites in our model system. Thus, the binding site for acetate ion, an inhibitor of human σ-alcohol dehydrogenase [10], and that for one of the ligands, the formate ion, coincided as shown in Fig. 2. The correspondence of ligand-binding sites revealed by our method and binding sites experimentally determined for some known inhibitors of alcohol dehydrogenase is also obvious.

Thus, the comparison of ligand-binding sites obtained in this study and experimental data on the structural details of the binding of inhibitor and water revealed coincident sites of complex formation. This confirms the validity of our approach and enables the determination of a limited set of requirements for a highly effective inhibitor (the pharmacophore model). This makes real the design of chemical compounds as potential drugs.

EXPERIMENTAL

Structure modeling. The modeling of structures of proteins, ligands, and their complexes and the calcula-

tions of intra- and intermolecular interactions were carried out using an approved program of molecular mechanics ICM [11] applicable to the calculation of complex protein systems [12, 13].

In program ICM, the structure of biological macromolecules is modeled on the basis of a set of internal coordinates: lengths of covalent bonds and valent and torsional angles. The three-dimensional structure of a biopolymer is represented as an oriented open (unclosed) graph (tree), with the numbered atoms at knot points. The algorithm permits the assumption of an arbitrary set of variables to be fixed. Coupled with the presentation of a molecule in internal coordinates, this approach allows the modeling of conformational rearrangements in biopolymers based on rigid geometry optimization, that is, at fixed values of all bond lengths and all valent and some torsional angles, for example, torsion angles that are responsible for the planarity of the peptide bond or aromatic rings. This approach significantly reduces the dimension of expanses of variables and provides for the efficiency of minimization of a function of system energy.

The calculations and visualization of the results were carried out on a graphical station SGI 4D70GT and by using a Pentium II PC.

Optimization of the experimentally determined structure of alcohol dehydrogenase. The model of human alcohol dehydrogenase was built on the basis of a 1HDY PDB structure (complex of alcohol dehydrogenase with NAD^+ and 4-iodopyrazole, at 2.5 Å resolution, the best achieved for human alcohol dehydrogenase). At the first step, a preliminary procedure was performed that involved an adjustment of bond lengths and valent angles to standard (ideal) values according to a model of fixed states and successive minimization of divergences between atoms of the model and of the experimental structure. This procedure provided the refinement of the structure used in modeling, in particular, the localization of hydrogen atoms, whose positions cannot be determined by X-ray studies. The mean square deviation of atomic coordinates of the experimental structure and those of the structure resulting from optimization was negligible (0.4 Å). The details of the procedure are described in [11].

Mapping of the enzyme AS. A method for the mapping of enzyme AS similar to that described in [7–9] has recently found use for the design of compounds that can interact with a target enzyme and serve as highly specific inhibitors. In this approach, the anchoring sites of a small ligand molecule interacting with a protein are considered to correspond to the local minima of energy of protein-pharmacophore interactions. A search for such sites is carried out by using functional groups characteristic of low-molecular mass organic compounds, ligands that might constitute fragments of a whole molecule of hypothetical pharmacophore and provide the necessary property of this model structure, inhibitor-enzyme binding.

Fig. 2. A comparison of ligand binding sites with inhibitor binding sites. Superposition of the ligand in the complex and of inhibitor in human σ-alcohol dehydrogenase. Numbers indicate: *1*, formate ion (complex 1.2, see table); *2*, acetate ion; *3*, cofactor NAD; and *4*, Zn atom. Oxygen atoms of carboxyl groups are indicated for acetate and formate ions; the hydrogen atom of formate ion is shown.

Selection of ligands. A variety of different fragments of organic compounds representing the most important functional groups of the putative inhibitor was used for mapping the alcohol dehydrogenase AS. Relatively simple molecules of molecular mass 17–32 D were selected. The small size of these molecules is important for an effective search for ligand-binding sites because it can help overcome the local minima of the conformational space available to a given ligand. When selecting ligands, the diversity of their physicochemical properties and the feasibility of ligand incorporation into the protein AS were taken into consideration. Ligands should provide the formation of the network of hydrogen bonds, which are usually generated with the involvement of nitrogen and oxygen atoms and are mainly responsible for the specific pharmacophore recognition by protein. The possibility for the application of a ligand to the subsequent design of a larger molecule is also important because the designed pharmacophore should further be chemically synthesized.

The following ligands were selected on the basis of these criteria: formate ion, ammonia, ammonium ion, methanol, and methylamine. Two of them (formate and ammonium ions) are oppositely charged, whereas methanol, methylamine, and ammonia are polar uncharged molecules. All this allows the search for different anchoring sites of ligand-protein binding.

Ligand docking at the alcohol dehydrogenase AS. The search for the anchoring sites of binding was carried out using the iterative procedure of docking the selected fragments (ligands) at the potential field of alcohol dehydrogenase AS.

Each fragment was docked by a simulation of chaotic motion. The procedure included a cyclic repetition of the following steps:

(1) Spatial transfer of ligand molecule in a random direction over a random distance limited by a given greatest distance.

(2) Rotation of ligand molecule around the axis of a random orientation by a random angle ranging from −180° to 180°.

(3) Variations of dihedral angles corresponding to protein atoms located in a close proximity to the region of ligand-protein globule contact. This set of atoms included protein atoms located within an ensemble of atoms inside the sphere of a given radius (sphere of a 5–9 Å radius is usually selected) around each of the ligand atoms, while the ligand torsional angles always remained flexible. Thus, the ligand and the nearby protein segment were considered flexible, while the rest of the protein molecule was considered to be fixed and uninvolved in the conformational changes.

(4) A search for the optimal conformation of the amino acid side chains located close to the ligand-protein contact by using the Monte Carlo method.

(5) A search for the optimal conformation of the polypeptide backbone (using the method of the local deformation) and the amino acid side chains located close to the point of contact by using the Monte Carlo method. The optimization procedure used in the ICM molecular mechanics program has been described in detail in [11].

Ligand orientation in the field of the alcohol dehydrogenase AS (free region between Zn atom of the active site, the nicotine ring of NAD, and Thr48 residue) was selected as a starting conformation. This region is sterically accessible for ligands that are close in size to ethanol. By imposing "penal" potentials, the calculating algorithm confines movements (floating) of the ligand tested to the enzyme AS.

The energy of the ligand-alcohol dehydrogenase interaction at each point of the conformational space was calculated as a sum of van der Waals forces, hydrogen-bond energy, and electrostatic interactions between ligand atoms, the alcohol dehydrogenase molecule, cofactor NAD, and Zn atom using the ECEPP/3 parameters [14–17]. Partial charges on the atoms of ligands, cofactor, and Zn atom were calculated by the method of semi-empirical analysis CNDO [18] using HyperChem 3.0 software [19]. After the calculation of the energy of the system, a new point is either accepted or rejected according to the Metropolis criterion [20] in accordance with system temperature, and the docking procedure moves to a new iteration.

The resulting positions of a ligand and its corresponding alcohol dehydrogenase complexes were sorted out according to their energy and proximity to the active site and each other; a position with the lowest energy and closest to the active site was selected, and its parameters were subjected to subsequent validation.

Comparison of model ligand-binding sites and inhibitor- and water-binding sites in the structures determined experimentally. To analyze the water-binding sites, the alcohol dehydrogenase structures whose X-ray data contain water molecules were taken from PDB. Twenty-two such proteins were found. All of these proteins were oriented the same way according to data on their pairwise spatial alignment from the bank of structural alignment FSSP [21]. The modeling of sites of complex formation was confined to a sphere of 10 Å radius centered at the catalytic Zn atom. All water-binding sites located in this region were then combined into groups. Water molecules were considered to be a group if their centroids fell within a sphere with a radius of 1.4 Å, which is usual for the approximation of the water molecule. By distributing all water-binding sites over such spheres, a total of 54 sites of preferable binding were revealed in the enzyme AS, from which 22 were chosen, including two or more water molecules from experimental structures.

The water-binding sites revealed were then compared with the inhibitor-binding sites experimentally determined for various alcohol dehydrogenases and with the ligand-binding sites calculated in our model. The comparison was performed according to the position of oxygen atoms. Initially, 13 protein structures containing inhibitors were selected, with 10 inhibitors having various chemical natures. It turned out that the inhibitor molecules occupy the sites of preferable water binding in 12 cases. A total of 4 regions were revealed. This implies that water-binding sites, which were taken as a starting point for the comparison of data between inhibitor and ligand binding, adequately reflect the energetic requirements for binding sites.

A similar procedure was applied to the comparison of all 11 complexes of the two ligands of the model structure containing oxygen (ligands 1 and 4). Seven of them coincided with the sites of preferable water binding. Six sites of preferable water binding were revealed in total. Three sites coincided among four sites corresponding to inhibitor binding and six sites corresponding to ligand binding.

As an additional control, the comparison between inhibitor-binding sites in the structures determined experimentally and ligand-binding sites in the model system was carried out based on the positions of oxygen and nitrogen atoms. All 5 ligands from all 22 model complexes and 11 molecules of inhibitors of various chemical natures from 16 protein structures, which were taken from PDB, were initially selected. Four model complexes and seven inhibitors turned out to coincide; these were acetate ion, iodopyrazole, cyclohexanol, *N*-cyclohexylformamide, *N*-formylpiperidine, dimethyl sulfoxide, and natural inhibitor, ethanol.

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