



HETEROLIGATION OF A MOUSE MONOCLONAL IgE ANTIBODY (La2) WITH SMALL MOLECULES, ANALYSED BY COMPUTER-AIDED AUTOMATED DOCKING

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Abstract—A mouse monoclonal anti-TNP IgE antibody (IgE-La2) was screened by a competitive-binding ELISA with a random pool of over 2000 small molecules, mostly drugs, drug derivatives and metabolites. Thirteen of these (naproxene, β -carboxy- α -naphthol, oxolinic acid, hymecromone, 8-aminoquinoline, β -naphthylamine, α -nitro-cinnamic acid, 1,5-diaminonaphthalene, pronium iodide, diaspirin, 3,4,5-trimethoxy-cinnamic acid, cycrimine, hemimellitic acid) were found to bind as strongly, or stronger, to the antibody as the immunizing hapten. We have used a Monte Carlo search technique for simulated docking of the DNP and non-DNP ligands to a model of the F₁ region of IgE(La2). The validity of structural predictions made by the AutoDock program were tested on IgG(ANO2), the three-dimensional structure of which had been obtained previously by X-ray crystallography and 2D-NMR. The rms differences between the experimentally determined and auto-docked complexes in the energetically most favored binding modes were 0.31–0.44 Å. Evaluation of structures of IgE(La2)–ligand complexes [including 2,4-dinitrophenol (DNP), 16 DNP amino acids, and the 13 non-DNP ligands listed above] obtained by computer-aided automated docking, suggested the existence of two *subsites* within an approximately 12 × 18 Å² groove extending between the H and L CDRs. Some of the ligands (DNP-Glu, 8-aminoquinoline, pronium-I, β -naphthylamine) were found to bind exclusively to *subsite* 1, others (DNP-Ala, α -nitro-cinnamic acid, hemimellitic acid, β -carboxy- α -naphthol) to *subsite* 2. The majority of DNP amino acids and other ligands (oxolinic acid, 3,4,5-trimethoxy-cinnamic acid, diaspirin, [R]-cycrimine) were found to occupy an overlapping area including subsites 1 and 2, while some of the compounds (DNP-Asn, DNP-Pro, hymecromone, 1,5-naphthylenediamine) were predicted to interact with either of these subsites with comparable probabilities. When all of the docked La2–ligand complexes were taken into account, five tyrosine residues (H33, L32, L91, L92, L96) were found to provide the majority (53.4%) of all observed contact points. Thus, a multitude of interactions with aromatic residues, and a combinatorial type of interaction within the binding region, seem to be the major factors to explain the mechanism of heteroligation by IgE(La2).

Key words: Multispecific IgEs, automated docking, simulated annealing.

INTRODUCTION

Earlier we screened a mouse monoclonal IgE with more than 2000 small molecules (mostly drugs and their metabolites) and found a large number of interactions with ligands that have no apparent structural similarity to the immunizing hapten (2,4-dinitrophenol, DNP) (Varga *et al.*, 1991a). Although heteroligation of antibodies has been demonstrated decades ago (reviewed by Richards *et al.*, 1975), the structures of antibody–heteroligand complexes are yet to be elucidated. Beyond its implications for conceptual immunology, information on these structures

could have relevance for interpreting the molecular mechanisms of multiple allergies and the highly prevalent allergic cross-reactions (Varga *et al.*, 1991a, 1991b). Determination of three-dimensional structures of IgE–ligand complexes by X-ray crystallography with such large number of ligands by currently available technology is considered to be too laborious to be practically feasible. In order to obtain structural information on IgE–heteroligand complexes, we have cloned and sequenced two mouse monoclonal anti-TNP IgEs (Kofler *et al.*, 1992), and predicted their three-dimensional structure by computer-aided molecular modeling (Droupadi *et al.*, 1994). The two IgEs were then screened with the same pool of small molecules as used before (Varga *et al.*, 1991a;

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Winger *et al.*, 1995) and a number of ligands were identified that bound to the antibody as strongly, or stronger than the immunizing hapten. The model structures of ligand-IgE(F_v) complexes were then computed and displayed by a modified automated docking protocol (Goodsell and Olson, 1990).

METHODS

Screening and affinity measurements

The mouse IgE(La2) hybridoma cell line was obtained from the American Type Culture Collection (ATCC-TIB142, Rockville, MD, U.S.A.). The hybridoma was generated by fusing spleen cells from ascaris suum-coupled 2,4,6-TNP-immunized BALB/c mice with the non-secretor P3X63Ag8.653 myeloma line (Rudolph *et al.*, 1981). The monoclonal antibody was produced in a serum-free medium, purified by ammonium sulphate precipitation and affinity chromatography (Wongyai *et al.*, 1991) and screened with a collection of more than 2000 compounds (listed in Varga *et al.*, 1991a) by a competitive ELISA (Winger *et al.*, 1995). The association constants of high-affinity ligand binding were measured by UV spectroscopy (Droupadi *et al.*, 1994).

Computer-aided molecular modeling

The model structure of the IgE(La2) F_v region was generated by homology modeling from sequence data (details are described in Droupadi *et al.*, 1994). For docking, the atomic coordinate file had to be further edited. Because an all-atom-approach with explicit hydrogens was chosen, non-polar hydrogens were added to the F_v model; this was done with the SYBYL-biopolymer-module (Tripos Associates, 1994). For the calculation of the electrostatic interaction, atomic charges were added to the coordinate file; charges from the AMBER all-atom force field (Weiner *et al.*, 1984, 1986) were used for this purpose. Finally, the file had to be edited to distinguish between "normal" hydrogens and hydrogens potentially capable of forming hydrogen bonds (*vide infra*). As for the haptens, 30 different molecules were investigated, 16 of which were DNP amino acids, the other 14 mostly aromatic compounds (see Fig. 2). The structures were generated with the tools provided by the modeling program SYBYL (Tripos Associates, 1994) and optimized with the Tripos Force Field to get a strain-free starting conformation for the docking process. Partial atomic charges were assigned using the Gasteiger-Hückel formalism. The compounds were used in the ionized form, which at physiological pH was estimated to be predominant. To generate a starting position for the docking search, the hapten coordinates were saved at freely chosen orientations somewhere above the center of the La2-CDR-region. The atom types were changed again in a way to mark potentially H bond-forming hydrogens.

Docking procedure

The docking searches were performed with the AutoDock program of Goodsell and Olson (1990). They use

the Metropolis Monte Carlo algorithm of simulated annealing for positional and conformational searching, in combination with a rapid energy evaluation through pre-calculated grids of molecular affinity potentials. This approach of energy calculation allows the inclusion of terms for the Van der Waals (VDW), electrostatic and hydrogen-bonding interaction. The program was slightly modified, in order to allow the definition of more than four atom types in the ligand, since a distinction was made between non-polar hydrogens and hydrogen atoms bonded to O or N, potentially able to form H bonds. For the latter, a 12,10 potential for the interaction with oxygen atoms was used. For the VDW interaction of all the other atom types, the Lennard-Jones 12,6 potential was applied. Potential parameters were taken as provided by the authors of the program and checked for applicability to our approach by comparison with parameters from other sources, as well as by docking tests on a known complex structure (*vide infra*). Electrostatic interaction was taken into account through the Coulombic potential, using a dielectricity constant of 40. The grids of affinity potentials were calculated over a volume of $30 \times 30 \times 30$ Å and centered on the CDR region; the spacing between the grid points was 0.5 Å. A set of simulation parameters was used which, in many tests reported in the literature (e.g. Goodsell and Olson, 1990; Stoddard and Koshland, 1993) and in tests carried out by ourselves, was able to reproduce experimental results and seems physically reasonable; thus the maximum step sizes for the random motion of the haptens were set to 0.2 Å for translation, 5° for rotation and 5° for torsions; the initial energy (RT in the Boltzmann factor) was set to 100 kcal/mol and reduced by a factor of 0.9 after a maximum of 30,000 accepted or rejected steps; the whole process of simulated annealing consisted of 50 such temperature-reduction cycles. While the structure of the antibody had to be assumed to be rigid, torsional degrees of freedom in the haptens could be taken into account by specifying rotatable bonds in the AutoDock input file and giving a set of constraints for interatomic distances in order to avoid intramolecular clashes or completely unfavorable conformations. For each hapten, 10 independent docking simulations (runs) were performed, one run taking between 15 and 60 min of CPU time on a SGI R4000 workstation, depending mainly on the size and flexibility of the hapten.

Analysis and interpretation of the docking output

The configurations obtained for one ligand in 10 AutoDock runs were analysed first for geometric similarity. Positions differing by less than 1 Å in the rms displacement were taken as identical and represented by the energetically top ranked of these solutions (the energy differences within them were generally very small). To this configuration a correspondingly higher occurrence frequency was assigned. The binding energy of the complexes thus found was re-evaluated with the same energy function as in AutoDock, but without the grid approximation, i.e. explicitly considering each protein atom. The

values obtained this way (additionally split into steric and electrostatic contribution) usually differed only slightly from the primary docking energies; in some cases, however, the ranking of the solutions changed. The obtained energies are to be interpreted only as scoring values for the docking of one hapten, their absolute values have an uncertain physical significance and are approximately one order of magnitude higher than free energies of binding measured experimentally. The energy differences within a set of solutions for one hapten thus serve only as a guideline for the quality of the geometry of interaction between ligand and receptor. The configurations were then analysed for VDW contacts (according to the definition of Sheriff *et al.*, 1987) and H bonds. As far as the latter are concerned, one should bear in mind that: (i) the protein was assumed to be static during the docking search; and (ii) no angle dependency was included in the H bond potential. Therefore, the geometry of the H bonds described below sometimes appears not to be ideal; nevertheless, these arrangements are reported as H bonds, since the real dynamic system could, in most cases, easily achieve better orientations (e.g. rotation of the OH of tyrosines). The work described here was done with the aid of two additional programs written in-house.

Predictive accuracy of the method: the structure of the Ab ANO2-hapten complex

We used the modified AutoDock program for the generation of DNP-Ab(ANO2) complexes to compare the modeled/docked structures to the one obtained by X-ray crystallography (Brünger *et al.*, 1991) and by nuclear magnetic resonance spectroscopy (Theriault *et al.*, 1991). By these studies, the substituted DNP hapten was found to be sandwiched between the indole sidechains of Trp91 of the L chain and Trp96 of the H chain [Fig. 1(A)]. We performed two tests, beginning the search from two different starting positions of the hapten, generated by moving it randomly away from the experimentally found location. The result was almost identical for both tests: in each case, 10 runs yielded two different results, one of which — the energetically favored solution — was nearly identical to the one observed experimentally (rms difference of 0.32 and 0.44 Å, respectively) [Fig. 1(A)]. The other, less favored, orientation was still located in the same site, with the aromatic system oriented in the same way, but the two ligands of the amino groups interchanged, i.e. the molecule rotated 180° around an axis through the unsubstituted atoms of the phenyl ring [Fig. 1(B)].

RESULTS

Heterologation by IgE(La2)

In agreement with earlier observations made on another mouse monoclonal IgE (clone SPE-7, Varga *et al.*, 1991a), the affinity of various DNP amino acids to IgE(La2) is greatly affected by the structure of amino acid sidechains. Thus, compared to DNP-OH, the alanil

moiety increases the strength of binding by almost an order of magnitude (Fig. 2). Generally, apolar sidechains (Ala, Pro, Val) tend to increase, while polar (Thr, Ser) and charged ones (Glu) decrease the energy of binding. In order to study heterologation by IgE(La2), 2078 compounds (listed in Varga *et al.*, 1991a) were screened by a competitive ELISA (Winger *et al.*, 1995); of these, 161 were mono-, di- or trinitro derivatives of aromatic compounds, which were all inhibitory at the > 90% level. In agreement with an earlier report (Furusawa and Ovary, 1988) we found that this antibody has a higher affinity to DNP than to the immunizing hapten (TNP). Out of the 1917 non-DNP compounds tested, 122 inhibited the binding of IgE(La2) to the polystyrene-bound DNP by more than 50%, when added in millimolar concentrations. The binding constants of selected compounds with > 90% inhibition were measured by a spectroscopic assay (Droupadi *et al.*, 1994) and are shown in Fig. 2. The affinity of some of the non-DNP ligands (naproxene and β -carboxy- α -naphthol) is higher by an order of magnitude than those of the strongest binding DNP amino acids, while others are generally in the same range. There is no obvious structural similarity between the immunizing hapten (TNP) and most of the ligands found by screening. While the majority of the screened non-nitrophenyl ligands are aromatic compounds, some aliphatics (e.g. prolonium iodide) were found with affinities comparable to DNP amino acids (Fig. 2).

Automated docking of DNP amino acids and non-DNP ligands to IgE(La2)

Docking of the haptens and ligands has revealed the existence of two spatially separate subsites. *Subsite 1* appears as a cavity formed by CDRs H2, L1 and L3; and *subsite 2* is formed by CDRs H1 and H3. Some of the ligands were found to occupy either subsite 1, or subsite 2; others were found to be accommodated in intermediate positions. A group of ligands was found to bind alternately to subsite 1 or subsite 2.

Ligands docking to subsite 1 (SS1)

The docking of 8-amino-quinoline takes place in SS1 [Fig. 3(A) and Table I]. The aromatic ring is sandwiched onto TyrL32 and further VDW contacts are made with TyrL27D, TyrL91 and TyrL92. In addition, a H bond is formed between one amino H atom of the ligand and the carbonyl-O of TyrL91, the distance being 2.13 Å. This conformation was found in eight out of the 10 runs and it is energetically more favorable than the other two minor solutions. The docking of β -naphthylamine takes place in SS1 in an identical orientation. Here again, the primary contact is with TyrL32 (π -stacking); VDW contacts are seen with TyrL27D, TyrL91 and TyrL92. Similarly to 8-aminoquinoline, a H bond is formed between the amino H atom of naphthylamine and the carbonyl-O of TyrL91 (which is at 2.04 Å). Again, this was the most favored conformation and was found in 8/10 runs. The binding of DNP-Glu also takes place in SS1, however, the orientation of the DNP ring is different from the previous

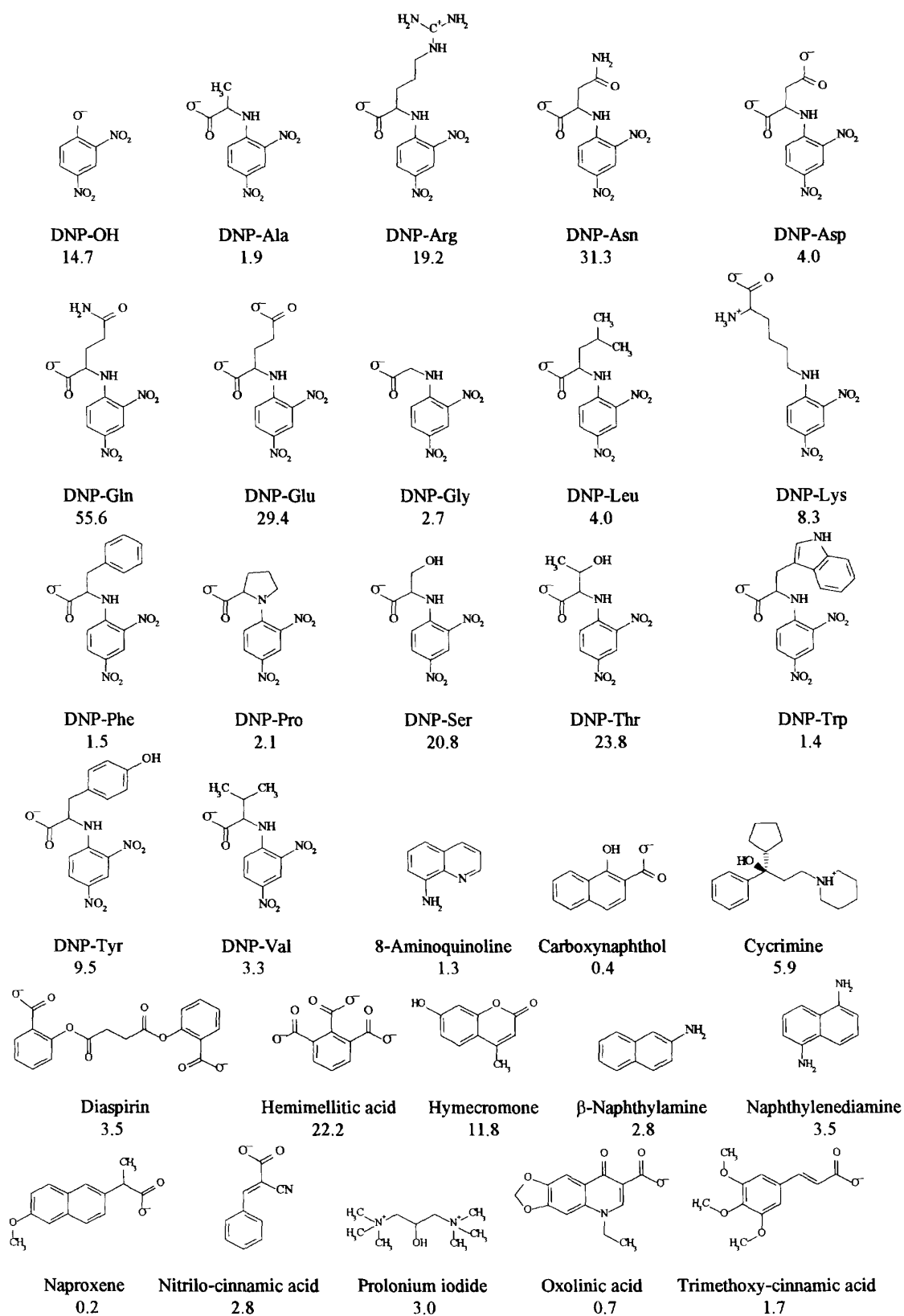


Fig. 2. Structures of La2-ligating DNP amino acids and non-DNP compounds found by screening; the K_D values (in μM) are shown under the names of compounds.

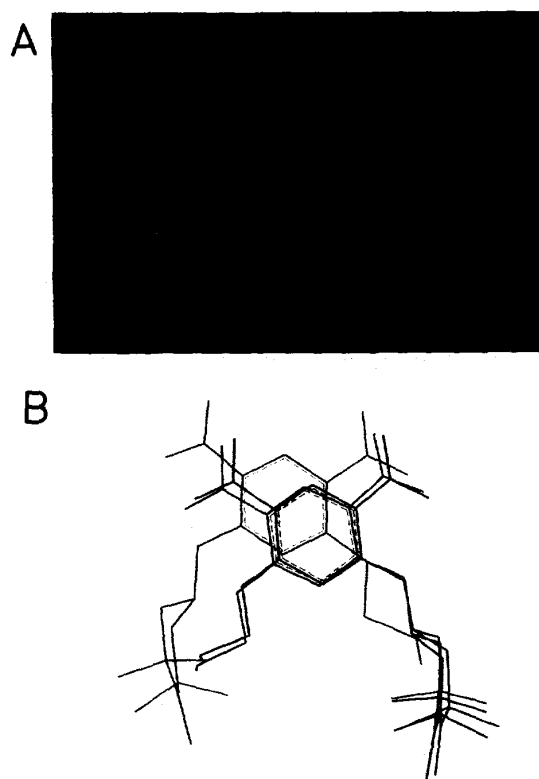


Fig. 1. Binding of the DNP ligand to IgG(ANO2). (A) Ligand position determined by X-ray crystallography (Brünger *et al.*, 1991) and 2D-NMR (Theriault *et al.*, 1991) (ligand in red) compared with the position obtained by automated docking (blue). (B) Superposition of the X-ray position (red) with the two docking results; energetically most favored in blue, less favored in green.

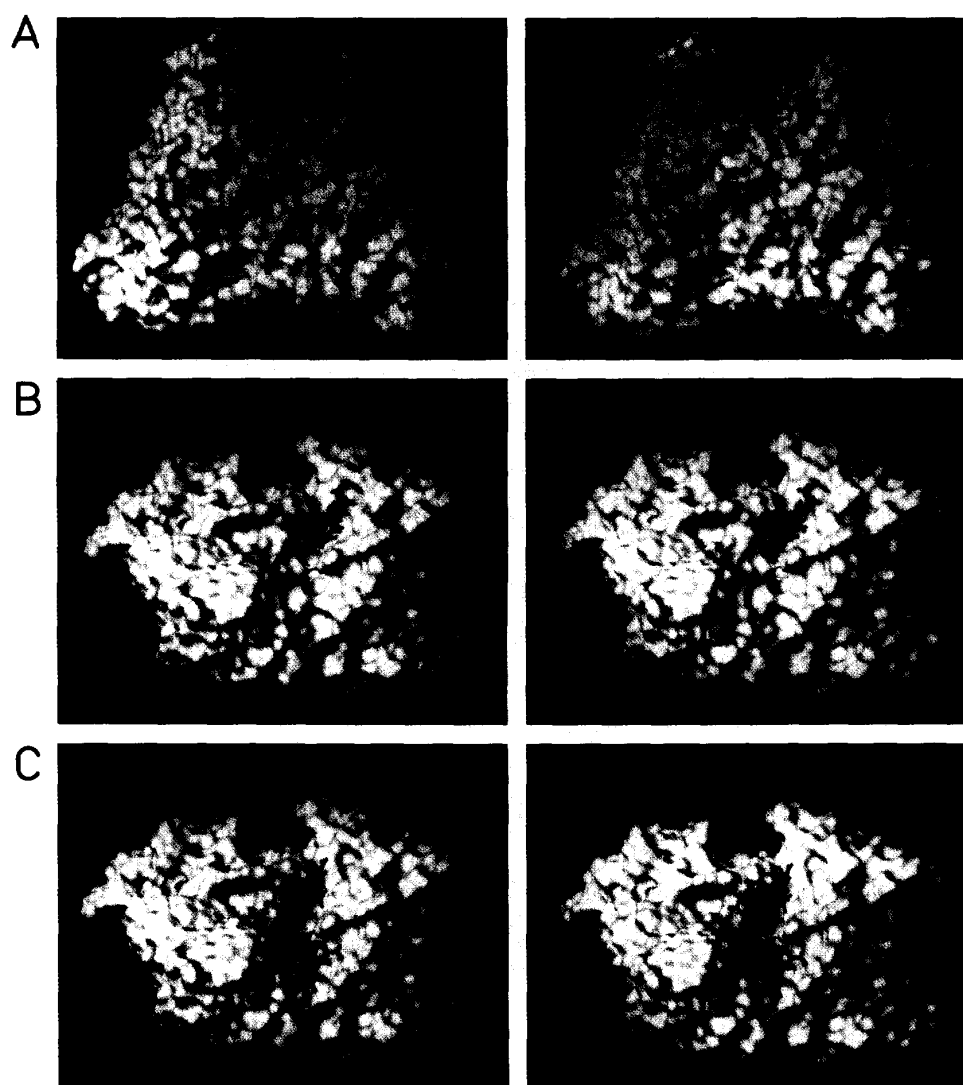


Fig. 4. Stereoview of ligand complexes (composite picture). (A) Ligands occupying SS1 (side view). (B) Ligands occupying SS2 (top view). (C) All ligands in SS1 and SS2 (top view).

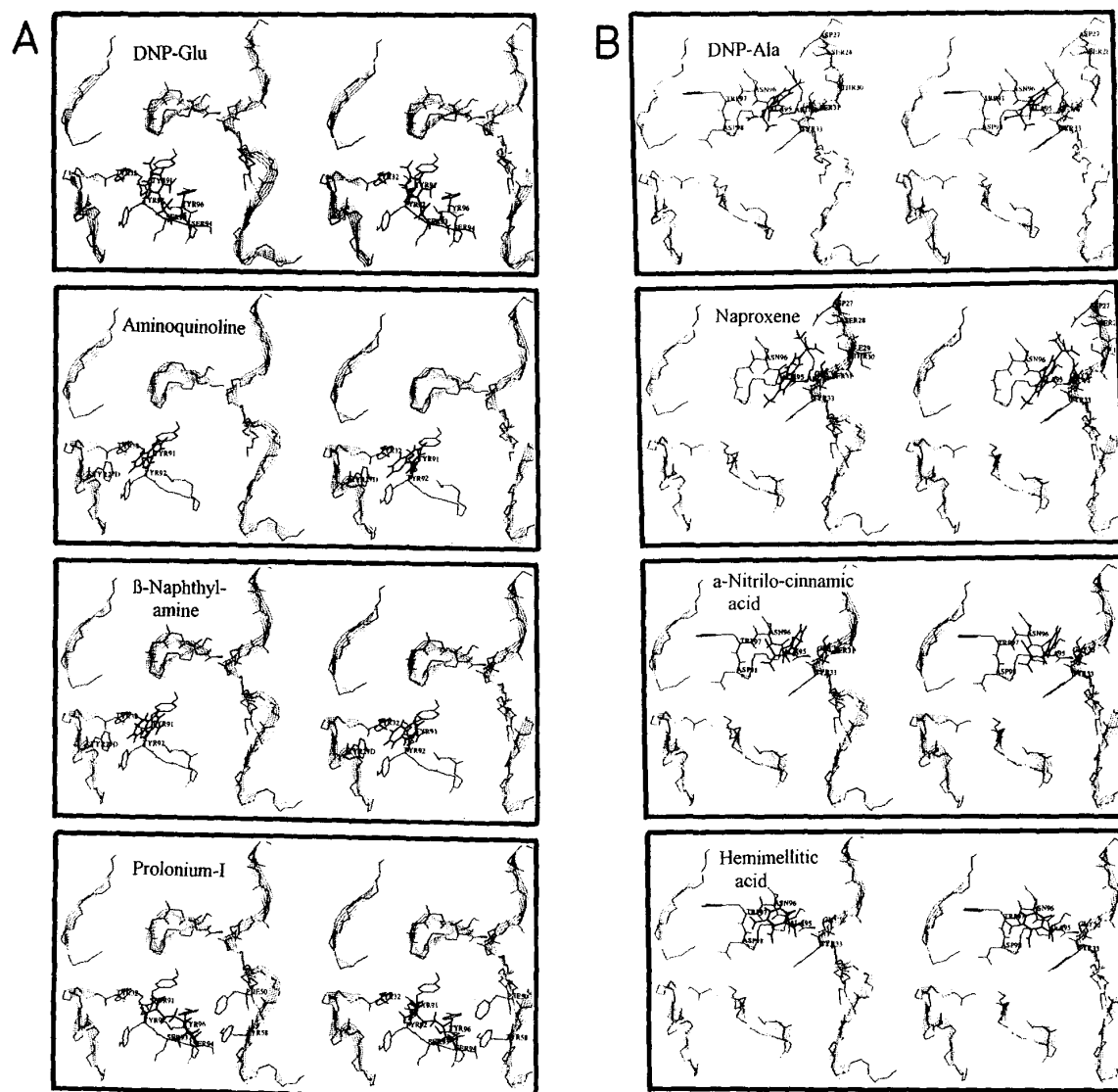


Fig. 3. Stereo view of ligand complexes. (A) Occupying SS1. (B) Occupying SS2.

examples, and there is no indication of π -stacking between the DNP ring and aromatic sidechains. Instead, VDW contacts are formed between the DNP moiety and TyrL32, TyrL91, TyrL92 and TyrL96; also, the Glu side-chain is contacted by SerL93 and SerL94. Hydrogen bond formation is suggested between the 2-nitro group and the phenolic H of TyrL96 [distance (d) = 2.0 Å] and the 4-nitro group and the phenolic H of TyrL32 (d = 2.28 Å), between the amino-H and the carbonyl-O of TyrL92 (d = 2.04 Å) and between an O of the terminal carboxylate group and the amido-H of SerL94 (d = 1.94 Å). This conformation was found with an occurrence frequency of 5/10 and was energetically distinctly more favorable than the other results. In one of the simulations, this ligand was found to occupy SS2, while in another one, it occupied an area between the two subsites; however, these conformations were energetically less favorable (by 2 kcal/mole and 10 kcal/mole, respectively). Two other

examples of DNP amino acids binding with preference to SS1 are *DNP-Leu* and *DNP-Ser*. The docking site of *prolonium iodide* is almost identical with that of *DNP-Glu*, but additional VDW contacts are made with PheH50 and TyrH58. Hydrogen bond formation is indicated between the O of the ligand's OH and the H of TyrL96 (d = 2.19 Å). The electrostatic interaction is unfavorable; indeed, no explicit negative countercharge can be seen in the vicinity of the docking location (the nearest negatively charged residue is AspH98 at approximately 7 Å). This conformation was found with a frequency of 7/10, 3 kcal/mole more favorable than the next solution. A composite picture of ligands docking to SS1 is shown in Fig. 4(A).

Ligands docking to subsite 2 (SS2)

The docking of *DNP-Ala* occurs through VDW contacts with CDR H1 (mostly Ser31, Gly32, Tyr33) and

Table 1. Overview of interactions shown by haptens docking to subsite 1 (left part) or subsite 2 (right part); every hapten is represented here by its energetically best result

	Amino-quinoline	DNP-Glu	Naphthyl-amine	Pronium iodide	Carboxy-naphthol	DNP-OH	DNP-Ala	Hemimellitic acid	Naproxene	Nitrilo-cinnamic acid
Subsite 1										
CDR H2	Phe H50				V (3)					
	Asn H52									
	Asn H56									
	Tyr H58				V (9)					
CDR L1	Tyr L27D	V (7)		V (8)						
	Tyr L32 (OL)	V (27), P	V (16), Hs	V (27), P	V (11)					
CDR L3	Tyr L91 (OL)	V (9), Hm	V (12)	V (19), Hm	V (5)	V (2), Hs	V (2)			
	Tyr L92	V (23), Hm	V (7), Hm	V (19), Hm	V (20), Hm					
	Ser L93		V (7)		V (13)					
	Ser L94		V (7), Hm		V (8)					
	Tyr L96		V (9), Hs		V (10), Hs					
Subsite 2										
CDR H1	Asp H27							V (1)		V (2)
	Ser H28							V (1)		V (5)
	Ile H29									V (2)
	Thr H30							V (2)		V (2)
	Ser H31							V (9)		V (10), Hm
	Gly H32							V (23)	V (7)	V (15)
	Tyr H33 (OL)							V (18)	V (13), Hm	V (3)
CDR H3	Arg H94							V (7), Hs		V (7), Hs
	Ala H95							V (10)	V (14)	V (10)
	Asn H96							V (6)	V (8)	V (11)
	Trp H97							V (5), Hm	V (21), Hm	V (9), Hm
	Asp H98 (OL)							V (3), Hm	V (10), Hm	V (5), Hm

V: Van der Waal's contacts (number of contacts); Hs: H-bonds with sidechain; Hm: H-bonds with mainchain; P: π stacking; OL: overlap-contacts between subsites.

CDR H3 (Arg94, Ala95, Asn96, Trp97, Asp98) [Fig. 3(B), Table 1]. In addition, H bond formation is suggested between nitro-O (of the 4-nitro group) and guanidino-H of ArgH94 ($d = 2.22 \text{ \AA}$), between carboxylate-O and amido-H of TrpH97 ($d = 2.01 \text{ \AA}$), and between carboxylate-O and amido-H of AspH98 ($d = 2.01 \text{ \AA}$). This conformation was found with a frequency of 6/10. The same ligand was found less frequently (2/10) in a different and slightly less favored ($\Delta E = 0.7 \text{ kcal/mole}$) orientation in SS2. Two of the simulations indicated two binding modes in SS1 with much less favorable energetics. *Naproxene* docks in the same narrow groove between the H1 loop, contacted by Asp27, Ser28, Ile29, Thr30, Ser31, Gly32, Tyr33. The major contacting residue for both naproxene and DNP-Ala is GlyH32. On the H3 side, the contact residues are Arg94, Ala95 and Asn96. Hydrogen bond formation is indicated between the carboxylate group and amido-Hs of SerH31 ($d = 2.25 \text{ \AA}$) and GlyH32 ($d = 1.97 \text{ \AA}$). This most preferred conformation was found with a frequency of 1/10. The other simulations have yielded different conformations in SS2, SS1-2 and in SS1; the best of the latter, however, with an energy of 6 kcal/mole less favorable. The docking of *hemimellitic acid* also takes place in SS2; however, in this case the H3 loop provides the primary contacts through Ala95, Asn96, Trp97 and Asp98. The H1 loop is contacted by the ligand through Gly32 and Tyr33. Hydrogen bond formation is indicated between the ligand carboxylates and the amido-Hs of TyrH33 ($d = 2.03 \text{ \AA}$), TrpH97 ($d = 2.06 \text{ \AA}$) and AspH98 ($d = 2.06 \text{ \AA}$). Although this conformation has occurred with a frequency of only 3/10, it had the most favorable energetics. The complex obtained with the highest frequency (5/10) occupies the same subsite in a slightly different orientation. In the first complex there is no indication for ion-pair interaction between the ligand carboxylates and the binding site, since the positively charged residues lie beyond reach (e.g. LysL30 at 7 $\text{\AA}$; ArgH94 at 5.5 $\text{\AA}$); in complex Nos 2 and 3, however, it should be taken into consideration that the carboxylate group of No. 2 has a distance of 3–4 $\text{\AA}$ from the guanidinium of ArgH94, whereas the ammonium group of LysL30 is separated from complex No. 3 by only 2–3 $\text{\AA}$. In any case, the electrostatic contribution to the total energy is highly attractive for all docking solutions found. The complex of α -nitro-cinnamic acid is held by a very similar set of contact residues as with hemimellitic acid: SerH31, GlyH32, TyrH33, AlaH95, AsnH96, TrpH97 and AspH98. Hydrogen bond formation is suggested between the carboxylate and the amido-H of AspH98 ($d = 1.94 \text{ \AA}$), as well as the amido-H of TrpH97 ($d = 2.15 \text{ \AA}$). This most favorable conformation was found with a frequency of 3/10; a very similar orientation of the ligand was found in the same subsite, with the same occurrence frequency, and slightly less favorable energetics. The remaining simulations yielded complexes in SS1 with energies clearly less attractive. *Carboxynaphthol* and *DNP-OH* also prefer SS2 as docking site, however they show already some minor contacts with TyrL91. The composite picture of ligands docking to SS2 is shown in Fig. 4(B).

Ligands docking to overlapping areas between SS1 and SS2

The majority of DNP amino acids and non-DNP ligands were found to dock in an overlapping area between SS1 and SS2 (Fig. 5, Table 2). One group of these compounds (e.g. cycrimine-[R], cycrimine-[S], diaspirin) occupy predominantly SS2, and some others (e.g. DNP-Gln, oxolinic acid) SS1 (Table 2). Representative examples are shown in Fig. 5.

The *DNP-Gln* lies mostly in SS1 (VDW contacts with TyrL32, TyrL91, TyrL92, TyrL96), extending with the aromatic ring into SS2 (VDW contacts with TyrH33 and AspH98). Hydrogen bond formation is suggested between the 2-nitro group and phenolic H of TyrL32 ($d = 2.11 \text{ \AA}$), between the 4-nitro group and amido-H of AspH98 ($d = 2.06 \text{ \AA}$), between the ligand carboxylate and phenolic H of TyrL96 ($d = 2.18 \text{ \AA}$), and between the

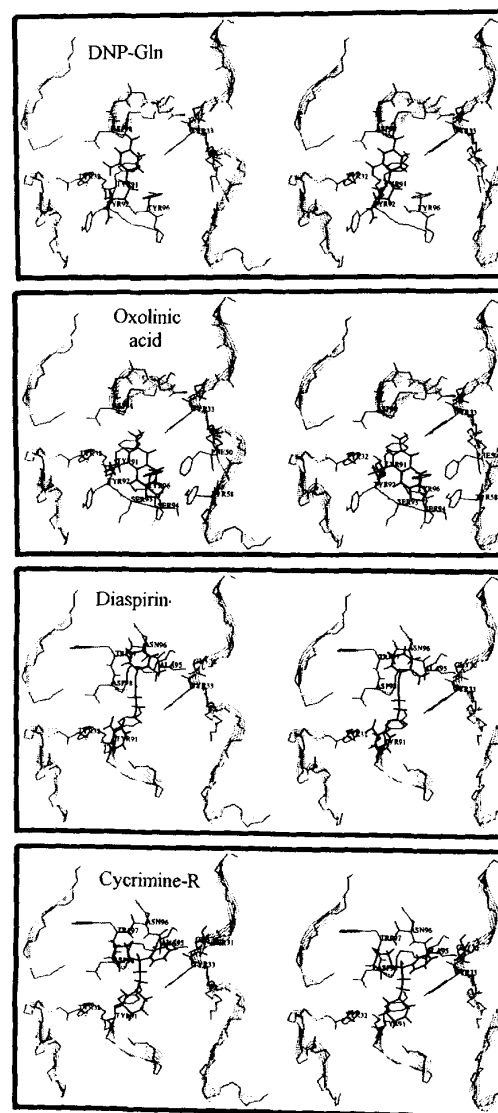


Fig. 5. Stereoview of ligand complexes occupying SS(1-2).

Table 2. Overview of interactions shown by haptens docking to overlapping areas between subsites 1 and 2; every hapten is represented here by its energetically best result

		Cycrimine [R]	Cycrimine [S]	Diaspirin	DNP-Gln	Oxolinic acid	Trimethoxy- cinnamic acid
Subsite 1							
CDR H2	Phe H50					V (12)	
	Asn H52						
	Asn H56						
	Tyr H58					V (3)	
CDR L1	Tyr L27D						
	Tyr L32 (OL)	V (3)	V (4)	V (14), Hm	V (14), Hs	V (13)	V (24), Hs
CDR L3	Tyr L91 (OL)	V (20), Hs	V (29), Hs	V (20)	V (23)	V (27)	V (19)
	Tyr L92			V (4)	V (23), Hm	V (13)	
	Ser L93					V (4)	
	Ser L94					V (6)	
	Tyr L96				V (4), Hs	V (12)	V (3)
Subsite 2							
CDR H1	Asp H27						
	Ser H28						
	Ile H29						
	Thr H30						
	Ser H31	V (1)					
	Gly H32	V (8)	V (8)	V (2)			
	Tyr H33 (OL)	V (31), P	V (33)	V (10), Hm	V (5)	V (8)	V (2)
CDR H3	Arg H94						
	Ala H95	V (9)	V (1)	V (9)			V (5)
	Asn H96	V (1)	V (8)	V (10)			V (1)
	Trp H97	V (12), Hm	V (6)	V (16)			V (5), Hm
	Asp H98 (OL)	V (26), Hm	V (15), Hm	V (16), Hm	V (16), Hm	V (1)	V (17), Hm

V: Van der Waals contacts (number of contacts); Hs: H-bonds with sidechain; Hm: H-bonds with mainchain; P: π stacking; OL: overlap-contacts between subsites.

ligand amido-H and carbonyl-O of TyrL32 ($d = 2.03$ Å). Occurrence frequency: 6/10; only one different orientation was found with lower (4/10) frequency and slightly less favorable energetics. Many other DNP amino acids are located in the SS1–2 area, namely *DNP-Arg*, *-Asp*, *-Gly*, *-ε-Lys*, *-Phe*, *-Thr*, *-Trp*, *-Tyr* and *-Val* (not shown). *Oxolinic acid* also occupies mostly SS1 (main VDW contacts with PheH50, TyrH58, TyrL32, TyrL91, TyrL92, TyrL96) extending into SS2 (VDW contacts with TyrH33 and AspH98). One-half of the runs have yielded this configuration, which is the most favored one, although no H bonds are seen. Different configurations were found in the rest of the runs, with minor differences in the calculated scoring energy. *Diaspirin* is one of the best examples for overlapping ligand docking, since one of the acetyl-salicyl groups lies in SS1 (VDW contacts with TyrL32, TyrL91, TyrL92), the other in SS2 (VDW contacts with GlyH32, TyrH33, AlaH95, AsnH96, TrpH97, AspH98). Hydrogen bond formation is suggested between the ester-O and amido-H of AspH98 ($d = 2.15$ Å), and between the two carboxylates and amido-H of TyrH33 ($d = 2.3$ Å) and phenolic H of TyrL32 ($d =$

2.22 Å), respectively. This complex was found with an occurrence frequency of only 2/10, but with the highest calculated binding energy. In the most frequently (4/10) obtained complex, the ligand assumed a different orientation in the same site with an energy of 5 kcal/mole lower. In the *cycrimine*-[R] complex, the piperidiny ring lies in SS1 (VDW contacts with TyrL32 and TyrL91), the cyclopentyl and phenyl rings in SS2 (VDW contacts with SerH31, GlyH32, TyrH33, AlaH95, AsnH96, TrpH97, AspH98). Hydrogen bond formation is suggested between the alcoholic O and amido-H of AspH98 ($d = 1.96$ Å), and between the amino-H and the phenolic O of TyrL91 ($d = 2.24$ Å). The aromatic ring of the ligand is sandwiched on to TyrH33. The π -stacking between cycrimine-[R] and Tyr H33 is suggested, which could be responsible for the charge-transfer complex signal observed experimentally (Droupadi *et al.*, 1994). From docking, only some interactions with the backbone of TrpH97 are suggested. A CT complex formation with this residue seems accessible only when rotation of the indolyl sidechain would be allowed. Every simulation has yielded different, mostly overlapping, ligand orientations,

but the complex shown in Fig. 5 had the largest calculated energy of binding (the two less favorable results are different orientations in SS1), the remainder of the simulations giving energies lower by 4–14 kcal/mole. *Cycrimine*-[S] and *trimethoxy-cinnamic acid* have all been found to occupy the SS1–SS2 binding region in similar, overlapping positions (Table 2).

Ligands occupying alternate binding positions

A group of ligands (e.g. DNP-Asn, hymecromone, 1,5-diaminonaphthalene) was found to dock in two spatially separate positions with no obvious preference for binding between SS1 and SS2 (Fig. 6 and Table 3). The *DNP-Asn* docks primarily to SS1 (VDW contacts with PheH50, AsnH52, AsnH56, TyrH58, TyrL32, TyrL91, TyrL92, TyrL96), H bonding with the phenolic H of TyrH33 ($d = 2.03$ Å), the phenolic H of TyrH58 ($d = 2.26$ Å), the phenolic H of TyrL96 ($d = 1.93$ Å) and the carbonyl-O of TyrL91 ($d = 2.08$ Å). The occurrence frequency of this configuration is 1/10. Alternatively, the same ligand may occupy primarily SS2 (VDW contacts with GlyH32, TyrH33, ArgH94, AlaH95, AsnH96, TrpH97, AspH98), H bonding with the amido-Hs of TrpH97 ($d = 1.87$ Å) and that of AspH98 ($d = 2.08$ Å), and the carbonyl-O of AspH98 ($d = 2.22$ Å). The occurrence frequency of this

configuration was 2/10, the energy of binding somewhat lower ($\Delta E = 2$ kcal/mole) than for the position in SS1. The remainder of simulations have yielded variations on these two themes, with less favorable energetics. The DNP-Pro was found to dock similarly to DNP-Asn (not shown). *Hymecromone* can also assume two spatially separate binding positions. In the energetically most favorable configuration this ligand docks exclusively to SS2 (VDW contacts with SerH31, GlyH32, TyrH33, AlaH95, AsnH96, TrpH97, AspH98), H bonding with the amido-Hs of TrpH97 ($d = 2.23$ Å) and AspH98 ($d = 1.97$ Å), as well as the carbonyl-O of AspH98 ($d = 2.10$ Å). Occurrence frequency: 1/10. In the alternate binding position the ligand occupies primarily SS1 (VDW contacts with PheH50, AsnH52, TyrH58, TyrL91, TyrL92, SerL93, TyrL96), H bonding with the carbonyl-O of TyrL91 ($d = 2.00$ Å). The occurrence frequency is 4/10; the energy is 2.5 kcal/mole less favorable. In a third complex the ligand assumed a very similar orientation (with an occurrence frequency of 3/10, and exactly the same energy). The docking of 1,5-diaminonaphthalene is very similar to that of hymecromone. The energetically most favorable conformation was obtained with low (1/10) occurrence frequency. In this position, the ligand occupies primarily SS2 (VDW contacts with GlyH32, TyrH33, AlaH95, TrpH97, AspH98), H bonding with

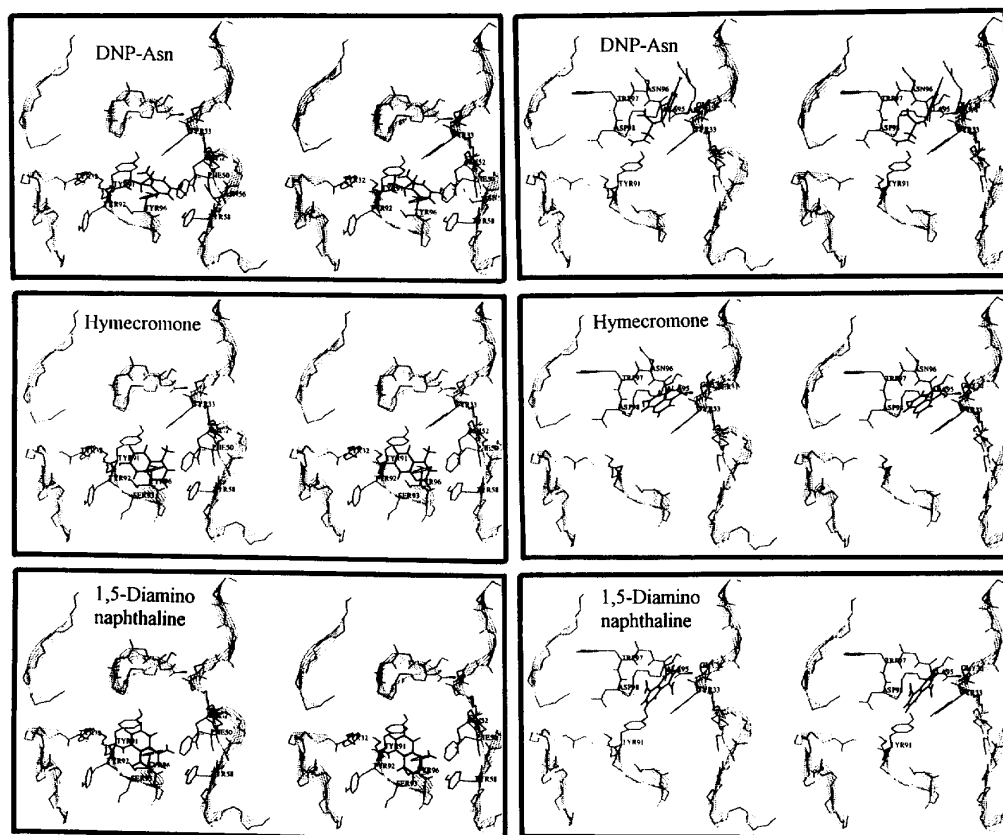


Fig. 6. Stereoview of ligand complexes occupying alternatively SS1 or SS2.

Table 3. Overview of interactions shown by haptens occupying alternate binding positions; numbers following the hapten name indicate the energetical rank of the result

		DNP-Asn (1)	DNP-Asn (2)	Hymecromone (1)	Hymecromone (3)	Naphthylene- diamine (1)	Naphthylene- diamine (3)
Subsite 1							
CDR H2	Phe H50	V (3)			V (13)		V (15)
	Asn H52	V (13), Hs			V (4)		V (1)
	Asn H56	V (7)					
	Tyr H58	V (14), Hs			V (11)		V (4)
CDR L1	Tyr L27D						
	Tyr L32 (OL)	V (3)			V (1)		V (1)
CDR L3	Tyr L91 (OL)	V (24), Hm	V (10), Hs		V (16), Hm	V (1)	V (15)
	Tyr L92	V (7)			V (11)		V (9), Hm
	Ser L93				V (1)		V (3)
	Ser L94						
	Tyr L96	V (11), Hs			V (8)		V (12)
Subsite 2							
CDR H1	Asp H27						
	Ser H28						
	Ile H29						
	Thr H30						
	Ser H31			V (7)			
	Gly H32		V (14)	V (5)		V (5)	
	Tyr H33 (OL)	V (14), Hs	V (21)	V (17), P	V (9)	V (35), Hs, P	
CDR H3	Arg H94		V (3)				
	Ala H95		V (16)	V (15)		V (11)	
	Asn H96		V (3)	V (6)			
	Trp H97		V (6), Hm	V (6), Hm		V (1)	
	Asp H98 (OL)		V (16), Hm	V (11), Hm		V (7), Hm	

V: Van der Waal's contacts (number of contacts); Hs: H-bonds with sidechain; Hm: H-bonds with mainchain; P: π stacking; OL: overlap-contacts between subsites.

the phenolic O of TyrH33 (2.03 Å), and the carbonyl O of AspH98 (1.93 Å); π stacking is possible between the naphthalene ring and TyrH33. The ligand was found in the alternate position with the highest occurrence frequency (6/10), but with lower (2.5 kcal/mole) energy of binding. In this position, it occupied exclusively SS1 (VDW contacts with PheH50, AsnH52, TyrH58, TyrL32, TyrL31, TyrL92, SerL93 and TyrL96), H bonding with the carbonyl-O of TyrL92 ($d = 2.17$ Å). The composite structure of all ligands docking to SS1 and SS2 is shown in Fig. 4(C).

DISCUSSION

Simulated annealing has been recently developed for the configurational search of protein–ligand (e.g. enzyme–substrate or antibody–hapten) complexes (Goodsell and Olson, 1990). Using a Metropolis Monte Carlo algorithm, the ligand is made to perform a random walk around a region of the protein that supposedly includes the binding site, in a search for maximal com-

plementariness between a relatively large static surface and a relatively small ligand. Regarding the predictive value of such a technique, the major question is about the correspondence between the static protein surface used as a template for docking, and the “real” (dynamic, flexible, malleable) environment of the binding site that exists under physiological conditions. So far, only the first steps have been made to bridge the tremendous gap between physical reality and computer-aided imagination.

Experimental controls and new information obtained by simulated annealing

Originally, the validity of structural predictions made by the AutoDock program was substantiated with one ligand–antibody (McPC 603), and three enzyme–substrate complexes (Goodsell and Olson, 1990). The energetically most favored conformations were found to be in good agreement with the X-ray crystallography data for the Ab–ligand and the enzyme–substrate complexes [rms difference 0.9–1.4 Å; with one exception (lysozyme–

α -N-acetylglucosamine), where the rms difference was about 4 Å]. In agreement with initial claims, in our hands the simulated annealing procedure correctly reproduced the experimentally observed binding mode of the substituted DNP ligand complexed to the anti-DNP antibody ANO2 (Fig. 1). Thus, auto-docking yields remarkably accurate reproductions with static binding site templates obtained by X-ray crystallography (Brünger *et al.*, 1991) which, in this last instance, is also in good agreement with the structure of the complex in solution (Theriault *et al.*, 1991). So far, few attempts have been made to exploit the capabilities of simulated annealing to generate new information. Thus, developers of this technique have successfully explored the binding of substrates to aconitase, which is difficult to do with crystallography (Goodsell *et al.*, 1993); and a combination of pre-existing shape complementarity and induced fit was predicted using a fragment docking approach of Ab(Fab)-peptide complexes (Friedman *et al.*, 1994). In all of these studies, experimentally obtained coordinates of proteins were used to create the ligand-binding templates.

Construction of Ab-ligand structures by auto-docking starting from Ab(Fv) amino acid sequence data

Clearly, the accuracy of structural predictions obtained by simulated annealing greatly depends on the authenticity of the protein template used. Thus, using templates produced by molecular modeling from sequence data instead of experimentally verified binding sites presents a quantum-jump in the potential source of errors, where relatively minor mistakes can result in a completely false picture. Regarding this complication, the Ig-fold family of proteins has definite advantages over most other protein families, mainly because in this case homology modeling can be done relatively accurately due to the stability of the Ig-fold, and the limited number of canonical structures from which the binding site is composed (Chotia and Lesk, 1987). Also, a relatively large number (so far about 30) of Ab-ligand structures have already been determined by X-ray crystallography and/or NMR, and the coordinates are available from the Brookhaven Protein Databank. Regarding our work, modeling of the Fv domain of IgE(La2) was relatively simple because five out of the six CDR loops represent clearly identifiable canonical structures (Kofler *et al.*, 1992) and CDR H3, which is generally the most difficult to model accurately, has some resemblance to a similar X-rayed loop (Droupadi *et al.*, 1994). The CDR H3 was modeled by the ABGEN program from position 94 to 102 (the sequence is RANWDVFAY) taking well into account the packing against the rest of the protein. This way, favorable interactions (salt bridges) between the sidechains of ArgH94 and AspH27 (CDR H1) as well as between AspH98 and LysL30 (CDR L1) can be observed. Thus, the La2(Fv) structure obtained by Droupadi *et al.* (1994) seems reasonable.

The probability of various Ab-ligand complexes

Altogether, we have carried out 31 docking experiments (i.e. 310 runs) with 30 ligands (two enantiomers,

R and S of cycrimine) and obtained 160 energetically feasible conformations for La2-ligand complexes. Regarding the accuracy of these predictions, we have to consider the following: each computed complex is the end result of a lengthy Monte Carlo trial-and-error procedure. Thus, the most important parameters of predictive accuracy are the coherence of predictions arrived at in successive simulations, characterized by the spread of occurrence frequencies of identical complexes, and the stability of the complexes, characterized by the calculated energy of binding. The probability score (p) for each identifiable complex may thus be given by the following formula:

$$p = \frac{H_n}{N} \exp\left(-\frac{E_n - E_1}{RT}\right)$$

where H_n = the occurrence frequency of the n th complex; N = the total number of runs for the ligand in question (usually 10); E_n = the energy of the n th run; E_1 = the maximal energy of the runs for the ligand in question; RT = 5.94 kcal/mole (heuristic); ($0 < p \leq 1$). The summary of probability scores for all energetically feasible La2-ligand complexes is shown in Table 4. Ligands that were found to occupy SS1 had at least one complex outside of this subsite; however, their probability score is generally an order of magnitude lower than sum- p in SS1. Nearly all ligands located in SS1 were found to dock in more than one orientation to the same subsite (marked by * in Table 4); however, the p of the predominant orientation is generally an order of magnitude higher than that of the next variant. Thus, it is about $10 \times$ more probable that 8-aminoquinoline, β -naphthylamine, prolonium-I and DNP-Glu occupy SS1 rather than SS2 or SS(1-2); and the existence of the predominant orientation [shown in Fig. 3(A)] is about $10 \times$ more probable than those of other complexes, found in the same subsite in different orientations.

Regarding the ligands that were found to dock predominantly in SS2, the probability of SS2 location is high; however, the identity of the most probable orientation within this subsite is, in most cases, uncertain. Thus, for DNP-Ala the most frequently found complex is roughly $3 \times$ more probable than the next variant, but for α -nitro-cinnamic acid and hemimellitic acid the existence of the next variant is only slightly less probable, and for naproxene the 10 runs yielded nine complexes, all in different orientations (only two runs gave identical orientations; however, these were energetically significantly less favorable than complex No. 1 shown in Fig. 3(B) (energy difference = 7.21 kcal/mole)).

According to our computations, oxolinic acid, DNP-Gln, trimethoxy-cinnamic acid, diaspirin and cycrimine are able to bind, most probably, to the SS(1-2) area; however, the predominant orientation only for oxolinic acid and DNP-Gln is obvious. With trimethoxy-cinnamic acid and diaspirin, the probabilities of the closest variants are only slightly lower than that of the one suggested in Table 4. Cycrimine is a special case, because for affinity measurements a racemic mixture of this compound was

Table 4. Probability scores of IgE(LA2)-ligand complexes

Ligand	SS1	SS1-2	SS2
8-Aminoquinoline	0.8, 0.082*		0.066
DNP-Glu	0.5, 0.03*	0.088, 0.019*	0.072
Naphthylamine	0.8		0.075
Protonium iodide	0.7, 0.063*, 0.044*		0.038
Carboxynaphthol	0.065		0.7, 0.081*, 0.068*
DNP-OH	0.02		0.4, 0.318*, 0.069*
DNP-Ala	0.021, 0.019*		0.6, 0.178*
Hemimellitic acid	0.016	0.045	0.3, 0.246*
Naproxene	0.059, 0.036*, 0.011*	0.044, 0.028*, 0.022*, 0.011*	0.1, 0.063*
Nitrilo-cinnamic acid	0.106	0.051	0.3, 0.236*, 0.046*
Cycrimine [R]	0.029, 0.013*, 0.009*	0.1, 0.053*, 0.036*, 0.03*, 0.024*	
Cycrimine [S]	0.014, 0.009*	0.1, 0.018*, 0.016*, 0.011*, 0.011*	0.003
Diasprin		0.2, 0.178*, 0.067*	
DNP-Gln	0.342	0.6	
Oxolinic acid	0.135, 0.076*	0.5, 0.063*	0.094
Trimethoxy-cinnamic acid	0.085	0.2, 0.217*, 0.041*, 0.037*, 0.023*	
DNP-Asn	0.1, 0.026*, 0.018*, 0.017*	0.012, 0.009*	0.128, 0.027*
Hymecromone	0.262, 0.197*, 0.056*		0.1, 0.053*
Naphthylendiamine	0.396, 0.054*		0.145, 0.1*

*Indicates results docking to the same subsite, but in a different orientation.

used. As we have shown earlier, the formation of the charge-transfer complex is indicated by the appearance of a marked spectral band in the 320–360 nm range (Droupadi *et al.*, 1994). Of the predicted complexes, only the one shown in Fig. 5 with cycrimine-R gives a possibility for charge-transfer complex formation, suggesting that this enantiomer is the active substance (i.e. the real K_D value is one-half of the one listed in Fig. 2). The high flexibility of this compound (six bonds with free rotation) may explain the wide spread of orientations of the computed complexes.

In the case of DNP-Asn, hymecromone, naphthylendiamine and DNP-Pro (analysed, but not shown), the *p*-scores of complexes found in SS1 and SS2 are of the same order of magnitude. The subsite-preference of DNP-Asn and DNP-Pro binding is quite uncertain; for hymecromone and naphthylene diamine SS1 is preferred, but binding to SS2 is also possible.

An interesting question arising from the alternate binding modes is whether the binding of the hapten could occur simultaneously to both sites. Considering only the distances between the two hapten positions, it would, in principle, be possible, i.e. the minimum distances between the two hapten positions are 3.3 Å for DNP-Asn, 5.3 Å for hymecromone and 2.6 Å for 1,5-diamino-naphthalene. There can be two explanations for these results. The finding of alternate binding sites can be an artifact, due to a low resolution of the method with these ligands. Alternatively, the existence of two non-exclusive binding positions of these ligands to the two subsites could be a physical reality, with obvious implications in conceptual immunochemistry. This question might be answered by

re-investigating these complexes with more AutoDock runs, and by physical studies.

Coherence of docking data

Some of the strengths and problems inherent in the simulated docking approach are clearly manifested in our studies of IgE-ligand complexes. As shown above, more than one complex structure was obtained with all ligands investigated (Table 4). Thus, if only one ligand were used for docking, the relevance of ligand location in the docked complexes to the “real” binding site would be questionable, even if the existence of one complex was more probable than others. By increasing the number of docking runs, the uncertainty could be reduced, but not completely eliminated. One approach to this problem is to use different analogs of the immunizing hapten with different intrinsic affinities. A correlation of experimentally measured and computed free energies of the complexes could validate the structural information obtained by docking. Further development of the docking program and an increase of computational capabilities to allow the simulation of dynamic changes upon complex formation (rotational/torsional movements of sidechains, solvent effects, entropy changes on binding, etc.) will, hopefully, make such analyses in the not-so-distant future possible. However, short of such a correlational analysis, coherence of docking data may allow some minimal conclusions to be drawn. Thus, the clustering of the docked structures of all DNP amino acids within a narrow groove of the H and L chain CDRs of IgE(LA2) makes it very likely that this area includes the “real” binding site of IgE(LA2).

This conclusion is supported by the finding that the energetically most favored conformations of all non-DNP compounds were clustered within the DNP binding site. Because we have employed a competitive binding assay for screening (Varga *et al.*, 1991b; Winger *et al.*, 1995), it is anticipated that all ligands found by screening should bind to the same site as DNP, or overlapping subsites, where steric hindrance prohibits simultaneous binding. The correctness of the structural information obtained by docking is further supported by the fact that identical binding modes were obtained for structurally very similar rigid compounds such as 8-aminoquinoline and β -naphthylamine.

The mechanism of heterologation

Regarding the mechanism of heterologation, these studies support earlier suggestions on the role of aromatic residues in the formation of multiple Ab–ligand complexes (Mian *et al.*, 1991; Droupadi *et al.*, 1994). The following five tyrosine residues of La2 provide the majority (53.4%) of contacts (% of all contacts provided by the residue is shown in parentheses): Tyr L91 (14.2), Tyr L32 (12.4), Tyr H33 (11.8), Tyr L92 (9.4) and Tyr L96 (5.6). In addition to the abundance of aromatic residues, perhaps the most important characteristic of the La2 binding site is that it is much larger than necessary for providing a complementary area for the immunizing hapten, or for any of the non-DNP ligands found by screening. Individual DNP and non-DNP ligands utilize the available contact points within this large area in different combinations (Tables 1–3). Thus, these results suggest a combinatorial-type binding mechanism to explain heterologation. The division of the combining region into two subsites may also contribute to Ab multispecificity as suggested long ago (Richards *et al.*, 1975); however, the existence of subsites does not seem to be necessary. In another heterologating IgE (clone Lb4) no spatially separate subsites could be identified (Winger *et al.*, 1995); thus, presence of separate subsites seems to affect only the degree of multispecificity.

Experimental verification of the predicted Ab–ligand structures by site-directed mutagenesis and physical studies such as 2D-NMR and X-ray crystallography is in progress. Publication of structures of Ab–ligand complexes at this stage, in the absence of additional experimental support, will allow an unbiased evaluation of the general validity of structural predictions for Ab–ligand complexes, made by simulated annealing.

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