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Comparative docking studies on ligand binding to the multispecific antibodies IgE-La2 and IgE-Lb4

Christoph A. Sotriffer*, Rudolf H. Winger, Klaus R. Liedl, Bernd M. Rode and Janos M. Varga

Theoretical Chemistry Department, Institute of General, Inorganic and Theoretical Chemistry, University of Innsbruck, Innrain 52 A, A-6020 Innsbruck, Austria

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Summary

A large comparative study is presented in which the binding of approximately 30 different ligands to two IgE antibodies (La2 and Lb4) is analyzed by means of an automated-docking procedure based on simulated annealing. The method is able to reproduce experimentally verified binding orientations, as shown by application to the Ig-AN02–hapten complex. The main address of the study is to investigate the concept of antibody multispecificity. Problems and usefulness of docking in this context are discussed. The results indicate reasons for multispecific binding properties and how they can be understood from the topology of the binding site. Though similar in general behaviour, the two antibodies show interesting differences in their binding characteristics. The binding sites of both antibodies are described and the main interacting residues revealed.

Introduction

Antibodies are used by the immune system to eliminate foreign molecules (antigens, haptens) from the organism. The first step in this process lies in the formation of antigen–antibody complexes, and thus in the ability of the antibody to recognize and bind a certain antigen. The antibody binding site faced with this task is formed by six so-called hypervariable loops or complementarity-determining regions (CDRs), three of which are contributed by the light-chain variable domain and the other three by the heavy-chain one. Although this binding region is relatively small compared to the complete antibody, antibody diversity is mainly due to differences in sequence, size and conformation of these CDRs, the rest of the antibody three-dimensional structure being mostly conserved. By genetic and somatic mechanisms a large diversity of antibody-combining sites is generated, thus making available a great repertoire of binding specificities and affinities, obviously able to successfully face the antigenic challenge [1–3] (for reviews concerning antigen–antibody complexes, see Refs. 4 and 5).

Regarding the specificity of antibodies, a commonly held notion is that antibodies react well only with the eliciting (i.e. immunizing) hapten, and when cross-reactions occur, they do so only by virtue of structural similarity of a compound with this hapten and tend to give weaker affinity constants. However, the unique three-dimensional structure of a single antibody does not necessarily imply high specificity, and indeed biophysical and biochemical evidence indicates that antibodies generally can be multispecific and cross-reactive [6–8], i.e., able to bind not only the inducing hapten or very similar ones, but also structurally different haptens, sometimes even with higher affinities.

Some important questions regarding the specificity of antibodies have for example been formulated and addressed by a comparative X-ray study [9] and an analysis of antibody-binding-site characteristics [6]: how do structurally different antigens bind to the same antibody? What are the limits of the antibody combining site's complementarity? Do antibody combining sites possess general properties that enable them to be multispecific? Here we focus on these questions by means of computer-aided

*To whom correspondence should be addressed.

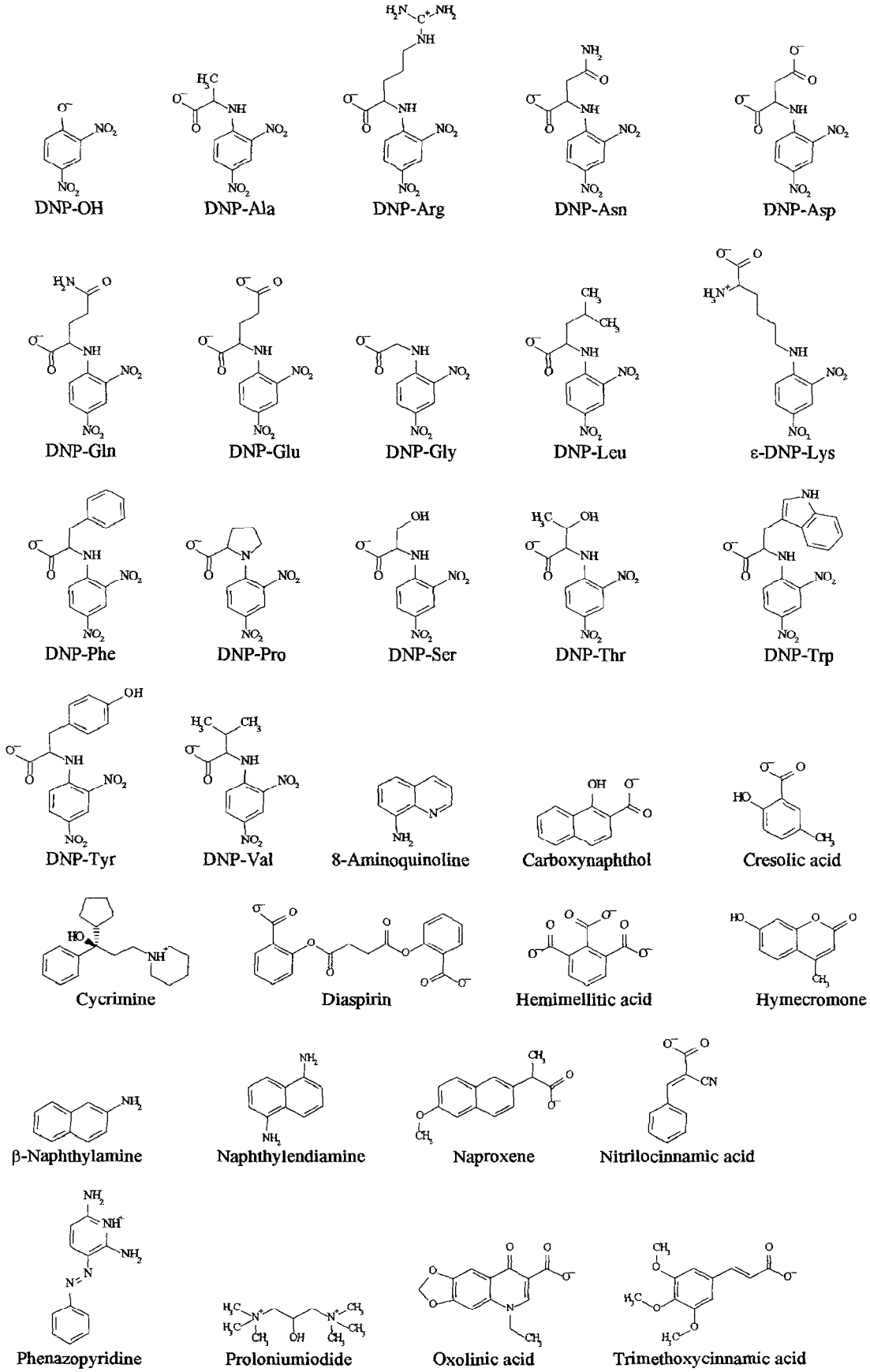

Fig. 1. Structures of the ligands docked to La2 and/or Lb4.

molecular modeling, investigating the complexes of two monoclonal IgE-antibodies, La2 and Lb4. These two antibodies have been shown to exhibit multispecific binding properties [10]: they are originally anti-trinitrophenyl antibodies, but were early recognized to be 'heteroclitic', i.e., they react with higher affinity to haptens other than the immunizing one. Experimental screening tests indicated entire sets of molecules bound by one or both of the antibodies.

A well-established automated-docking program was used in the work at hand to analyse the binding of about 30 ligands to these antibodies. The obtained results allow the formulation of some predictions, but need also to be critically discussed on the basis of known problems regarding the docking methodology. Immunological aspects of this work are discussed elsewhere [11,12].

Materials and Methods

Structures of antibodies and ligands

In the context of ligand binding only the variable domains (F_v) of antibodies have to be considered: these F_v parts are refered to as 'antibody structures' in the following. The structure of the two antibodies La2 and Lb4 had been determined earlier by homology modeling from sequence data, as described in Ref. 10. The method of homology modeling is reliable for the structure prediction of antibodies, due to the special characteristics of this class of proteins (stability of the Ig-fold, limited number of canonical structures for the single CDRs, as shown by Chothia and Lesk [13]). Examination of the amino acid sequences of La2 and Lb4 revealed that key residues are indeed present, known to create specific canonical loop conformations. In the 'parent' loop structures thus selected for modeling, no insertions or deletions of amino acids were required for either La2 and Lb4 models, a fact which not only facilitated the modeling process, but also increased the reliability of the obtained antibody structures.

For the docking process nonpolar hydrogens were added to the structural data files of both antibodies using the SYBYL Biopolymer module [14]. Polar hydrogens connected to O or N were specially marked as potentially able to form hydrogen bonds (vide infra). Charges from Weiner and Kollman's all-atom AMBER force field [15, 16] were assigned to each atom.

The ligands investigated were selected with respect to the relatively high binding affinity shown in a screening essay of more than 2000 compounds and subsequently in the experimental determination of their binding constants, which are in the μM affinity range [10]. For La2 and Lb4, 30 and 26 ligands were included in this work, respectively; out of these, 24 ligands are bound by both antibodies. The number of investigated ligands was actually 31 and 27, respectively, since cycrimine was docked in both stereochemical configurations. The structures of the ligands are given in Fig. 1. About half of these molecules are amino acids attached to a dinitrophenyl residue (DNP). Most of the remaining compounds are of aromatic type. The structures were generated with the SYBYL modeling tools and optimized with the TRIPOS force field; atomic charges were assigned by the Gasteiger–Hückel formalism. Acidic or basic compounds were modeled in the form predominant at physiological pH. In analogy to the antibodies, potentially hydrogen-bonding H-atoms were marked in the coordinate file.

Docking method

To search for favourable interaction geometries between ligand and antibody, the program AutoDock written by Goodsell and Olson was used [17] (the original version was slightly modified in order to allow the definition of more than four atom types). It combines a rapid energy evaluation through precalculated grids of affinity potentials with a Monte Carlo search algorithm for simulated annealing. The program is described in detail elsewhere [17].

A grid of points was laid over the receptor region of interest (the antibody thus being assumed to be rigid); at every point the interaction energy of a probe-atom/probe-charge with the whole macromolecule was calculated, supplying a 'map' of affinity potentials for each defined atom type, as well as a map for the electrostatic potential (these maps served as look-up tables for the calculation of the interaction energy during the docking process). The grid was centered on the CDR region and had the dimension of $30 \times 30 \times 30$ Å^3, with a grid spacing of 0.5 Å. The energy was calculated using 12,6-Lennard-Jones potentials for van der Waals (vdW) interactions and Coulomb potentials for electrostatic interactions. To account for hydrogen bonding, a modified Lennard-Jones (12,10-) potential was used for hydrogen-bond-forming atoms, with parameters chosen to describe a minimum of −5 kcal/mol at a distance of 1.95 Å between the hydrogen and receptor atom. The vdW and hydrogen-bond potential parameters were used as provided by the authors of the program, who took them from the AMBER force field [15–17]. A dielectric constant of 40 was used for the Coulomb potential.

TABLE 1
AN02 DOCKING RESULTS

Result number	Frequency of occurrence	Docking energy (kcal/mol)	Rmsd to experimental position (Å)
Starting position 1			
1	3	−87.46	0.44
2	7	−81.23	7.25
Starting position 2			
1	2	−87.21	0.32
2	8	−81.16	7.21

TABLE 2
La2 DOCKING RESULTS

Hapten type	N[a]	Energetically top-ranked result					Other results		
		E[b] (kcal/mol)	f[c]	p[d]	ss[e]	Major contacting residues	Rmsd to top result[f] (Å)	E (kcal/mol)	p
Aminoquinoline	3	–43.0	8	0.80	1	Tyr L32, Tyr L92, Tyr L91	4.1–13.1	–41.8––40.4	0.082–0.066
Carboxynaphthol	4	–51.4	7	0.70	2	Asp H98, Asn H96, Trp H97	2.0–11.8	–50.2––48.9	0.081–0.065
Cycrimine (*R*)	8	–74.2	1	0.10	1/2	Tyr H33, Asp H98, Tyr L91	1.6–10.1	–70.4––59.7	0.053–0.009
Cycrimine (*S*)	8	–76.2	1	0.10	1/2	Tyr H33, Tyr L91, Asp H98	6.0–10.0	–66.2––55.5	0.018–0.003
Diaspirin	3	–75.5	2	0.20	1/2	Tyr L91, Trp H97, Asp H98	5.7–9.0	–73.1––70.7	0.178–0.067
DNP-OH	4	–47.0	4	0.40	2	Ala H95, Asn H96, Asp H98	3.2–11.3	–45.6––37.3	0.318–0.020
DNP-Ala	4	–61.4	6	0.60	2	Gly H32, Tyr H33, Ala H95	6.1–14.8	–60.8––51.7	0.178–0.019
DNP-Arg	4	–74.9	5	0.50	1/2	Tyr L91, Gly H32, Ala H95	8.1–10.9	–73.1––62.1	0.074–0.025
DNP-Asn	8	–72.8	1	0.10	1	Tyr H91, Tyr H33, Tyr H58	0.8–13.3	–70.2––58.8	0.128–0.009
DNP-Asp	8	–65.2	2	0.20	2	Asp H98, Tyr H33, Ala H95	6.1–9.8	–59.1––53.8	0.036–0.015
DNP-Gln	2	–66.2	6	0.60	1	Tyr L91, Tyr L92, Asp H98	5.3	–65.2	0.342
DNP-Glu	5	–64.1	5	0.50	1	Tyr L32, Tyr L91, Tyr L96	5.1–12.2	–62.2––54.4	0.088–0.019
DNP-Gly	6	–53.3	1	0.10	1	Tyr H58, Tyr H33, Phe H50	5.6–9.9	–52.7––47.6	0.210–0.038
DNP-Leu	4	–61.1	1	0.10	2	Trp H97, AspH98, TyrL32	6.2 –10.6	–60.6––56.8	0.636–0.048
DNP-Lys	7	–81.5	1	0.10	1/2	Tyr L91, Tyr H33, Asp H98	1.8–11.6	–81.4––68.6	0.197–0.011
DNP-Phe	3	–68.9	1	0.10	2	Tyr H33, Gly H32, Ala H95	8.2–8.4	–67.0––66.0	0.217–0.122
DNP-Pro	4	–57.9	1	0.10	2	Asp H98, Tyr H33, Ala H95	7.7–10.6	–52.6––50.7	0.119–0.041
DNP-Ser	5	–63.9	1	0.10	2	Gly H32, Ala H95, Asp H98	7.7–14.4	–62.8––54.6	0.327–0.021
DNP-Thr	6	–63.5	1	0.10	2	Gly H32, Tyr H33, Trp H97	6.5–10.0	–60.6––58.2	0.162–0.059
DNP-Trp	8	–71.1	2	0.20	2	Tyr H33, Ala H95, Asp H98	4.7–13.8	–68.5––58.9	0.064–0.013
DNP-Tyr	4	–71.1	5	0.50	1/2	Tyr L91, Asp H98, Tyr L96	5.1–8.3	–70.6––62.8	0.092–0.046
DNP-Val	7	–66.4	2	0.20	2	Tyr H33, Asp H98, Trp H97	6.6–11.0	–61.8––53.7	0.092–0.012
Prolonium iodide	4	–48.3	7	0.70	1	Tyr L92, Ser L93, Tyr L32	2.3–10.3	–45.6––42.5	0.063–0.038
Hemimellitic acid	4	–59.6	3	0.30	2	Trp H97, Ala H95, Tyr H33	4.3–12.0	–55.4––48.8	0.246–0.016
Hymecromone	5	–47.5	1	0.10	2	Tyr H33, Ala H95, Asp H98	4.0–12.1	–45.0––43.7	0.262–0.053
Naphthylamine	3	–44.6	8	0.80	1	Tyr L32, Tyr L91, Tyr L92	10.3–12.9	–42.9––42.7	0.075–0.072
Naphthylen diamine	4	–47.0	1	0.10	2	Tyr H33, Ala H95, Asp H98	4.7–9.9	–45.1––43.3	0.396–0.054
Naproxene	9	–65.1	1	0.10	2	Gly H32, Tyr H33, Ser H31	7.6–16.0	–62.4––52.2	0.063–0.011
Nitrilocinnamic acid	5	–47.5	3	0.30	2	Gly H32, Asn H96, Ala H95	5.4–13.6	–46.1––42.9	0.236–0.046
Oxolinic acid	5	–55.9	5	0.50	1	Tyr L91, Tyr L92, Tyr L32	4.8–14.2	–55.6––53.2	0.135–0.063
Trimethoxycinnamic acid	6	–61.1	2	0.20	1/2	Tyr L32, Tyr L91, Asp H98	4.4–8.4	–59.1––52.4	0.217–0.023

[a] N = number of different results.
[b] E = docking energy.
[c] f = frequency of occurrence in 10 runs.
[d] p = probability score.
[e] ss = subsite.
[f] Rmsd = root-mean-square deviation.

To search for suitable sites of interaction, the ligand was moved through the receptor-near space by small random displacements along translational, rotational and torsional degrees of freedom. Evaluation of the interaction energy at every step was followed by application of the Metropolis algorithm [18] to decide on the acceptance of the new position, and thus on the point from which the search had to proceed. Since this was coupled to a process of simulated annealing, a wide region of conformational space could be searched while avoiding to finish in local minima next to the starting position. We began the runs at a temperature corresponding to RT = 100 kcal/mol and reduced it by multiplying with 0.9 after a maximum of 30 000 accepted or rejected steps; 50 such temperature-reduction cycles were carried out till the end of the search. For each hapten 10 independent runs of this scheme were performed.

The program has been shown in many cases to successfully reproduce experimentally found binding modes [17, 19–21] (one may speak of reproduction if the root-mean-square deviation (rmsd) is below 1–2 Å [22]; this is a reasonable assumption, given the nature of the affinity potential and the grid-point approximation, as well as the experimental uncertainties in structure determination). As an additional control, we applied the same procedure as described above to a hapten–antibody complex solved by X-ray crystallography, namely the F_{ab}-AN02 complexed with its hapten, a substituted dinitrophenyl compound [23]. The $30 \times 30 \times 30$ Å^3 grids were again centered over the CDR region and the hapten was removed from its binding position by translation and rotation. It was kept in its experimental conformation during the docking simulations. Two sets of 10 independent runs were carried out, using two different starting positions. The results,

given in Table 1, are essentially the same for both. Only two different orientations were obtained. The one with a considerably more favourable energy reproduces the experimental result exactly, while the second result is not as different as the large rmsd value may suggest: the aromatic system lies in exactly the same plane as in the experimental orientation, but the two large substituents in positions 1 and 3 are interchanged, i.e., the molecule is rotated by 180° around an axis through the unsubstituted carbons of the phenyl ring (Fig. 2). However, it was this particular position that was more often reached in the 10 runs (seven and eight times, respectively).

Analysis of the results

To analyse the large number of results the following method was applied: the interaction energy was recalculated without grid approximation by taking into account explicitly every atom–atom interaction. The results of the 10 runs were then reranked according to the new energy value. Afterwards, geometrical similarity was checked; results differing by less than 1 Å rmsd in all atom positions were regarded as identical and were represented by the energetically most favourable result, which was assigned an accordingly higher occurrence frequency. The recalculated interaction (docking, pseudo-) energy used to rank the results is definitely not the 'final' criterium for binding (concerning this important point: see Discussion) and is not suited to assign lower-ranked results to lower relevance in the real binding process. Rather, one has to include the energetically very similar results into the discussion. As an additional aid for judging the significance of a result we therefore introduced a scoring factor, which takes into account the frequency of occurrence of a certain result, since for a mimimum position to be occupied easy accessibility must be fulfilled as well, i.e., the potential barriers surrounding the position must be surmountable within a reasonable time. A configuration occurring more often is thus more likely to be easily accessed and should thus be regarded with greater attention. In the scoring factor or probability score, p, the frequency of occurrence is combined with a factor containing the energy difference to the energetically top-ranked result according to the following formula:

$$p = \frac{H_n}{N} \exp\left(-\frac{E_n - E_1}{E_0}\right) \qquad (1)$$

where H_n is the occurrence frequency of the nth result, N the total number of runs (10), E_n the energy of the nth run and E_1 the most favourable energy of the runs for the ligand in question. For E_0 the heuristic value of 5.94 kcal/mol was used; this is motivated by the comparison of docking energies with free energies of binding: since they differ by one order of magnitude, this factor was also chosen 10 times larger in comparison to room-temperature conditions (E_0 is comparable to RT in the Boltzmann factor).

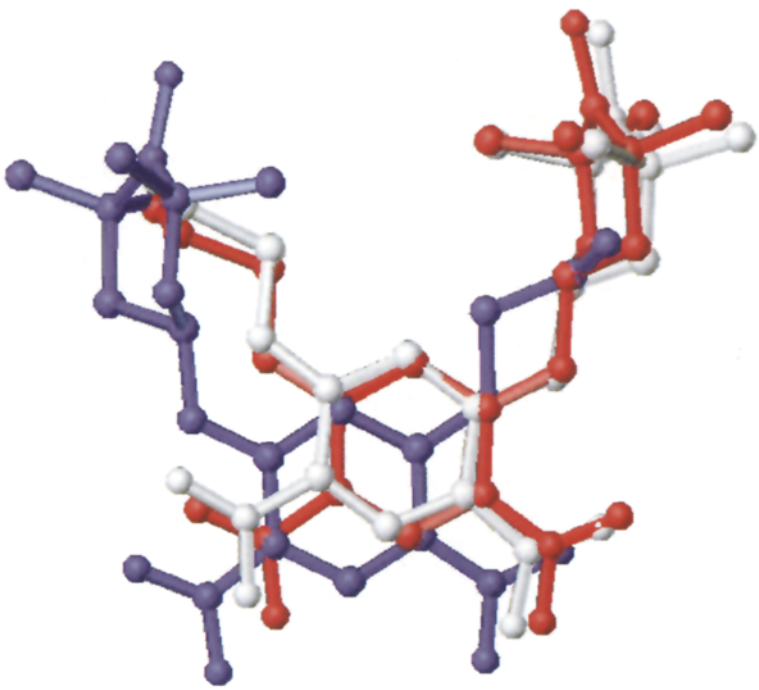

Fig. 2. Comparison of the positions of the AN02–hapten complex determined by X-ray crystallography (white) and by docking with AutoDock: the red structure corresponds to result number 1 in Table 1; the blue to result number 2 in Table 1.

As final steps the location in the antibody binding site, the vdW contacts (as defined in Sheriff et al. [24]), the Coulombic interaction and the possibility of hydrogen bonding were analysed for each result.

At this point some comments should be given concerning Tables 2 and 3, which summarize the results obtained by this procedure. As previously mentioned, the 10 independent runs for one ligand generally did not result in a single position; instead, between two and nine distinct docking orientations differing by more than 1 Å rmsd were obtained. The number of such different orientations is reported in the first column of the tables (only energetically favourable results were taken into account). For La2, some of the runs were trapped in unfavourable positions, yielding a positive, i.e. repulsive binding energy. For DNP-Asn, DNP-Lys, DNP-Ser and DNP-Trp one run did not 'converge'; for (*R*)-cycrimine, (*S*)-cycrimine and DNP-Pro two runs; for diaspirin three; and for DNP-Phe four runs, respectively. No such behaviour was observed for Lb4, which means that all 27×10 runs ended with attractive binding energies.

For the energetically most favourable result some details are given in Tables 2 and 3, namely the calculated interaction energy between ligand and antibody, the number of times this result was obtained in the 10 runs ('frequency of occurrence' f), the scoring factor p (for the top-energetical result it is equal to the relative occurrence frequency, i.e. f/10), the three most important amino acids interacting with the ligand, and – for La2 only – a classification refering to in what subsite the result was found (vide infra). For other results of each ligand, only the ranges of some parameters of the results are given. This means that the values presented in the last three columns do not necessarily belong to the same result, but are simply the minimum and maximum values observed for the ligand in question. For example, in the case of La2, the result of DNP-Gly with E = −52.7 kcal/mol actually has an rmsd value of 9.9 Å with p = 0.180. This demonstrates that a low rmsd does not imply a more favourable energy and p values are not only determined by the interaction energy, but by the occurrence frequency as well.

TABLE 3
Lb4 DOCKING RESULTS

Hapten type	N[a]	Energetically top-ranked result				Other results		
		E[b] (kcal/mol)	f[c]	p[d]	Major contacting residues	Rmsd to top result[e] (Å)	E (kcal/mol)	p
Carboxynaphthol	2	−60.4	7	0.70	Tyr H50, Trp H95, Gly L91	3.3	−58.2	0.207
Cresolic acid	3	−51.2	4	0.40	Trp H95, Tyr H50, Leu H97	2.1–3.7	−50.1–−48.8	0.416–0.067
Cycrimine (*R*)	6	−86.5	1	0.10	Tyr H52, Tyr H50, Val L94	2.2–8.1	−79.5–−59.5	0.125–0.001
Cycrimine (*S*)	8	−80.5	1	0.10	Tyr H52, Glu H58, Tyr L32	1.9–7.2	−76.2–−67.3	0.097–0.011
Diaspirin	9	−83.0	1	0.10	Tyr H52, Tyr H56, Glu H58	2.0 –10.0	−82.2–−75.1	0.101–0.026
DNP-OH	2	−50.4	2	0.20	Val L94, Tyr H50, Trp H95	3.8	−48.3	0.560
DNP-Ala	3	−59.9	2	0.20	Trp H95, Leu H97, Gly L91	5.6	−57.3–−55.2	0.453–0.046
DNP-Arg	2	−72.8	8	0.80	Tyr H52, Tyr H50, Ile H57	3.0	−71.6	0.164
DNP-Asn	5	−72.6	1	0.10	Val L94, Tyr L32, Ser L92	3.2–5.1	−68.7–−65.4	0.205–0.029
DNP-Asp	5	−69.9	2	0.20	Val L94, Tyr H50, Trp H95	3.9–5.9	−64.4–−57.2	0.120–0.012
DNP-Gln	8	−75.3	1	0.10	Leu H97, Trp H95, His L27D	5.0–7.8	−71.9–−57.7	0.113–0.005
DNP-Glu	4	−68.2	2	0.20	Tyr H52, Val L94, Tyr H50	6.2–7.9	−66.9–−61.0	0.406–0.052
DNP-Gly	3	−61.0	6	0.60	Val L94, Tyr H50, Trp H95	4.6–4.7	−57.2–−54.8	0.108–0.072
DNP-Leu	4	−65.6	4	0.40	Val L94, Tyr L32, Trp H95	2.9–7.2	−65.0–−61.0	0.330–0.046
DNP-Lys	8	−80.4	1	0.10	Tyr H52, Gly H91, Tyr H50	7.5–8.3	−78.5–−70.0	0.163–0.017
DNP-Phe	7	−72.2	1	0.10	Tyr H50, Leu H97, Val L94	4.9–6.9	−71.7–−65.2	0.276–0.031
DNP-Pro	3	−59.2	6	0.60	Leu H97, Val L94, Trp H95	3.7–4.4	−58.8–−56.7	0.198–0.094
DNP-Ser	2	−71.8	8	0.80	Val L94, Tyr H50, Tyr L32	3.7	−62.4	0.041
DNP-Thr	5	−62.8	2	0.20	Leu H97, His L27D, Trp H95	6.0–6.2	−60.6–−54.2	0.324–0.024
DNP-Trp	7	−79.5	2	0.20	Leu H97, Trp H95, Asn L28	2.3–10.6	−75.7–−64.9	0.105–0.009
DNP-Tyr	7	−76.5	1	0.10	Tyr H52, Trp H95, Tyr H50	2.8–7.6	−73.9–−65.9	0.254–0.017
DNP-Val	3	−61.7	1	0.10	Leu H97, Trp H95, Tyr H50	6.6–6.9	−61.6–−59.0	0.788–0.064
Phenazopyridine	3	−62.6	7	0.70	Tyr H52, Tyr H50, Glu H58	4.4–7.8	−61.9–−60.2	0.177–0.067
Prolonium iodide	5	−52.7	1	0.10	Tyr H52, Glu H58, Tyr H50	6.0–7.8	−52.1–−47.2	0.538–0.039
Hemimellitic acid	3	−57.9	4	0.40	Val L94, Gly L91, Tyr H50	3.9–4.4	−53.9–−53.1	0.221–0.051
Naphthylamine	2	−48.9	6	0.60	Val L94, Tyr H50, Tyr L32	4.4	−48.2	0.357
Nitrilocinnamic acid	2	−49.4	4	0.40	Val L94, Tyr H50, Gly L32	4.7	−49.3	0.589

[a] N = number of different results.
[b] E = docking energy.
[c] f = frequency of occurrence in 10 runs.
[d] p = probability score.
[e] Rmsd = root-mean-square deviation.

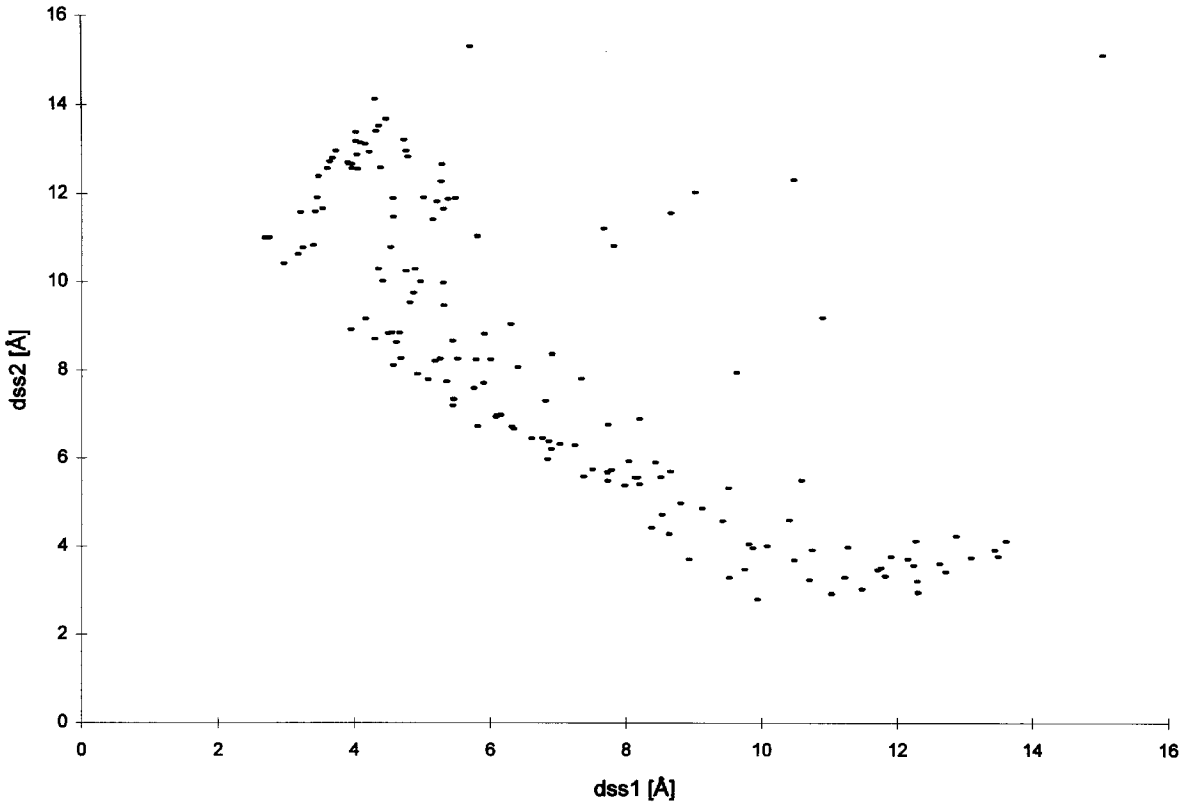

Fig. 3. Scatter plot of distance 1 (dss1; in Å) versus distance 2 (dss2; in Å) for all 160 La2 docking results.

Results

The docking results obtained for La2 and Lb4 are summarized in Tables 2 and 3. To analyse the results in more detail the two antibodies La2 and Lb4 are discussed separately. Residues are numbered according to the system of Kabat et al. [25], with H and L indicating heavy and light chains, respectively.

La2 binding site

The six CDRs of La2 are oriented relative to each other in such a way, that a shallow groove of approximately 20 Å length and 10–12 Å width is formed. Upon visual inspection of the antibody structure, the most striking feature is a pocket surrounded by seven tyrosine residues, mainly belonging to the CDRs L1 and L3 (Tyr L27D, L32, L91, L92, L96, and additionally Tyr H33 and H58); it is this part of the groove which at a first glance appears as a suitable binding site. The analyses of the docking results confirm this expectation, but also reveal a second important site of interaction, namely a region surrounded by residues of CDR H1 (Ser H31, Gly H32, Tyr H33) and H3 (Arg H94, Ala H95, Asn H96, Trp H97, Asp H98). The former part of the binding site, the 'tyrosine pocket', will be termed 'subsite 1' (ss1) in the following, the latter 'subsite 2' (ss2), while the intermediate region will be named 'ss1/2'. Explicitly charged residues are almost absent in the binding site; exceptions are Arg H94, Asp H98 and Lys L30. However, they are not occupying central positions in the binding site, and Asp H98 and Lys L30 are spatially neighbouring with a strong saltbridge between them.

The energetically most favourable results of eight ligands – mainly the smaller ones – are accommodated in ss1, whereas 16 other ligands preferably dock to ss2 (Table 2). Some of the largest ligands, such as cycrimine and diaspirin, extend into both subsites, because they are too bulky to be located in one subsite alone. Not all positions found for one ligand lie in the same subsite as the energetically most favourable one. In fact, the most striking feature is that the results of many ligands cover a wide range of locations, differing by up to 16 Å rmsd, as in the case of naproxene. Remarkably, some of the different positions yield almost equally favourable interaction energies: the energetically optimal position of oxolinic acid, for example, corresponds to an energy of –55.9 kcal/mol and differs by only 0.3 kcal/mol from the second-ranked result, which has a completely different orientation within the antibody binding site, indicated by an rmsd of 14.2 Å with respect to the first result. Comparing only the first two results with respect to energy for a single ligand, there are 14 other cases in which the energetical difference between them is less than 2 kcal/mol, but the corresponding rmsd is more than 4 Å, the most

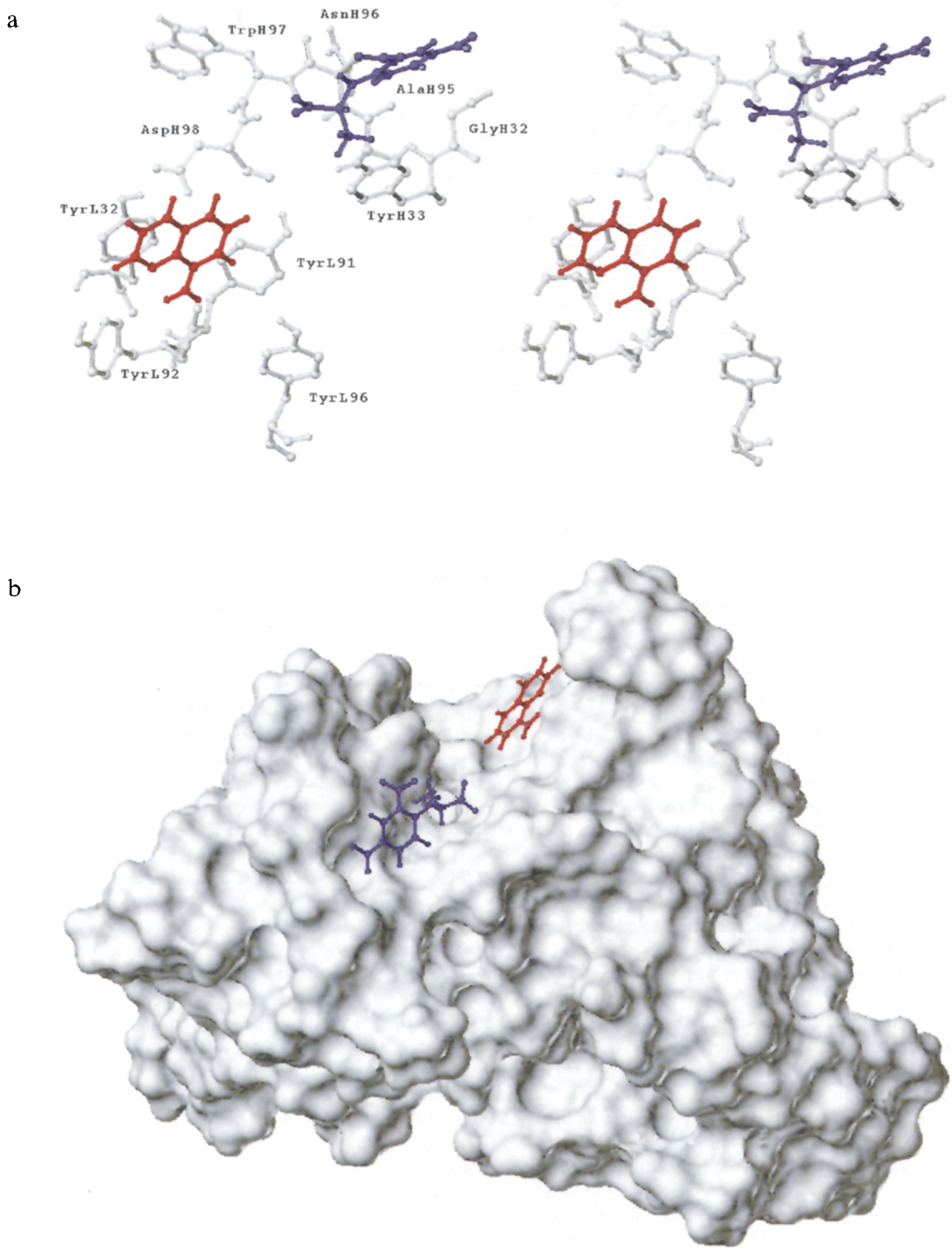

Fig. 4. (a) Stereoscopic view of the top results of the two ligands aminoquinoline (in ss1) and DNP-Ala (in ss2), displayed simultaneously within the framework of the 10 most important residues of the La2 binding site (according to Table 4); (b) the top results of the two ligands aminoquinoline (in ss1) and DNP-Ala (in ss2), displayed simultaneously within a Connolly surface model of the La2 variable domain.

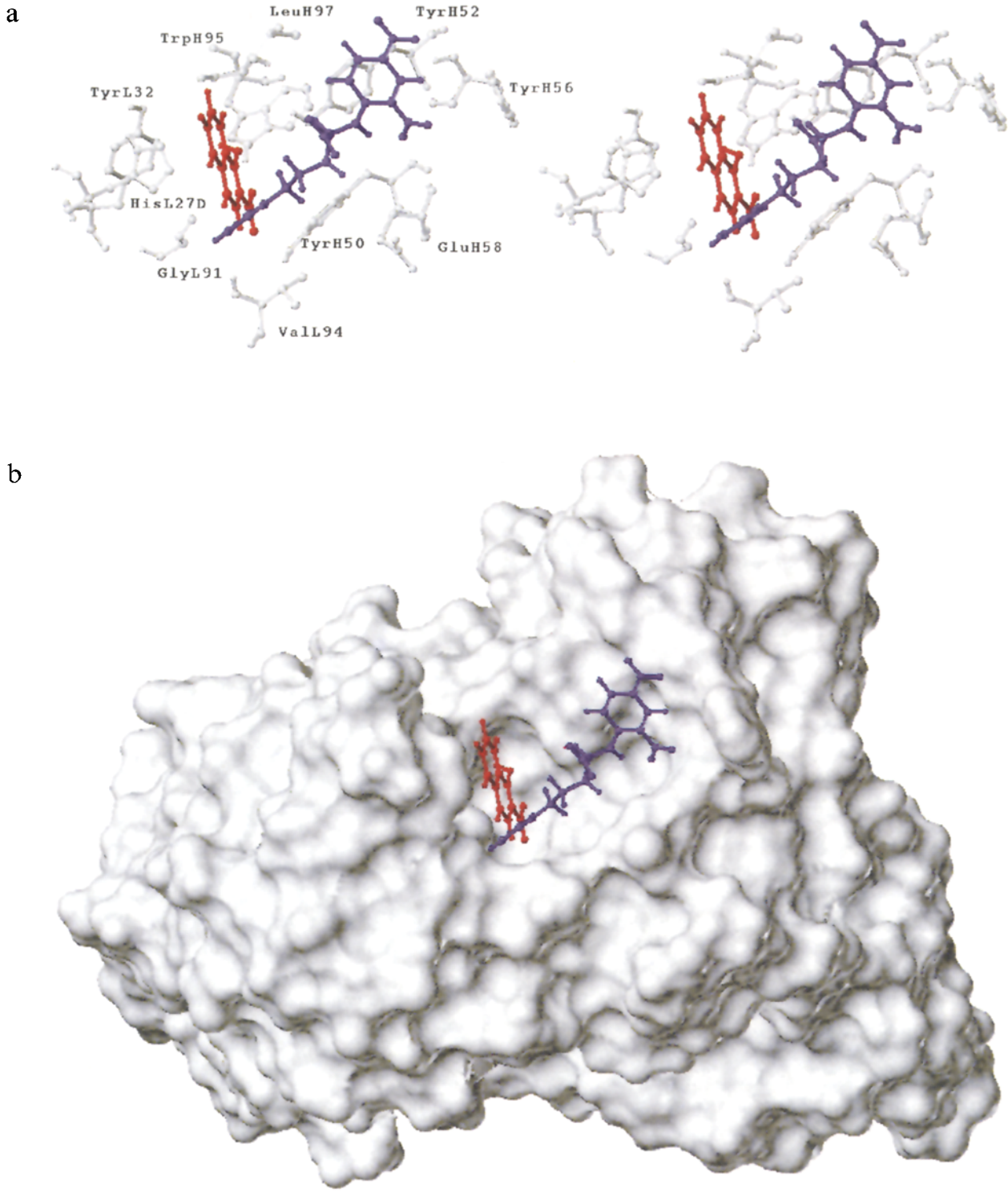

Fig. 5. (a) Stereoscopic view of the top results of the two ligands carboxynaphthol and DNP-Arg, displayed simultaneously within the framework of the 10 most important residues of the Lb4 binding site (according to Table 6); (b) the top results of the two ligands carboxynaphthol and DNP-Arg, displayed simultaneously within a Connolly surface model of the Lb4 variable domain.

prominent examples being naphthylamine (12.9 Å, 1.7 kcal/mol), DNP-Glu (12.2 Å, 1.9 kcal/mol), DNP-Leu (10.6 Å, 0.5 kcal/mol) and DNP-Gly (9.9 Å, 0.6 kcal/mol). Rms deviations of more than 9–10 Å often indicate a result in an alternate subsite. For aminoquinoline, carboxynaphthol, DNP-Asn, DNP-Glu, DNP-Leu, DNP-Ser, hymecromone, naphthylamine, naphthylen diamine and oxolinic acid there exist orientations in the other subsite differing by less than 3 kcal/mol in comparison with the first result.

TABLE 4
STATISTICS OF VAN DER WAALS CONTACTS FOR THE La2 BINDING SITE

Hypervariable region	Residue	Number of results with contacts to residue	Sum over weighted contacts ('importance value')
CDR L1	Tyr L27D	19	19.2
	Asn L28	2	0.4
	Gln L29	1	0.5
	Lys L30	27	12.5
	Tyr L32	111	180.0
CDR L2	Trp L50	2	2.4
	Tyr L53	2	0.5
CDR L3	Tyr L91	122	205.7
	Tyr L92	80	135.9
	Ser L93	42	41.2
	Ser L94	33	30.9
	Tyr L96	84	81.5
CDR H1	Asp H27	9	2.6
	Ser H28	7	2.4
	Ile H29	1	0.2
	Thr H30	7	2.6
	Ser H31	20	15.1
	Gly H32	54	72.0
	Tyr H33	124	171.7
CDR H2	Phe H50	55	40.3
	Asn H52	25	16.6
	Tyr H53	5	1.4
	Ser H54	5	0.8
	Asn H56	9	3.4
	Tyr H58	41	31.2
CDR H3	Arg H94	21	17.5
	Ala H95	86	91.8
	Asn H96	67	67.2
	Trp H97	77	69.9
	Asp H98	102	134.2

Although there is a considerable spread of results for one ligand, clustering of results in certain regions ('subsites') of the antibody's binding site is also observed, i.e., many different ligands are found to dock into the same or very similar positions. In some cases closely related ligands show almost identical positions ('binding modes'). To give a general impression of the distribution of all ligands results (160) two points were defined, approximately centered in ss1 and ss2, respectively, and the distances between these points and the ligand's center were calculated (with a distance between the two points of 10.6 Å). The centers were calculated as centroids, i.e., centers of mass with all masses set to unity. For ss1 the Tyr L32, L91, L92, L96, H33 and H58 were used to evaluate the centroid, and for ss2 the Ser H31, Gly H32, Tyr H33, Arg H94, Ala H95, Asn H96, Trp H97 and Asp H98. A large distance to point 1 ('distance 1') and a short distance to point 2 ('distance 2') therefore indicates binding to ss2. A scatter plot of distance 1 versus distance 2 (Fig. 3) for each result shows the clustering of results in regions of small distance 1 and large distance 2 (ss1), large distance 1 and small distance 2 (ss2) and of medium distances 1 and 2 (ss1/2). A few 'outliers' are observed: these correspond to positions outside the usual region, found only in a few cases. The majority of these outliers are still in ss1, but contacting mainly residues of CDR H2.

As far as the energy differences within the results for one ligand are concerned, they are usually (in 26 out of the total 31 cases) below 4 kcal/mol between the first and the second result, in 17 cases even below 2 kcal/mol. The other results sometimes show slight differences too, as can be seen in the case of DNP-Gly. In such situations the probability score p prooves its usefulness, because it contains the additional criterium of occurrence frequency to judge the relevance of a single result. With the value of E_0 chosen as indicated above, for DNP-Gly this leads to the highest p value for the fifth result, since it is observed three times, that is more often than any other result. Eight cases are observed in which energetically less favourable results give significantly higher probability scores than the top result (DNP-Gly, DNP-Leu, DNP-Lys, DNP-Phe, DNP-Ser, DNP-Thr, hymecromone and naphthylen diamine). In all these examples the energetically best result occurs only once in 10 runs, and the energy

difference with the closest other results is relatively small. Hence, it seems not sufficient to describe the docking behaviour of these ligands simply by taking the energetically best results into account. In other words, a result is highly relevant or 'predictive' when it is energetically most favourable and also most often observed. With La2, such behaviour was found most clearly for the ligands aminoquinoline, carboxynaphthol, DNP-Ala, DNP-Glu, prolonium iodide and naphthylamine, for which the top result was obtained in more than half of the runs. In the following, aminoquinoline and DNP-Ala will serve as two examples of binding to La2 in ss1 and ss2, displayed simultaneously in Figs. 4a and 4b.

Aminoquinoline is representative for docking to ss1. It is observed in 8 out of 10 runs at this position, with the most favourable energy in this subsite. The molecule is placed between the four tyrosines L27D, L32, L91 and L92, oriented almost coplanarly with the aromatic ring of Tyr L32. Hydrogen bonding of the amino group to the carbonyl oxygen of Tyr L91 contributes to the interaction energy. For similar ligands, such as napthylamine, naphthylen diamine and hymecromone, almost identical binding geometries are observed; however, only in the case of naphthylamine this geometry represents the energetically prefered result.

As an example for ligands in ss2, DNP-Ala is observed with high significance in this subsite. The main contacting residues are Gly H32, Tyr H33, Ala H95, as well as Ser H31 and Arg H94. A weak hydrogen bond of one nitro group to the guanidino part of Arg H94 is found, as well as strong hydrogen bonding (O-H distance of 2.0 Å) of the ligand carboxylate group to the peptidic NH functions of Trp H97 and Asp H98. The orientation of the Trp H97–Asp H98 backbone is apparently one of the major characteristics of ss2: the ligands interacting with these two residues normally do not have any contact with their side chains (pointing outside the subsite), but most often show hydrogen bonds with the backbone functionalities. More details regarding single ligand positions are given in Ref. 11.

TABLE 5
STATISTICS OF SPECIFIC HYDROGEN BONDS FOR THE La2 BINDING SITE

Hypervariable region	Residue	Sum over probability scores of results showing specific hydrogen bonds to residue
CDR L1	Lys L30	1.37
	Tyr L32	4.64
CDR L2	–	–
CDR L3	Tyr L91	2.87
	Tyr L92	0.27
	Tyr L96	5.84
CDR H1	Ser H28	0.06
	Thr H30	0.06
	Ser H31	0.17
	Tyr H33	1.02
CDR H2	Asn H52	0.28
	Ser H54	0.12
	Asn H56	0.04
	Tyr H58	0.41
CDR H3	Arg H94	2.46

Although these two examples are to some extent representative for general characteristics of ligand binding to La2, there is a substantial number of different orientations for other ligands, as indicated above. To obtain a general measure of important sites of interaction, a statistical evaluation was carried out, including all 160 results weighted according to their probability score, i.e., with a higher weight for more favourable and more frequently occurring results. In a first analysis regarding the vdW contacts, for each residue in the CDRs the numbers of contacts with each ligand were multiplied by the scoring factor of the respective result and summed to give an 'importance value', indicating the degree to which a residue is involved in ligand binding. Thus, the more ligand positions are found near a particular residue, the closer these ligands contact the residue and the more favourable the respective result is, the higher the calculated 'importance value' will be. Table 4 shows the result of this procedure: Tyr L91, Tyr L32 and Tyr H33 are most often and most significantly involved in ligand binding; these three residues indeed form the 'border line' between the two subsites and can be contacted from either side. Tyr L91 and Tyr H33 show interactions with the ligand in more than 75% of all the 160 configurations. Further important residues are mainly those of CDR H3 and the additional two tyrosines in CDR L3.

A similar analysis was carried out concerning hydrogen bonding. Only so-called 'specific hydrogen bonds' were taken into account, i.e., those which are formed with the amino acid side chain and not with the backbone of the peptide chain (this distinction is of importance for the selection of residues for mutagenesis experiments). In this case only the scoring values of the results showing a specific hydrogen bond with a certain residue were summed to give the 'importance value'. As displayed in Table 5, specific hydrogen bonds are observed for 14 amino acids, half of them are of very low relevance. The most important are Tyr L96 and Tyr L32, followed by Tyr L91 and Arg H94. Out of the important CDR H3 residues only the side chain of Arg H94 is involved in hydrogen bonding, although Asn H96, Trp H97 and Asp H98 could form hydrogen bonds with their side chains too. However, as mentioned above, these residues point outside the ss2 binding site and thus contribute to it primarily through their backbone.

Lb4 binding site

The binding site of Lb4, as revealed by the docking

TABLE 6
STATISTICS OF VAN DER WAALS CONTACTS FOR THE Lb4 BINDING SITE

Hypervariable region	Residue	Number of results with contacts to residue	Sum over weighted contacts ('importance value')
CDR L1	Gln L27	1	0.5
	His L27D	64	69.7
	Ser L27E	1	0.2
	Asn L28	15	10.6
	Gly L29	8	3.0
	Tyr L32	88	115.5
CDR L2	Lys L50	1	0.3
CDR L3	Gly L91	114	133.0
	Ser L92	79	56.6
	His L93	34	10.1
	Val L94	82	166.3
	Leu L96	62	35.3
CDR H1	Tyr H50	117	238.1
	Tyr H52	114	226.0
	Tyr H54	18	5.6
	Gly H55	11	4.3
	Tyr H56	49	58.3
	Ile H57	48	55.9
	Glu H58	73	101.8
CDR H2	–	–	–
CDR H3	Trp H95	106	167.4
	Leu H97	95	136.1
	Ile H98	77	31.1
	Phe H99	6	1.3

procedure, appears as an approximately 10 Å deep cavity with an opening of about 16 Å × 15 Å, formed primarily by the CDRs L3 (Gly L91, Ser L92, His L93, Val L94, Leu L96), H2 (Tyr H50, Tyr H52, Tyr H56, Ile H57, Glu H58) and H3 (Trp H95, Leu L97, Ile L98), and to a minor extent also L1 (His L27D, Tyr L32). The most important contact residues were determined in the same way as described for La2 and are reported in Table 6. Again, three aromatic amino acids play a key role, namely Tyr H50, Tyr H52 and Trp H95. Three apolar residues immediately follow in importance, namely Val L94, Leu H97 and Gly L91. This may explain the experimental finding that the DNP analogues with apolar side chains show higher affinity constants for Lb4 than those with polar and charged side chains [10]. Concerning hydrogen bonds, the statistical procedure shows that besides the hydrogen bonding to the protein backbone, Trp H95 and Tyr H52 most often form 'specific' hydrogen bonds with their side chains, followed by two other tyrosines at positions H50 and L32 (Table 7).

For Lb4 the docking results of any single ligand generally show a 'sharper' distribution in comparison with the La2 results. This can easily be recognized from Table 3: although again the 10 runs for each ligand gave between 2 and 9 different positions (> 1.0 Å rmsd), there are many (13 out of the total 27) cases with only two or three different outcomes; additionally, the rmsd values of the results relative to the energetically top-ranked one are in general below 8.5 Å (this maximum value is only exceeded in two cases, namely for diaspirin and DNP-Tyr). Thus, the ligand positions are considerably less spread in location, in contrast to the La2 binding site. This is true not only for the different results for one ligand, but also for the total of all 121 results. Indeed, they are all found essentially in the same pocket in some different orientations with common features (Figs. 5a and 5b).

To emphasise that all ligands are accommodated in a certain way in this framework of residues, a central point was defined as the center of the 10 amino acids most important for binding (as listed in Table 6). The position of this point is found 1.8 Å away from the phenolic oxygen of Tyr H52. Calculation of the distances of the ligands centroids to this point shows that they are all within 5.5 Å, except for six results, in which they are at maximum 7.1 Å away.

Within the above described binding site the most often encountered orientation for the ligand is near CDR L1 and L3, though still allowing to form strong hydrogen bonds with Trp H95 and Tyr H50. Hydrogen bonds with the backbone are often observed at the amido group of Val L94. Normally, the aromatic system of the ligand is sticking into this site in a vertical orientation, often roughly parallel to the H-L-domain interface; eventually present side chains of the aromatic system are pointing

outside the cleft and are oriented in different ways, according to the molecular functionalities and flexibility. Many of the small ligands exclusively show this binding mode, such as carboxynaphthol, cresolic acid, DNP, hemimellitic acid and naphthylamine. The two variations of this binding scheme are given on the one hand by an altered (rotated) orientation of the aromatic plane leading to a deviation from the vertical direction, and on the other hand by a reversed orientation in the sense that the side chain points into the cleft where normally the aromatic ring lies, whereas the aromatic part points outwards.

This last variant is closely related to a somewhat more different binding mode, shown mainly by larger ligands: they do not enter the pocket as deeply as described above, but seem to prefer a position shifted more towards the amino acids of CDR H2 (Tyr H50, Tyr H52, Tyr H54, Tyr H56, Ile H57 and Glu H58). If the ligand is a DNP amino acid, the DNP part normally points towards CDR H2. The side chain of Tyr H52 is frequently contacted through hydrogen bonds, while Tyr H56 and Ile H57 allow hydrogen bonding through their peptide backbone. Val L94 is quite often involved in stabilizing the position as well. Examples which prefer an orientation of this kind are DNP-Arg, DNP-Val and phenazopyridine. More details regarding single ligand positions are given in Ref. 12.

The Lb4 energy data may be compared to those of La2: the energy differences between the first and second result are in 50% of the cases below 2 kcal/mol and exceed the 4 kcal/mol limit only for five of the 27 ligands. High predictivity, i.e., highest frequency of occurrence of the energetically most favourable result, is obtained for carboxynaphthol, DNP-Arg, DNP-Gly, DNP-Pro, DNP-Ser, phenazopyridine and naphthylamine. Examples such as DNP-Val, prolonium iodide and nitrilocinnamic acid should also be mentioned, of which the first and the second result are energetically almost identical, but the second result occurs with considerably higher frequency. This leads to a clearly higher scoring factor.

TABLE 7
STATISTICS OF SPECIFIC HYDROGEN BONDS FOR THE Lb4 BINDING SITE

Hypervariable region	Residue	Sum over probability scores of results showing specific hydrogen bonds to residue
CDR L1	Gln L27	0.07
	His L27D	0.03
	Ser L27E	0.01
	Asn L28	0.03
	Tyr L32	1.48
CDR L2	Lys L 50	0.10
CDR L3	His L93	0.17
CDR H1	–	–
CDR H2	Tyr H50	4.49
	Tyr H52	10.22
	Tyr H56	0.09
	Glu H58	0.01
CDR H3	Trp H95	14.68

Discussion

The basic method used in this work is the docking program AutoDock. With reference to a classification presented in Ref. 26, the currently exploding spectrum of docking methods may be split into new programs with improved algorithms overcoming some basic assumptions of current docking technology, and somewhat older methods which are frequently used in studies oriented towards application. AutoDock may be regarded as belonging to the second group: this means that some basic assumptions are still present, but also that it has already been applied many times and proven to work well.

What are the approximations of the AutoDock method and their consequences for the results? First of all, the antibody is treated as rigid, due to the enormous number of degrees of freedom of a macromolecular system. The assumption that no – or at least no large – conformational rearrangements occur upon complex formation is often true for the binding of small ligands, but not generally valid. In the case of hapten–antibody complexes experimental results provide examples for the whole range of possibilities: from no conformational changes upon ligand binding [27–29], over side-chain and loop rearrangements [30–32] up to domain movements [33].

A second approximation is the reduction of the available search area to a predefined region of interest. This is no problem in the context of antibodies, since the binding site is generally formed by the six CDR loops. A box of $30 \times 30 \times 30$ Å^3 centered over the CDR region is therefore large enough to cover the whole space of potential binding sites and also assure an unbiased search.

The simplifications concerning the energy evaluation have to be mentioned as third major approximation. The thermodynamically correct criterium to score and evaluate protein–ligand configurations would be the free energy (enthalpy) of binding, which, however, is not directly accessible. Instead, the intermolecular terms of forcefields (vdW, electrostatic and eventually explicit hydrogen-bonding terms) are applied for the purpose of finding favourable geometries of interaction. Other terms accounting for solvation–desolvation, the hydrophobic effect, polarization effects and entropic contributions would be important to the free energy of binding and a correct ranking of docking results, but are usually neglected (for an attempt to partially solve this problem, see Ref. 22). Consequently, the experimental binding energy can not be predicted quantitatively: the force-field energies are one order of magnitude too high (favourable)

and are not comparable for different ligands. This study clearly underlines this, showing that the docking energies of the top results of every ligand do not correlate with the values of the binding energy determined by experiment [10] and differ approximately by a factor of 8–10 in absolute value (while the experimental binding constants imply ΔG values in the range from –5.8 to –9.2 kcal/mol, the docking energies of the most favourable results lie between –43.0 and –86.5 kcal/mol). Nevertheless, the force field is highly effective as far as geometries are concerned and is able to provide likely binding sites or orientations for interactions resulting from the relatively steep nature of the used potentials, which are very sensitive to unfavourable contacts on the one hand. By comparison, the neglected terms are considerably less geometrically specific. Thus, the docking energies (which must always be seen as 'pseudo-energies') work well in providing insight into the topology of the binding site and give good initial models of the complexation, which may then be refined further by more sophisticated computational methods or investigated by experimental techniques.

Despite of all these approximations and limitations, the program has been shown in many cases to successfully reproduce binding modes found by experiment [17,19–21]. This is also suggested by a test with the AN02 antibody carried out by ourselves. The result is interesting not only because it reproduces the experimental binding mode, but also in comparison with the La2/Lb4 results and their distribution. While in the AN02 case the two docking results refer essentially to the same position, differing only by the interchange of two side chains, the ligands docking to La2 or Lb4 are often found in strongly deviating positions, but nevertheless with energies less different than the AN02 results. This can be seen as a strong indication that the behaviour observed for La2 and Lb4 is not an artefact of an unspecific docking method, but is indeed determined by the architecture of the binding site and its 'multispecific' properties. Furthermore, an inspection of the AN02 binding site shows that the hapten is sandwiched between two Trp residues which form the central part of a much narrower pocket than that of La2 and Lb4. Clearly, in such a molecular framework there are far less possibilities for the favourable accommodation of a ligand and therefore higher specificity may be observed.

The unusual distributions of the docking results for Lb4, and especially for La2, thus indicate that these two antibodies are indeed not highly specific, but able to accommodate a wider range of different small molecules. This leads to a better understanding of the experimental observation gained by screening essays and subsequent spectroscopic determination of binding constants [10,12] showing that these antibodies bind large sets of ligands in the range of medium and low affinity, which is still of physiological relevance as shown by the stimulation of mediator release [34]. Further support for the results comes from the study of Mian et al. [6] who concluded that frequent occurrence of Tyr and Trp residues in the binding site is of major importance for multispecific behaviour. As the present work clearly shows, aromatic residues play a key role in the binding sites of the two antibodies (Tables 4–7).

Two hitherto generally accepted assumptions are thus questioned by our results: on the one hand, that antibodies are generally highly specific systems and on the other hand, that for a given ligand there exists only one true binding mode to a certain receptor. Concerning the first point, experimental evidence for multispecific behaviour of antibodies is already available for a considerable amount of systems [6–9] and models exist which describe the specificity of the immune response, in general, and of the antigen–antibody reaction in particular, as a population-and-threshold phenomenon [12,34,35]. However, little structural information is available upon which an explanation of multispecific behaviour may be based. The results of this study indicate how it may be understood: the binding site is provided by a wide valley and not a narrow cleft; numerous tyrosines and tryptophanes are present and they are oriented in a way to enable several good possibilities for hydrogen bonding as well as to provide large contact surfaces; charged side chains are almost absent in the binding sites of both antibodies; charged ligands, especially of negative sign, can nevertheless be accommodated and their charge be compensated by polar side chains/oriented dipoles instead of explicit countercharges [6]. The ligands thus show some recurring features of interaction (such as hydrogen bonding to the backbone of Trp H97 and Asp H98, coplanar stacking to Tyr L32 or hydrogen bonding to the phenolic groups of Tyr L96 in the case of La2; hydrogen bonds with the Trp H95 side chain or the Val L94 backbone, or the close contact to CDR L1/L3 in the case of Lb4), but utilize different combinations of the potentially available interaction contacts. This variability can be achieved by different orientations in a single site (as in the case of Lb4, where the positions of all haptens are overlapping to a certain degree) or even by binding to different subsites (as observed for La2). Thus, given a set of ligands experimentally known to bind to an antibody, one can map the antibody binding site and reveal the important features of interaction.

Concerning the second point that there is always only one 'true' orientation of the ligand within the receptor, it has to be stated that this is a rather simplifying view of the potential energy surface represented by a protein binding site and a too much static concept. The systems are definitely in continuous movement at ambient temperature, and fluctuations around an average position occur [36] (an additional reason why it makes no sense to discuss results differing by less than 1 Å rmsd separately). Furthermore, it seems reasonable that large binding sites,

as in this study, with their high-dimensional potential energy surfaces, possess many local minima of almost equal energy for the interaction with a ligand. These minima may be populated to a varying degree, ligands could even be trapped to a certain extent in less favourable minima, if the barriers around them are too high to be surmounted with sufficient probability in the course of constant fluctuations. Experimental results – either structure determination or evaluation of binding energies – are in fact always weighted averages over a range of molecular configurations, reporting essentially one final outcome, although several events occur at the microscopic level. Computational docking simulations deal with a single system and not with large ensembles; a reproduction of the experimental result is therefore by no means stringent and lack of agreement with experimental findings may not only be attributed to simplifications in the docking method, although this is of course a major source of uncertainty. In any case, a single docking run is useless and the frequency of occurrence of a result is indeed an important measure. In this context, 10 runs for a single ligand are also still very few runs. However, they already yield insight into the nature of the binding-site potential energy surface: in the case of AN02 the two single results, almost completely overlapping, model the experimental finding very well and indicate the preference for the experimental position by the relatively large energy difference; the higher occurrence frequency of the less favourable result may be rationalized by the fact that once the hapten has arrived to the sandwiched position, the barriers for rotation into the more favourable configuration become very high, and hence it remains in its initial orientation. A larger number of runs and variable starting positions could result in an altered frequency distribution. By comparison, the La2 results for oxolinic acid or DNP-Gly reveal completely different characteristics of the binding site, since almost equally favourable, but nevertheless non-overlapping positions are accessible. In general, our docking results often do not provide a clear indication of what position would most probably be observed experimentally; more runs could be of use here. However, they suggest that alternative binding orientations may be populated by a single ligand and that subsites may not only increase the spectrum of ligands to be bound, but also augment the possibilities of interaction for a single ligand.

Conclusions

The docking results presented in this work invite to rethink certain concepts of antibody specificity. It can be rationalized why antibody binding sites, such as those of the two antibodies La2 and Lb4, show multispecific characteristics: the binding pocket is relatively large and thus able to accommodate ligands of different shapes and sizes; the most important interaction partners are the numerous aromatic amino acids, and thus ligands of different molecular functionalities can be bound well rather easily.

The ligand orientations are observed to vary considerably, more than what is usually found in common docking studies. Predictions regarding well-defined locations of ligands that will eventually be experimentally observable within the binding site seem feasible only in cases in which the high-occurrence frequency of a result is coupled to the most favourable interaction energy. In other cases the existence of alternative binding possibilities should be taken into account.

Experiments of structure determination, as well as further computational studies to get more insight into the phenomena,indicated by this initial docking study, are in progress. Experiments of site-directed mutagenesis may soon yield additional information about important sites of interaction. Besides the possibility to formulate predictions for changes (or undisturbed behaviour) in the binding energy of single ligands out of the vast amount of docking data, by statistical considerations based on all docking results, it is anticipated that, for example, mutations of Tyr L96 in the case of La2, and of Trp H95 or Tyr H52 in the case of Lb4, should have a remarkable effect upon the binding for the majority of the ligands. However, the results also suggest that the single alteration of one residue could have a less dramatic effect than expected, in that alternative favourable binding positions may still be present and limit the decrease in binding energy by being higher populated under the changed circumstances.

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