and 100 μM EN, and 1 h incubation at 37 °C. EN-**3** formation was performed in HEPES buffer (10 mM, pH 7.0, 150 mM NaCl), with a final EN concentration of 400 μM at 37 °C for 1 h. Mn-**3**, Ni-**3**, and EN-**3** provided the expected results by MALDI-TOF MS and base composition analysis (see Supporting Information).

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Keywords: DNA hairpin · metal complex · metallosalen – DNA · nucleic acids · template synthesis

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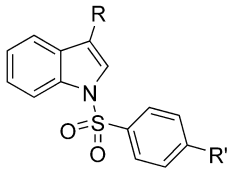
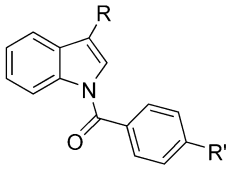
An Unprecedented Catalytic Motif Revealed in the Model Structure of Amide Hydrolyzing Antibody 312d6

Fabio Benedetti,*[a] Federico Berti,*[a] Kevin Brady,[b] Alfonso Colombatti,[c, d] Alessandro Pauletto,[c] Carlo Pucillo,[c] and Neil R. Thomas*[b]

While catalytic antibodies are well established as artificial catalysts with enzyme-like properties for a variety of reactions,[1] few amidase antibodies have been obtained so far.[2] Recently, we have described the preparation and kinetic characterization of amidase antibody 312d6.[3] The antibody was raised against the sulfonamide hapten **2a**, designed to induce catalysis by transition-state mimicry and torsional activation, and has been found to accelerate the hydrolysis of amides **3a**, **4a** by a factor of 10^3 (Scheme 1).





1 R = H
2 R = CHO

3 R = H $k_{cat}/k_0 > 750$ (**3a**)
4 R = CHO $k_{cat}/k_0 = 1120$ (**4a**)

R' = **a**: CH_3; **b**: H; **c**: OCH_3; **d**: Cl; **e**: NO_2

Scheme 1. *Sulfonamide haptens (**1**, **2**) and carboxamide substrates (**3**, **4**) of antibody 312d6.*

Here we report the results of a docking analysis carried out on the homology model of the variable fragment (Fv) region of antibody 312d6. A novel catalytic motif based on an arginine dyad, unprecedented in antibody hydrolases, is identified by this study.

[a] *Prof. F. Benedetti, Dr. F. Berti*
Dipartimento di Scienze Chimiche, Università degli Studi di Trieste
Via Giorgieri 1, 34127 Trieste (Italy)
Fax: (+39) 040-558-2402
E-mail: benedett@units.it
berti@dsch.univ.trieste.it

[b] *Dr. K. Brady, Dr. N. R. Thomas*
School of Chemistry, University of Nottingham
University Park, Nottingham, NG7 2RD (UK)
Fax: (+44) 115-951-3564
E-mail: neil.thomas@nottingham.ac.uk

[c] *Prof. A. Colombatti, A. Pauletto, Prof. C. Pucillo*
Dipartimento di Scienze e Tecnologie Biomediche
Università degli Studi di Udine
P.le Kolbe 4, 33100 Udine (Italy)

[d] *Prof. A. Colombatti*
CRO-IRCCS, Aviano
33081 Aviano (Italy)

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The amino acid sequence of the Fv region (Table S1 in the Supporting Information) indicates that antibody 312d6 is substantially different from hydrolase antibodies raised against phosphonate or phosphonamidate haptens[4] and does not contain the "canonical binding array"[4c] commonly found in those antibodies.

Homologous sequences corresponding to antibodies of known structure[5] have been retrieved from the Protein Data Bank and used to build a model of the Fv of 312d6. Modelling of the variable light chain (V_L) was straightforward due to the high similarity of the V_L to that of anti-HIV protease antibody F11.2.32.[5] Modelling of the variable heavy chain (V_H) required a multistep procedure: anti-fullerene antibody 1-10F-8a was used to provide V_H. The complementarity-determining region (CDR) H3 loop of anti-4-hydroxy-5-iodo-3-nitrophenyl caproic acid antibody B1-8 was then grafted on in place of the less homologous CDR H3 of antibody 1-10F-8a.[5] The residues of the heavy chain were then mutated to those found in sequence 312d6, and the whole structure was optimized in order to relax clashes with the heavy chain framework.

After obtaining the starting models of the two chains, the Fv dimer was constructed by using the crystallographic structure of antibody 25.3 (PDB 1AFV) as a template. For a protein of known structure 25.3 has the highest degree of sequence homology with 312d6, if both the V_L (89%) and V_H chains (69%) are considered. Its loops also fall into the same Chothia's canonical classes[6] (L1 undefined; L2, L3 and H1 all Class 1; H2 Class 2). Although the resolution of this particular structure is comparably low (3.7 Å), the backbone position is sufficiently well resolved for the purpose of Fv dimer construction, which consists primarily in finding the relative orientation of V_L and V_H and hence involves the peptide backbone positions only. The two chains of antibody 312d6 were thus aligned by sequence homology to the two chains of 25.3, and a starting geometry for the dimer was obtained. The final structure was obtained by optimization of the V_H-V_L interface, followed by relaxation of the whole dimer. The dimer was then equilibrated in a box of 7000 TIP (transferable intermolecular potential) water molecules by a 3000 ps molecular dynamics run at 300 K and the lowest-energy equilibrium structure was used to obtain the final optimized geometry (Figure S1). A PROCHECK[7] analysis assessed the structural validity of the final model.

A putative binding site for sulfonamide **1a** was identified by mapping the antibody binding area with GRID 19[8] (Molecular Discovery Ltd.). The GRID sulfonamide probe immediately identified the Arg50H, Arg98H dyad as the most promising interaction site for the sulfonamide group. Mapping with the hydrophobic, aromatic and methyl probes identified a small hydrophobic pocket located under the two arginines, lined by Leu101H and the methylene groups of Ser91L and Asp94L, with Trp96L at the bottom (Figure 1). The hapten was then docked onto the interaction map with the mobile side chain docking routine of GRID 19, and the resulting structure was optimized (Figure 1A). Strong electrostatic and hydrogen bonding interactions between the sulfonamide group of the hapten and the same arginine dyad are also identified by docking **1a** with the FlexX[9] module of Sybyl 6.8 (Tripos Inc.). Thus it seems safe to assign a key role in hapten binding to Arg50H and Arg98H. All the contact residues identified by the GRID/FlexX approach are commonly used by hapten-binding antibodies,[10] including amidase antibodies,[11] to establish interactions with the hapten.

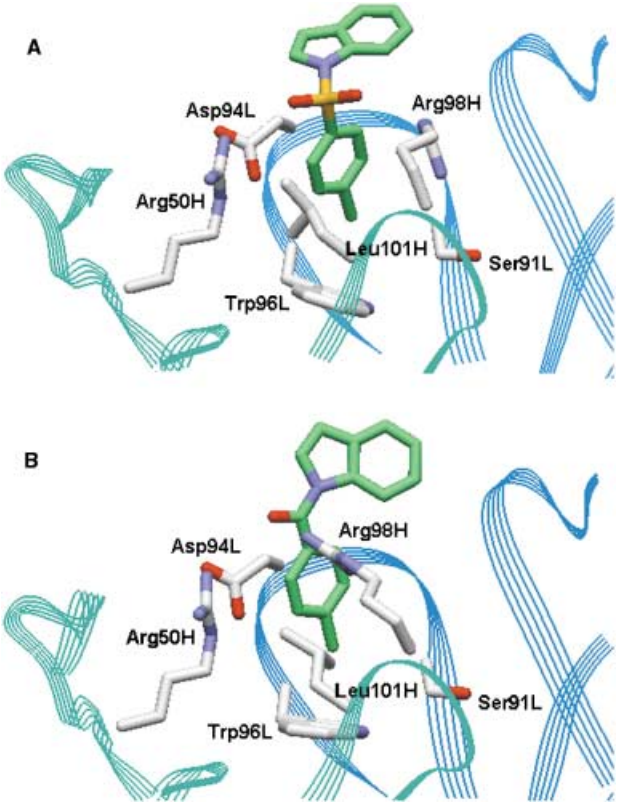

Figure 1. *A) Complex between catalytic antibody 312d6 and sulfonamide* ***1a*** *showing important active-site interactions. The CDRs are shown in green (V_H) and blue (V_L); the rest of the sequence is omitted for clarity. B) Complex with amide* ***3a****.*

The complex with substrate **3a** was obtained similarly (Figure 1B). The binding mode of the amide closely resembles that of the sulfonamide hapten, with Arg98H flipped towards the carbonyl oxygen leaving enough space for the incoming water molecule. The catalytic role of the arginine dyad may thus be to activate the amide bond by polarization and hydrogen bonding. Involvement of arginine residues in catalysis is consistent with the experimental results; this indicates that the rate is pH-independent between pH 7.5 and 9.0.[3]

Substrate destabilization was another element taken into account in the design of the sulfonamide hapten **2a.** The aromatic rings of **2a** are nearly orthogonal and it was thus proposed that anti-**2a** antibodies, on binding the amide substrates, would force the substrates to adopt a similar conformation, with a twisted amide bond.[3] Indeed, in the complex with 312d6, in order to bury the phenyl ring in the hydrophobic pocket and at the same time establish a hydrogen bond with Arg98H, substrate **3a** must adopt a distorted conformation with the phenyl ring and the carbonyl nearly orthogonal, as predicted (Figure 1B). However, the wide space available at the top of the site allows the indole ring to rotate with the carbonyl, thus minimizing the twist of the amide group. Calculations at the B3LYP/6-31G(d,p) level indicate that the

conformation adopted by **3a** in the binding site is destabilized by 1.5 kcal mol^{-1} with respect to the ground state, against a maximum destabilization of 9 kcal mol^{-1} calculated for complete twisting of the amide bond out of planarity. This may explain why the acceleration observed in 312d6-catalyzed hydrolysis of amides **3a** and **4a** is modest compared with the rate enhancements that can be obtained by constraining an amide bond in a twisted conformation.[12]

Antibody 312d6 was found to be highly specific for *p*-tolyl-substituted indole sulfonamides and carboxamides.[3] The docking analysis was thus extended to *p*-substituted carboxamides **3b**–**e** and sulfonamides **1b**–**e**. Relative complexation energies for the two sets of ligands were calculated and are reported in Table 1. The data confirm that the model accounts qualitatively for the observed specificity, at least when the methyl is replaced by larger groups.

Table 1. *Relative complexation energies for binding of 312d6 with sulfonamide (**1**) and carboxamide (**3**) ligands.*

R′	Sulfonamide	$\Delta E_{compl,rel}$[a] [kcal mol^{-1}]	Carboxamide	$\Delta E_{compl,rel}$ [kcal mol^{-1}]
CH_3	**1a**	0.0	**3a**	+8.16
H	**1b**	+0.15	**3b**	+8.08
OCH_3	**1c**	+23.41	**3c**	+30.18
Cl	**1d**	+4.62	**3d**	+14.21
NO_2	**1e**	+7.92	**3e**	+13.47

[a] $\Delta E_{compl,rel} = (E_{PL} - E^{o}_{L}) - (E_{P1a} - E^{o}_{1a})$, where E_{PL}, E^{o}_{L} are the Amber4.1 energies for the optimized geometries of the protein–ligand complexes and for the ground state conformations of the free ligands, respectively. E_{P1a}, E^{o}_{1a} are the corresponding values for the reference ligand **1a**.

In conclusion, a unique catalytic motif, based on an arginine dyad lining a hydrophobic pocket, has been found in antibody 312d6. The strong electrostatic field generated by the dyad is likely to be used by 312d6 to activate the amide bond towards nucleophilic attack and stabilize the transition state.[13] The second arginine may also play a role as a general base, activating the hydrolytic water molecule.[3] The homology model also confirms that substrate destabilization plays a role in the catalytic activity of 312d6 and is consistent with the binding specificity displayed by the antibody. Crystallization trials on the fully functional antigen binding fragment (Fab) are currently underway.

Experimental Section

Sequencing: mRNA from freshly subcloned hybridoma 312d6 cells was isolated and first-strand cDNA was synthesized.[14] cDNAs encoding the antibody variable domains were amplified by PCR by using Taq polymerase. V_H was amplified with the forward primer VHFor (5′-TGAGGAGACGGTGACCGTGGTCCCTTGGCCCCAG-3′), which hybridizes to the γ-chain constant domain, and with the reverse primer VH1Rev (5-AGGTSMARCTGCAGSAGTCWGG-3 where M = A or C, S = C or G, R = A or G, W = A or T), which hybridizes to the signal sequence region of the antibody γ-chain. The primers for amplifying V_L were VkFor (5′-GTTAGATCTCCAGCTTGGTCCC-3′), which hybridizes to the κ-chain constant domain, and VkRev (5′-GACATTGAGCTGACCCAGTCTCCA-3′), which hybridizes to the signal sequence region of the mouse antibody κ-chain. cDNA fragments were ligated into pGEMT, and recombinant plasmids were purified by alkaline lysis. Sequences of cloned DNA encoding the V_H and V_L inserts were determined by Taq-Dye-Terminator-Cycle-Sequencing, by using two independent batches of RNA to ensure accuracy. The sequences have been deposited at GenBank (Accession numbers: V_L: AY349608; V_H: AY351681).

Modelling: calculations were carried out on SGI Octane R12000 workstations with the Cornell version of the AMBER force field, as implemented in Sybyl 6.8 (Tripos Inc.). New AMBER parameters for the sulfonamide group and for chlorine were developed as described by Geremia and Calligaris.[15] The coordinate file of the homology model has been deposited and may be obtained from the PDB on quoting the depository number, 1OK5. B3LYP/6-31G(d,p) calculations on substrate **1a** were carried out by using Spartan 02 (Wavefunction Inc.).

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Keywords: amides · catalytic antibodies · homology model · hydrolysis · sulfonamides

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