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A MUTATIONAL STUDY OF A DIELS–ALDERASE CATALYTIC ANTIBODY

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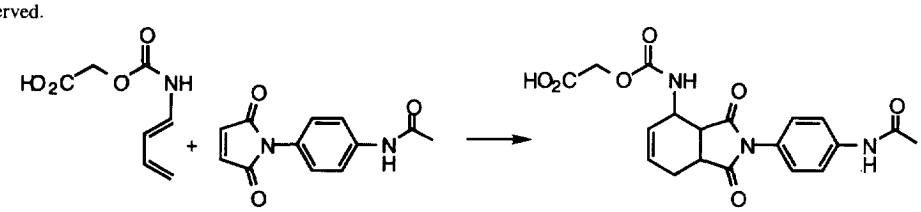
Abstract: Site-directed mutagenesis was used to probe the mechanism of antibody 39-A11, which catalyzes the Diels–Alder reaction of substrate **1** and **2** to give cycloadduct **3**. Mutations that lead to improved packing interactions with the diene afford an order of magnitude increase in catalytic activity.



1 **2** **3**

Recent studies of the structures, mechanisms, and immunological origins of catalytic antibodies are providing important insights into the molecular basis of antibody binding and catalytic activity.[1-9] This more detailed understanding of antibody catalysis should also afford a basis for generating antibodies with enhanced activity. Here we report mutagenesis studies of antibody 39-A11, which was generated to transition state analogue **4** and catalyzes the cycloaddition reaction of diene **1** and dienophile **2** to give the Diels–Alder adduct **3**.[10] Mutations in the active site to improve packing interactions with the boatlike transition state of the Diels–Alder reaction have resulted in an order of magnitude increase in catalytic rate.

Antibody 39-A11 binds hapten **4** with a dissociation constant sec^{-1} (K_d) of 10 nM and catalyzes the Diels–Alder reaction of **1** and **2** with a k_{cat} of 0.67 sec^{-1}, K_m(**1**) = 1200 μM and K_m(2) = 740 μM (Table 1). The three dimensional crystal structure of the 39-A11 Fab•hapten **4** complex shows that the "dienophile-like" succimido portion of the hapten is tightly packed against the hydrophobic side chain of Trp^{H50} with the buried carbonyl group hydrogen-bonded to the carboxamide side chain of Asn^{H35} (Figure 1). The *N*-phenyl substituent is packed against the backbone of residues Gly^{H33} and Glu^{H96}, and the methylene groups of Arg^{H97}. In contrast, the "diene-like" portion of **4** is only loosely packed with residues of the light chain: the closest contacts are from Val^{L91} and Pro^{L96} at 4.2 and 4.6 Å, respectively (Figure 1). This difference in the degree of packing interactions with these two regions of hapten **4** is consistent with the relative binding constants for the dieneophile and diene substrates **1** and **2**, respectively (Table 1). Interestingly, Val^{L91} is a somatic mutation—Ser^{L91} is the

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corresponding residue in the germline antibody. The kinetic constants for the germline antibody are: $K_a(4)$ = 400 nM, k_{cat} = 0.17 s^{-1}, $K_M(1)$ = 1400 μM, and $K_M(2)$ = 450 μM.[3,5,11] The 40-fold increase in binding and the 4-fold increase in catalysis were found to have resulted almost exclusively from the SerL91Val somatic mutation (Table 1).

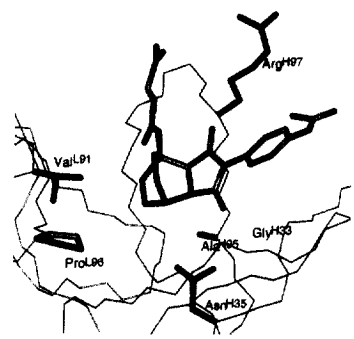

Figure 1. Sites of mutation in the active site of the 39-A11 Fab•hapten **4** complex.

Table 1. Catalytic constructs for wild-type and mutant 39-A11 Fab fragments.

Fab fragment	$K_a(4)$	k_{cat}	$K_M(1)$	$K_M(2)$
germline	400 nM	0.17 s^{-1}	1400 μM	450 μM
$Ser^{L91}Val$ (germline)	16 nM	0.67 s^{-1}	1200 μM	740 μM
39-A11	10 nM	0.67 s^{-1}	1200 μM	740 μM
$Val^{L91}Tyr$ (39-A11)	4 nM	6.3 s^{-1}	2100 μM	420 μM

Based on this structural data a number of site-directed mutants of antibody 39-A11 were generated in order to further probe the mechanism of this antibody-catalyzed pericyclic reaction. First an attempt was made to increase the dienophilicity of **2** by introducing an amino acid whose side chain could form hydrogen bonds to the succinimido carbonyl groups of hapten **4**.[12] Based on the crystal structure and modeling we reasoned that by mutagenizing[13] the complementarity determining region 3 (CDR3) residue Arg^{H97} to Gln or Ser, a new hydrogen bond to the solvent exposed succimido group of hapten **4** could be formed. In addition, to assess the potential contributions of Arg^{H97} to catalysis, this residue was also substituted with alanine. To assay the mutants, specific activities were determined at several substrate concentrations (Table 2).[10] All of the mutants have decreased catalytic activities, roughly paralleling the decreasing length of the side chain. The crystal structure shows that Arg^{H97} is involved in van der Waals interactions with the phenyl substituent of hapten **4**; the loss of this interaction may lead to a less-favorable binding geometry for substrates **1** and **2**. Mutants were also generated at residues proximal to the buried carbonyl group of hapten **4**, which is hydrogen-bonded to the side chain carboxamide of Asn^{H35} (mutants of Asn^{H35} lead to a significant loss in activity[9]). Modelling

suggested that in addition to Asn^{H35}, mutation of Gly^{H33} and Ala^{H95} to Asn or Ser could result in an additional hydrogen bond to the buried carbonyl group, again increasing the dieneophilicity of substrate **2**. We produced the mutants Gly^{H35}Asn, Gly^{H35}Ser, Ala^{H95}Asn and Ala^{H95}Ser; again, all mutants showed reduced activities (Table 2), possibly due to deleterious structural perturbations.

The crystal structure of the 39-A11 Fab•hapten **4** complex suggests that packing interactions between the antibody and substrates also play an important role in binding the dienophile and diene in a reactive orientation, restricting translational and rotational entropy.[14,15] Whereas the structure reveals extensive van der Waals interactions between the antibody and the dienophile, the diene is more loosely packed in the active site. This observation is consistent with the fact that bicyclic hapten **4** can mimic either the *endo* or *exo* transition states for the cycloaddition reaction. In order to improve van der Waals packing interactions with the kinetically favored *endo* transition state, the Val^{L91}Phe, Val^{L91}Tyr, Val^{L91}Ile, Pro^{L96}Phe, Pro^{L96}Tyr and Pro^{L96}Ile active site mutants were generated.[13] In contrast to the results at the previous two positions, three of the packing mutants, Val^{L91}Phe, Val^{L91}Tyr and Pro^{L96}Phe, showed increased catalytic activity (Table 2). To determine if the mutations were additive, the double mutants Val^{L91}Phe/Pro^{L96}Phe and Val^{L91}Tyr/Pro^{L96}Phe were generated and assayed. Each double mutant had a slightly reduced activity relative to the faster of its respective single mutants, suggesting that these side chains may interact with each other.

Table 2. Specific activities of wild-type and mutant 39-A11 Fab fragments.[a]

Fab fragment	Specific activity $\times 10^4$, (μmol min^{-1} mg^{-1})
germline	0.09
39-A11	0.53
Arg^{H97}Gln	0.41
Arg^{H97}Ser	0.16
Arg^{H97}Ala	0.08
Gly^{H35}Asn	0.43
Gly^{H35}Ser	0.32
Ala^{H95}Asn	0.53
Ala^{H95}Ser	0.53
Asn^{H35}Glu[b]	0.11
Val^{L91}Phe	1.6
Val^{L91}Tyr	4.2
Val^{L91}Ile	0.60
Pro^{L96}Phe	3.1
Pro^{L96}Tyr	0.53
Pro^{L96}Ile	0.53
Val^{L91}Tyr / Pro^{L96}Phe	1.9

[a]Assays contained 1 μM protein, 250 μM **1** and 250 μM **2**, and were carried out run at pH 7.4 and 25 °C.
[b]Assayed at pH 5.0

The most active mutant, Val^{L91}Tyr, was fully characterized: K_a(**4**) = 4 nM, k_{cat} = 6.3 s^{-1}, K_M(**1**) = 2100 μM and K_M (**2**) 420 μM (Table 1).[10] The order of magnitude increase in k_{cat}, a decrease in affinity for **1**, and an increase in affinity for the transition state analog hapten **4**, implies that the Val^{L91} to Tyr mutation results in enhanced catalytic activity from increased packing interactions in the transition state relative to the ground state. As noted earlier, residue L91 is also the site of somatic mutation during the process of affinity maturation. Further optimization at this site by in vitro directed-mutagenesis results in improved catalytic function. Attempts to increase activity by generating mutations at other active site residues were unsuccessful. We are currently carrying out additional rounds of in vitro affinity maturation to identify other sites that may allow further improvements in the binding and catalytic function of this novel biological catalyst.

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