Structural Basis for a Disfavored Elimination Reaction in Catalytic Antibody 1D4

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Murine antibody 1D4 selectively catalyzes a highly disfavored β-elimination reaction. Crystal structures of unliganded 1D4 and 1D4 in complex with a transition-state analog (TSA) have elucidated a possible general base mode of catalysis. The structures of the unliganded and liganded Fabs were determined to 1.80 and 1.85 Å resolution, respectively. The structure of the complex reveals a binding pocket with high shape complementarity to the TSA, which is recruited to coerce the substrate into the sterically demanding, eclipsed conformation that is required for catalysis. A histidine residue and two water molecules are likely involved in the catalysis. The structure supports either a concerted E2 or stepwise E1C-like mechanism for elimination. Finally, the liganded 1D4 structure shows minor conformational rearrangements in CDR H2, indicative of induced-fit binding of the hapten. 1D4 has pushed the boundaries of antibody-mediated catalysis into the realm of disfavored reactions and, hence, represents an important milestone in the development of this technology.

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Introduction

The catalytic antibody field has matured rapidly since its inception 15 years ago.¹² Currently, the production of catalytic antibodies is the most effective technology available for generating de novo enzyme catalysts. In brief, antibodies are raised against a stable, small molecule mimic of the transition state for a target reaction. In principle, antibodies that bind this so-called transition-state analog (TSA) may also bind and stabilize the transition state of the target reaction, thus lowering the energy of activation barrier.³ The elicited antibodies are screened for catalysis, and often one or more of them possess some catalytic potential. Many catalytic antibodies have been elicited that may impact public health directly,⁴,⁵ be of general chemical utility,⁶ or provide insights into enzymatic reactions through transition state design.⁷ To date, this technology has been used successfully to generate a variety of antibodies that catalyze ester or amide cleavages,¹ elimination,⁶ cyclization,⁹ aldol,⁸ Diels-Alder reactions,¹⁰ acyl transfers¹¹ and concerted rearrangements.¹² Some antibodies have rate accelerations that are comparable to their natural enzyme counterparts,⁶ while others catalyze reactions for which there is no known enzyme available.¹⁰ Notably, antibodies have been used to catalyze disfavored reactions, such as the anti-Baldwin ring closure reaction.¹³ Catalytic antibody 1D4 is a prototypic example of an antibody that catalyzes a highly disfavored elimination reaction for which there is no chemical or natural counterpart. This antibody helped establish the proof of principle that antibodies are capable of catalyzing highly disfavored reactions.¹⁴

Antibody 1D4 selectively catalyzes the disfavored syn-elimination of HF on an acyclic substrate to form a cis (Z) olefin (Figure 1).¹⁴ Although the catalyzed reaction proceeds slowly (kcat = 2.95 × 10⁻³ min⁻¹ and Km = 212 μM), the background elimination reaction to the cis product is undetectable in the absence of antibody; the competing anti-elimination reaction dominates the background reaction, such that the product distribution is entirely trans. This background elimination to the trans product is highly favored because of reduced steric inter-
actions in the staggered transition state. Hence, the antibody successfully overcomes the large mechanistic barriers that are associated with the syn-elimination to alter the product distribution predominantly towards the cis product. In the absence of any measurable background syn-elimination to the cis product, we can only speculate that the overall rate acceleration is $10^4$ and possibly higher. Hence, the antibody-combining site likely utilizes the majority of its binding energy towards overcoming the extreme steric barriers, and hence stabilizing the eclipsed transition state. Comparison of the background elimination rate ($2.48 \times 10^{-4}$ min$^{-1}$) of the trans product with the syn-elimination of the antibody further suggests that the abzyme uses some form of chemical catalysis to further accelerate the elimination reaction.

In order to catalyze formation of the cis-product, the TSA design incorporated a rigid bicyclic ring structure to constrain the two phenyl groups of the substrate to the eclipsed conformation that characterizes the transition state (Figure 1). In addition, the $\alpha$-keto proton that is abstracted during elimination was replaced with a positively charged amine group in the hapten to elicit a complementary general base in the antibody-combining site. An estimated energy difference of approximately 5 kcal/mol (1 cal = 4.184 J) between the eclipsed and staggered transition states makes this elimination one of the most energetically demanding reactions yet catalyzed by an antibody.$^{14}$ Hence, we sought to understand the interactions that confer specificity to the eclipsed transition state and to identify residues involved in catalysis.

**Results**

Native 1D4 crystals and co-crystals with the TSA were grown in space group $P2_1$ with one Fab molecule per asymmetric unit (Table 1). The crystal structures were determined by molecular replace-

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**Figure 1.** The target $\beta$-elimination reaction. The two possible elimination reactions for the 1D4 substrate are shown with the relevant transition states represented as Newman projections. The favored anti-elimination leads to the trans product, while the highly disfavored syn-elimination leads to the cis product. The rigid bicyclic ring structure in the transition state analog constrains the phenyl rings (blue) to the eclipsed conformation, while the amine group (red) replaces the abstracted hydrogen atom (red) in order to elicit a general base in the active site. The hapten used in co-crystallization with 1D4 was synthesized without the linker to KLH.

**Table 1.** Data collection and refinement statistics

<table>
<thead>
<tr>
<th></th>
<th>Unliganded 1D4</th>
<th>Liganded 1D4</th>
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<td>Unit cell dimensions (Å)</td>
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<td>98.5 % (98.8 %)*</td>
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* Parentheses denote outer-shell statistics.
* Val$^{35}$ is located in a $\gamma$ turn observed in all antibodies.$^{16}$
ment and refined to an $R_{\text{cryst}}$ of 20.9% and $R_{\text{free}}$ of 22.9% at 1.80 Å resolution and an $R_{\text{cryst}}$ of 20.6% and $R_{\text{free}}$ of 24.7% at 1.85 Å resolution for the unliganded and liganded Fabs, respectively. Data parameters, and scaling and refinement statistics are summarized in Table 1.

**General description of the Fab complex**

The 1D4 Fab heterodimer is composed of a $\gamma_2\alpha$ heavy-chain and a $\kappa$ light-chain, in which the variable and constant IgG domains ($V_H$, $V_L$, $C_H1$, and $C_L$) associate along pseudo-2-fold rotation axes (Figure 2). The binding pocket is located between the six loops comprising the heavy and light-chain complementarity determining regions (CDRs), denoted CDR H1, H2, H3, L1, L2, and L3. CDR L1 has a five amino acid residue insertion at position 27, CDR L3 has a single insertion at position 95, and CDR H3 has a two residue insertion at position 100, following the Kabat & Wu numbering scheme for antibodies. A full canonical analysis for L3 loops with this size insertion has not been reported. All of the CDRs have well-defined electron density, and follow the known canonical classes closely, with the exception of CDR H2, which deviates slightly from other structures. The base of CDR H3 has the typical $\beta$-bulge at Asp H101 caused by a salt-bridge interaction between Arg H94 and Asp H101. This $\beta$-bulge is a conserved motif in antibodies with these two CDR H3 residues.

**Selectivity for the eclipsed transition state**

The electron density map (Figure 2) unambiguously shows the TSA nestled between the CDR loops, which form a binding pocket burying 248 Å$^2$ or 92% of the TSA. A total of 71 van der Waals contacts are made between the antibody and TSA. CDRs L3, H2, and H3 supply 25%, 29%, and 39% of the total contacts, respectively. The remaining contacts are derived from framework residue Trp H47 and from Ser H33 in CDR H1. Therefore, 75% of the total contacts are derived from the heavy chain. Analysis of the contacts reveals that the dominant recognition elements for the antibody are the two hapten phenyl rings, which stack between His H58 and the aliphatic portion of Arg H100 (Figure 3(a) and (b)). This stacking interaction accounts for 42% of the contacts. In total, 58 of the 71 van der Waals contacts are made between the antibody and these two aromatic rings, and hence, ~80% of the binding energy derived from van der Waals contacts may be attributed to interactions with the two phenyl rings. The extensive interactions between Arg H100 and the hapten prevent further interactions between the hapten and CDRs L1 and L2. While interactions between the substrate phenyl rings and either side of the binding pocket are monopolized by His H58 and Arg H100, the base of the binding pocket is uniquely adapted to accommodate the two phenyl rings. Specifically, Leu H96 is located between the two rings, forming contacts with each and defining a ridge that separates the bottom of the binding pocket into two distinct slots (Figures 3(a) and 4). The phenyl rings fit into each of these slots exquisitely (Figure 4). This high degree of shape complementarity between the antibody and hapten, therefore, accounts for the selectivity for the eclipsed transition-state of the reaction.

**Electrostatic and cation-π interactions**

The majority of the residues in the binding pocket are hydrophobic, with the exception of two charged residues, Arg H56 and Arg H100. These resi-
dues give an overall electropositive potential to the antibody-binding pocket (Figure 4). Interestingly, the guanidinium moiety of Arg H100 directly abuts the aromatic face of Tyr L37 and is slightly off-centered over one of the hapten phenyl rings. In fact, the aliphatic portion of ArgH100 is centered directly over the hapten phenyl ring. However, since cation-π interactions are inherently electrostatic in nature,20 they may still exist, albeit more weakly, when the cation is slightly off-centered from the π system.21 Hence, it would appear that a cation-π sandwich is formed, where two of the participating moieties are derived from the antibody and one is derived from the hapten (Figure 3(a) and (b)). The electrostatic energy (∆E_{es}) for the Tyr L37 - Arg H100 interaction was estimated at −4.1 kcal/mol, while ∆E_{es} for the hapten phenyl moiety Arg H100 interaction was estimated to be lower, at −1.6 kcal/mol, which reflects the off-centering of the guanidinium group.21 The Tyr L37 - Arg H100 electrostatic interaction is considered strong and constitutes a significant cation-π interaction. Assignment of a cation-π interaction to the weaker hapten phenyl-Arg H100 interaction is more ambiguous. Indeed, ∆E_{vdw} is estimated at −3.4 kcal/mol,21 which indicates that overall, the relative contribution from van der Waals (vdw) interactions is of more significance. This observation of the apparent cation-π sandwich further underscores the importance of this interaction in antibody-antigen recognition. To our knowledge, this type of interaction has been described only for a couple of antibody structures.22,23 It is tempting to speculate that this is an interaction frequently employed by the immune system, which hitherto has been largely unappreciated.

**Hydrogen bond interactions**

No direct hydrogen bonds are made between the hapten and the antibody; however, a water molecule and an apparent hydroxide ion in the binding site form water/hydroxide-mediated hydrogen bonds between the hapten and CDR H2 (Figure 3(a)). The water molecule forms hydrogen bonds between the hapten carbonyl group and the backbone carbonyl group of Arg H100, and the hydroxide ion forms salt-bridge/hydrogen bond interactions.

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† http://capture.caltech.edu/
interactions with $N$ atom of ArgH56, the hapten amine group, and $N^2$ of HisH58 (Figure 3(a)). Our assignment of hydroxide to this electron density is based solely on the chemical environment of the binding pocket, since it is impossible to distinguish unambiguously between hydroxide and water from refinement alone. In summary, the primary amine group on the hapten carries a positive charge (crystallization at pH 7.5) and the nearest antibody residue is ArgH56. Surprisingly, no other counter ion exists in the binding pocket, which carries net electropositive potential (Figure 4). Assignment of the density to some other counter ion, such as chloride, may be excluded safely by B-factor refinement (assuming full occupancy), since chloride has nearly twice as many electrons as oxygen. Finally, hydroxide could be formed by deprotonation of $H_2O$ by HisH58, which is within hydrogen bonding distance (3.0 Å). Hence, a hydroxide ion has been modeled in this electron density, based on the surrounding chemical environment.

Comparison between the liganded and unliganded structures

The liganded and unliganded structures superimpose very closely, with a backbone RMSD of 0.2 Å for $V_L$, 0.4 Å for $V_H$, and 0.45 Å for the entire Fab molecule. Both structures have similar elbow angles of 176.1° and 175.0° for the unliganded and liganded Fabs, respectively. Little difference was observed in the buried surface area or contacts at the $V_L/V_H$ interface (unliganded 1360 Å², 126 contacts, and liganded 1411 Å², 122 contacts), which is consistent with comparative analysis of other structures. The most significant difference between the two structures is located in CDR H2 (Figure 5), where the backbone RMSD of the two structures is 0.7 Å. This conformational difference could be caused by subtle differences in crystal packing, a ligand-induced conformational change, or inherent flexibility. In summary, we believe that all three mechanisms may be at play, although unraveling the relative contributions is challenging.

In order to determine whether the conformational difference in CDR H2 is induced by ligand binding or a difference in crystal packing, we have analyzed seven of the residues, TyrH52-HisH58, with the largest RMSD difference in greater detail. Two of these residues, AsnH53 and PheH55, make crystal contacts; AsnH53 forms identical crystal contacts in both the liganded and unliganded structure, while PheH55 forms unique crystal contacts in each structure. None of the other residues in this loop forms any other direct crystal contact. Two residues, TyrH52 and HisH58, make direct ligand contacts, while ArgH56 forms a solvent-mediated ligand contact. TyrH52 also contacts PheH55 in the unliganded structure, but this contact is no longer present in

Figure 4. Electrostatic and shape complementarity of the hapten in the antibody combining site. The Fab 1D4 binding pocket is shown with the electrostatic potential mapped to the molecular surface. The z-slicing plane has been positioned to highlight the electrostatic surface in the interior of the binding pocket. In this coloring scheme, atoms near a red surface experience an electronegative potential while a blue surface corresponds to an electropositive potential. The relative position of the hydroxide group (red sphere) in the binding pocket is shown. The other water molecule is occluded by the surface, and, therefore, is not visible in this view. The Figure was generated in GRASP.

Figure 5. Comparison of the active sites of the unliganded and liganded 1D4 structures. The $V_L$ and $V_H$ domains for the unliganded structure are shown in dark red and blue with brown side-chains, while the liganded structure is represented in lighter shades of the same colors with yellow side-chains. The largest difference in the two superimposed structures is in CDR H2, where ligand binding probably triggers the conformational rearrangement. Notably, PheH55 adopts a completely different rotamer. ArgH56 is disordered beyond the $C^\alpha$ atom in the native structure. Four water molecules are present in the native active site (denoted W1-W3, the hapten superimposes on W4, rendering it invisible in all views that include the hapten), while two different OH− and HOH molecules are present in the complex.
the liganded structure, where Phe\textsuperscript{H55} adopts a new rotamer, which appears to be stabilized by the unique set of crystal contacts discussed previously. We believe that the new Phe\textsuperscript{H55} rotamer may be triggered by the slight shift in Tyr\textsuperscript{H55} that occurs on ligand binding. Higher than average B-values are observed in CDR H2 for both the liganded and unliganded structures, and hence CDR H2 may have some inherent flexibility in 1D4. The quality of the electron density on CDR H2 is comparable in both structures, except for residue Arg\textsuperscript{H56}. In the unliganded structure, no visible electron density exists beyond the C\textsubscript{b} position of Arg\textsuperscript{H56}, while in the liganded structure, interpretable electron density is manifest for the entire side-chain. Hence, binding of the ligand seems to order this residue. Finally, a hydrogen bond is lost between CDRs H1 and H2 in the liganded structure; in the unliganded structure, the backbone nitrogen atom of Gly\textsuperscript{H31} hydrogen bonds to the side-chain of Asn\textsuperscript{H53} (Figure 5). In concert with the other observed differences, this hydrogen bond could help to preferentially stabilize one loop conformation over another, while ligand binding results in a conformational change that disrupts this hydrogen bond interaction.

Although the majority of the water molecules in the liganded and unliganded Fab structures are nearly superimposable, the distribution of water molecules differs markedly within the binding pocket (Figure 5). The unliganded structure has four water molecules in the binding site, none of which superimposes on the water/hydroxide molecules in the liganded structure. Therefore, in addition to the conformational rearrangements in CDR H2 that occur upon ligand binding, at least two of the water molecules in the unliganded structure must be displaced. Overall, this lack of preorganization in the water molecules and in CDR H2 could explain, in part, why 1D4 is a fairly slow catalyst. It is possible, however, that the loss of conformational entropy that occurs in binding the substrate could be balanced, in part, by the gain in entropy that occurs on returning two molecules of H\textsubscript{2}O to the bulk solvent.

**Discussion**

Elimination reactions can be classified broadly as E1, E2, and E1cB (unimolecular elimination via conjugate base). E1 elimination is characterized by loss of leaving group and formation of a carbocation, followed by spontaneous loss of hydrogen to form the double bond. In contrast, E1cB elimination is characterized by loss of hydrogen and formation of the conjugate base (often stabilized through formation of an enolate or enol intermediate), which collapses to eject the leaving group and form the double bond. E2 elimination lies between the extremes of E1 and E1cB, and is concerted with bond cleavage and formation occurring in a single step. The geometric constraints for E2 elimination are strict, wherein the leaving groups must be either syn- or anti-periplanar.\textsuperscript{25} Although many enzymatic reactions are believed to proceed by an E1cB-like mechanism, clear discrimination between the E1cB and E2 mechanisms is often challenging.\textsuperscript{25}

**Modeling of the substrate**

Superimposing the substrate on the TSA structure affords further mechanistic insights (Figure 6). Interestingly, the antibody buries around 220 \textmum\textsuperscript{2} of the substrate, assuming no further structural rearrangement arises from substrate binding. This buried surface area differs by only 25 \textmum\textsuperscript{2} from that of the TSA. Hence, very little binding energy appears to be directed towards the bicyclic ring portion of the hapten, and specificity for the eclipsed transition state may be explained by the extensive interactions formed between the antibody and the two phenyl moieties of the hapten.

The most intriguing mechanistic insight is the observation of the apparent hydroxide ion near the z-keto proton that must be abstracted during elimination and the water molecule near the substrate carbonyl group. A solvent molecule near the hapten amine group suggests solvent-accessibility to the z-keto proton during the actual reaction. That this solvent molecule could be hydroxide during catalysis is inferred from the alkaline buffer condition (pH 9.0) used for kinetic characterization, where the reaction occurs optimally.\textsuperscript{14} The formation and stabilization of this hydroxide is probably assisted by hydrogen bonds between Arg\textsuperscript{H56} and the substrate carbonyl group, possibly lowering the pK\textsubscript{a} of the abstracted proton.

![Figure 6. Substrate modeling by superposition on the hapten. Most of the antibody interactions with the hapten and substrate alike are directed towards the two phenyl rings. The putative hydroxide ion is positioned to abstract the hydrogen atom from the substrate, while the water molecule forms a hydrogen bond with the substrate carbonyl group, possibly lowering the pK\textsubscript{a} of the abstracted proton.](image-url)
Comparison with β-elimination enzymes

In assessing the catalytic efficiency of many natural enolase enzymes, it has been proposed that the conjugate acid of the general base and the α-keto hydrogen must have similar pKₐ values. The measured pKₐ of an α-keto hydrogen atom is typically 16-20, which should, in principle, be inaccessible for abstraction by solvent-exposed side-chains. Therefore, the pKₐ of both the general base and the carbon acid must be perturbed by several units to effect catalysis. The enolase superfamily of enzymes abstracts a carboxylic acid α-proton to form a reactive enolate, which is harnessed to effect a variety of chemical transformations. For this family of enzymes, perturbation of the pKₐ of the α-hydrogen atom can be achieved by protonation of the carbonyl group by a general acid and/or electrostatic interactions between the α-carboxylic acid and a conserved divalent metal ion. This form of electrophilic catalysis decreases the pKₐ of the α-proton by ~8 units. This family appears to have little homology to 1D4, however, due to the conserved divalent metal ion coordinated in the active site.

A classical example of a natural enzyme that catalyzes a β-elimination reaction is the 3-dehydroquinate dehydratase (DHQase) involved in the shikimate biosynthesis pathway that is critical for the growth of microorganisms and plants. The substrate, dehydroquinate, is cyclic and, hence, already geometrically restrained, making the reaction less energetically demanding than 1D4. Two types of enzymes that perform this elimination have been characterized structurally. Type 1 enzymes invoke a catalytic lysine residue to form a reactive Schiff base intermediate leading to syn-elimination, while the type 2 enzymes invoke a catalytic histidine residue to form a reactive enolate intermediate, leading to anti-elimination. The reaction product is the same for both the syn and the anti-elimination reactions in the two types of enzyme. The type 2 enzyme appears to have the greatest biochemical homology to 1D4, despite its mechanistic preference for the anti-elimination pathway. We propose an analogous role for HisH58 in 1D4, except that HisH58 appears to act through an intervening water molecule.
action between the water molecule and the hapten carbonyl group appeared to be serendipitous, as this interaction was not pre-programmed explicitly in the original hapten design. Second-generation haptns for β-elimination reactions could attempt to elicit stronger hydrogen-bond donor groups by replacing the carbonyl group in the hapten with a more electronegative group, or a negatively charged moiety.

Despite the relatively low efficiency of 1D4, the antibody clearly catalyzes the syn-elimination of an acyclic substrate well above the syn-elimination background reaction, which is immeasurably slow, and tenfold faster than the relatively facile anti-elimination reaction. This antibody represents an important proof of principle for the field of catalytic antibodies, because 1D4 demonstrates clearly that the large energy barriers associated with disfavored reactions are surmountable. Hence, 1D4 successfully recruits binding energy to trap the eclipsed transition state conformation, and further recruits water-mediated hydrogen bonds and a histidine residue to yield a possible elimination mechanism that is reminiscent of scytalone dehydratase, dehydroquinase dehydratase, and the enolase mechanism that is reminiscent of scytalone dehydratase, dehydroquinase dehydratase, and the enolase superfamily of enzymes.

Materials and Methods

Digestion and purification

Murine IgG antibody was obtained from cultured hybridoma cells. The IgG was digested by standard protocols40 in 1 % (w/v) papain for an hour and stopped with 200 mM iodoacetamide. The Fab was purified by affinity chromatography (protein A column (Repligen) and G column (APBiotech)), followed by ion-exchange chromatography (MonoS column) yielding four Fab fractions. The last of these fractions to elute from the MonoS column crystallized.

Crystallization and data collection

1D4 Fab was concentrated to 16 mg/ml in 0.2 M sodium acetate buffer, pH 5.5. Unliganded and liganded crystals were grown from 18-21 % (w/v) PEG 4000, 0.1 M isopropanol, 0.1 M Hepes (pH 7.5). An approximately tenfold molar excess of TSA was used for co-crystallization. The crystals form needles of various size in space group P2₁ with cell parameters a = 67.7 Å, b = 48.2 Å, c = 69.3 Å, β = 106.2°. Crystallization was facilitated by streak seeding,41 which routinely yielded high-quality crystals. Smaller crystals generally gave diffraction with the least mosaicity, probably because the larger crystals suffered from inter-growth and/or twinning. Crystals were cryo-cooled in liquid nitrogen after soaking in mother liquor substituted with 20 % (v/v) glycerol. Data were collected at SSRL on beamlines 7-1 and 9-2 (Table 1) and integrated and scaled with HKL2000.42

Structure solution

The structure was determined by molecular replacement using Merlot43 to rapidly screen ~100 Fabs for potential solutions to the rotation function, and then with AMoRe44 using murine anti-progesterone Fab DB345 (from Merlot) as the search model for rotation (correlation coefficient 22.5, 9.9 noise) and translation functions (correlation coefficient 37.2, Rcryst 48.3 %, compared to 12.0 and Rcryst 56.1 % noise). Coordinates for a structurally related TSA were obtained from the Cambridge Structural Database (CSD).46 The linker of the CSD analog was removed and the nitro moiety was substituted with an amino moiety. This model was subsequently subjected to 1000 iterations of least-squares minimization with the X-PLOR parameter files in Discover (ACCELERYS, San Diego, CA). The resulting model could be fitted easily into the initial Fv – F₀ map with slight changes in a few dihedral torsion angles. The model was refined in CNS,47 and refinement of the Fab complex converged to an Rcryst of 20.6 % and Rfree of 24.7 % for all data. The unliganded 1D4 was determined using the refined TSA-1D4 structure as a model. Model bias was avoided by simulated annealing steps during refinement. The unliganded Fab was likewise refined in CNS and converged to an Rcryst of 20.9 % and Rfree of 22.9 % for all data.

Protein Data Bank accession codes

The atomic coordinates and structure factors for the unliganded and liganded Fab have been deposited in the RCSB Protein Data Bank with accession codes 1JGV and 1JGJ.

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