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Review

Canonical binding arrays as molecular recognition elements in the immune system: tetrahedral anions and the ester hydrolysis transition state

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Abstract

The structures, obtained by X-ray crystallography, of the binding sites of catalytic antibodies raised to bind different phosphonates are compared. Although the amino acid sequences differ, all exhibit a tetrahedral array of hydrogen bond donors (a 'canonical binding array') complementary to the tetrahedral anion, which represents a 'transition state epitope' for the basic hydrolysis of esters and amides. Antibodies for phosphates, arsonates, and sulfonates are found also to possess the tetrahedral anion canonical binding array.

Keywords: Canonical binding array; Molecular recognition; Immune system; Tetrahedral anion

1. Introduction

The ability of the immune system to produce proteins that selectively bind to almost any foreign antigen is amazing. It is known that the immune system possesses a naive repertoire of greater than 10^8 antibody binding sites (derived from only 10^3–10^4 gene fragments) in the absence of antigen [1]. When the immune system is challenged by an antigen, the process of affinity maturation produces new antibodies that bind to antigens with increased affinity and specificity [2].

Following a strategy suggested by Jencks [3], antibody catalysts [4–11] for many organic reactions have been produced by challenging the immune system with stable mimics [12] of putative transition states. Since the mature antibodies produced in this manner bind to transition state analogs (TSAs), catalysis is often achieved through selective stabilization of a transition state over other species on the reaction's potential energy surface. Even in cases that do not strictly conform to this paradigm, transition state complementarity is responsible for some fraction of catalysis, and recent computational studies on antibody-catalyzed reactions have revealed intimate details of antibody–transition state interactions [13–24].

Catalysis requires selective binding of the transition state. Modifications of the TSA should cause a change in the binding site and attenuation of the catalyst proficiency. We have explored the structures of 10 catalytic antibodies for ester and amide hydrolysis and have compared these to antibodies that bind phosphonates, arsonates, and sulfonates. We have found that the immune system binds all of these tetrahedral anions by a recurring motif that we refer to as a 'canonical binding array'. The implications for production of antibody catalysts are discussed.

2. Results and discussion

2.1. Binding sites for tetrahedral anionic haptens

The majority of all known catalytic antibodies hydrolyze esters or amides or mediate closely related reactions such as transesterification or ester aminolysis [4–11]. As a

Abbreviations: BSA, bovine serum albumin; TSA, transition state analog; TSE, transition state epitope; CDR, complementarity determining region

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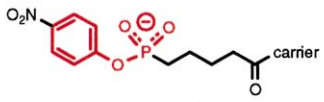
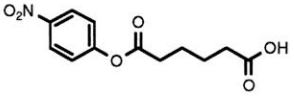
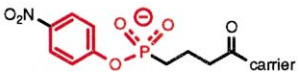
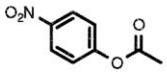
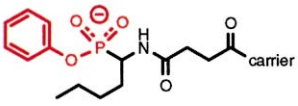
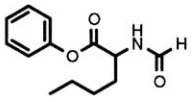
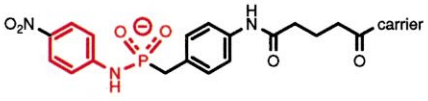
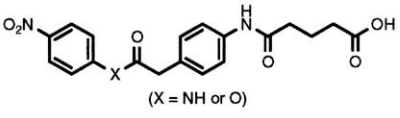
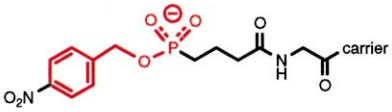
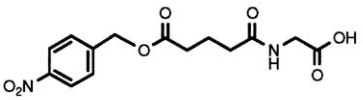
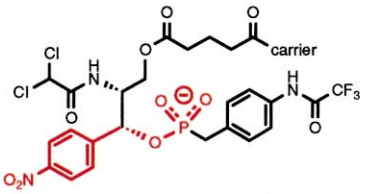
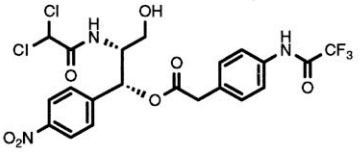
result, much of our understanding of how antibodies catalyze chemical reactions has been derived from experiments on hydrolysis.

X-ray structures of greater than 10 hydrolytic antibodies have been described in the literature [25–60]. The haptens used to elicit these antibodies are shown in Table 1, along with kinetic data on catalysis and some information about the X-ray structures. All of the haptens are aryl or benzyl phosphonate or phosphonamidate TSAs and were conjugated to bovine serum albumin (BSA) or keyhole limpet hemocyanin carrier proteins during immunization. Fig. 1 shows the binding sites for these antibodies. Hapten binding involves recognition of the anionic phosphonate oxygens by several hydrogen bond donating residues and sequestration of the aromatic moiety in a hydrophobic pocket (Fig. 1). It was suggested in 1996 by MacBeath and Hilvert that the combining sites of four of these independently derived antibodies – 48G7 [25–29], CNJ206 [30–33], 17E8 [34–42], and 43C9 (at the time only available in a modeled structure based on the sequence) [43–51] – represent 'variations on a theme' which may be general for antibody-catalyzed hydrolysis [61] based on sequence analysis and the observation that many of the same residues were found to contact bound hapten in 48G7, CNJ206, and 17E8 (Fig. 1). Subsequent structures of the germline precursor to 48G7 [25–29] and another antibody from the same immunization as 17E8, 29G11 [34–42], show similar patterns of hapten recognition (Fig. 1). In Fig. 1, we emphasize relative orientations of the four or more hydrogen bond donors observed to contact the anionic oxygens of the phosphonate haptens.

At the time of the 'variations' proposal [61], the X-ray structure of 43C9 was not available. The structure of this antibody complexed with *para*-nitrophenol was, however, reported recently [51]. Although the hapten (a phosphonamidate), substrate (an amide), and mechanism of catalysis (thought to involve the formation of a covalent adduct) differ from those of 48G7, CNJ206, 17E8, and their rela-

Table 1
Haptens and substrates for structurally characterized hydrolytic antibodies[a]

antibody	hapten	substrate	k_{cat}/k_{uncat}	model resolution
48G7			1.6×10^4	2.0 Å w/ hapten 2.7 Å w/o hapten
germline precursor			10^2	2.1 Å w/ hapten 2.1 Å w/o hapten
CNJ206			1.6×10^3	3.2 Å w/ hapten 3.0 Å w/o hapten 2.1 Å w/product
17E8			8.3×10^3	2.5 Å w/ hapten
29G11			2.2×10^3	2.2 Å w/ hapten
43C9		 (X = NH or O)	2.7×10^4 (O) 2.5×10^5 (NH)	2.1 Å w/PNPOH 2.1 Å w/o PNPOH
D2.3			1.3×10^5	1.9 Å w/ hapten
D2.4			3.6×10^4	3.1 Å w/ hapten
D2.5			2.5×10^3	2.2 Å w/ hapten
6D9			9.0×10^2	1.8 Å w/ hapten
7C8			7.1×10^2	2.2 Å w/ hapten

[a]References may be found in the text. PNPOH refers to *para*-nitrophenol.

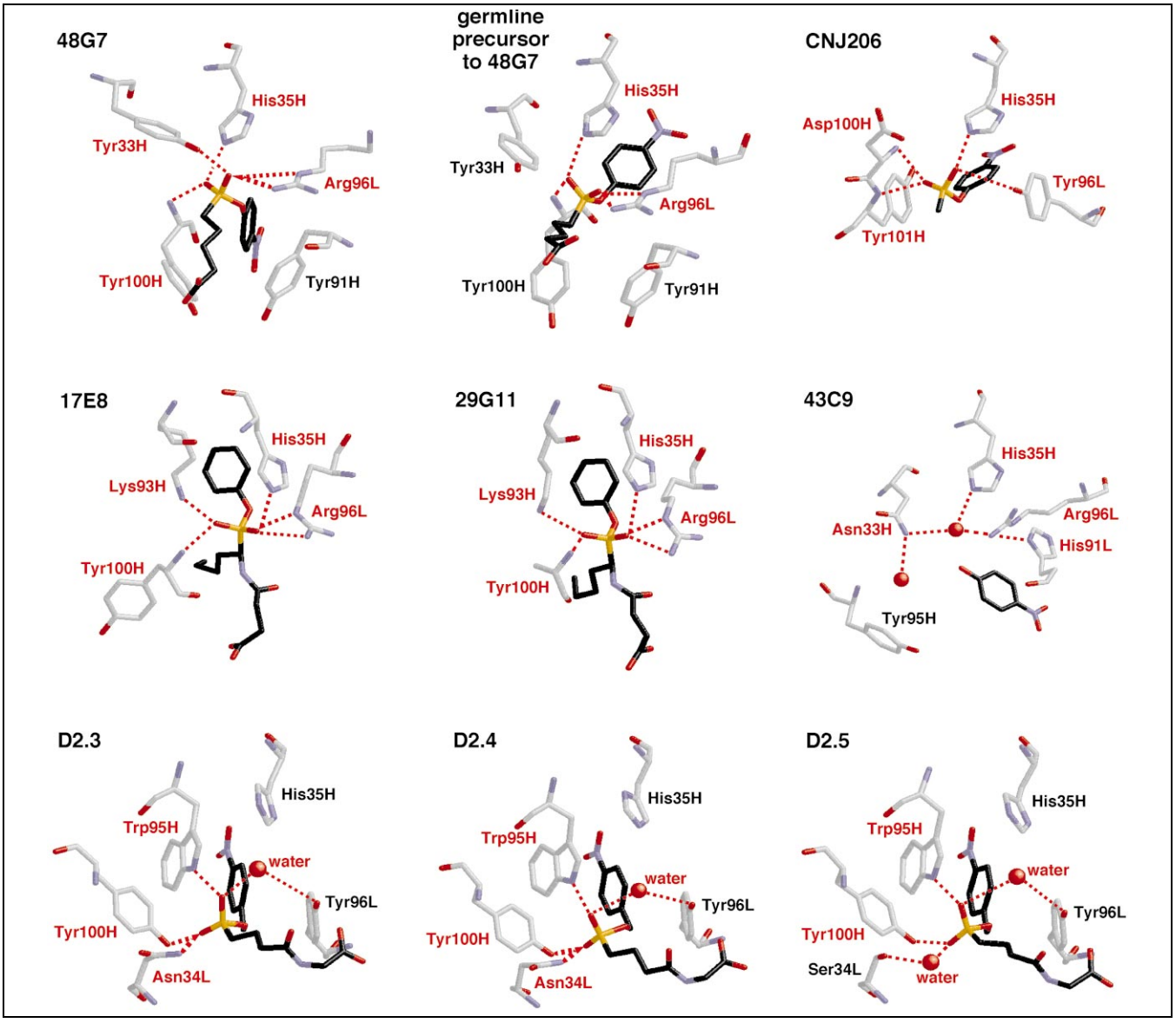


Fig. 1. Crystallographically determined binding sites of hydrolytic antibodies. Bound hapten (Table 1) is shown in each case, except for 43C9 where only the *para*-nitrophenol-bound structure was available; in this structure, the two water molecules shown are presumed to be located in approximately the same areas as the phosphonamidate oxygens would be. Residues that hydrogen bond to phosphonate oxygens are labeled in red. In the 17E8 and 29G11 structures, the orientation of the imidazole ring of His35H has been flipped by 180° from that reported.

tives, the binding site of 43C9 does indeed show a similar pattern of residues involved in hapten recognition (Fig. 1).

Crystal structures of additional hydrolytic antibodies (D2.3, D2.4, and D2.5) [52–55] show that the 'variations' proposal can be extended to benzyl phosphonate recognition as well [62]. The pockets in D2.3, D2.4, and D2.5 are slightly more spacious than those of the 'variations' antibodies – most likely due to the fact that they were elicited against benzyl rather than aryl phosphonate haptens – and hapten recognition is mediated by water molecules in several places. Nonetheless, antibodies D2.3, D2.4, and D2.5 do possess binding sites which are similar to those observed for CNJ206, 48G7 and 17E8 (Fig. 1).

The similarity of these binding sites can be assessed in several ways: in terms of the binding surface presented by the antibody and in terms of the actual residues that contact the bound hapten. Fig. 2 provides a comparison of combining site residues from the former perspective. It is clear from this sort of comparison that combining sites elicited against similar phosphonate haptens contain many similar – often identical – residues at particular positions. All of these antibodies have His35H at site a and a tyrosine residue at site c (Figs. 1 and 2). The residues at sites b and d are more variable, yet still somewhat conserved.

Fig. 3 delineates the types of contacts actually found for the pro-*R* and pro-*S* oxygen atoms of the haptens. In this figure the residues are grouped according to their position

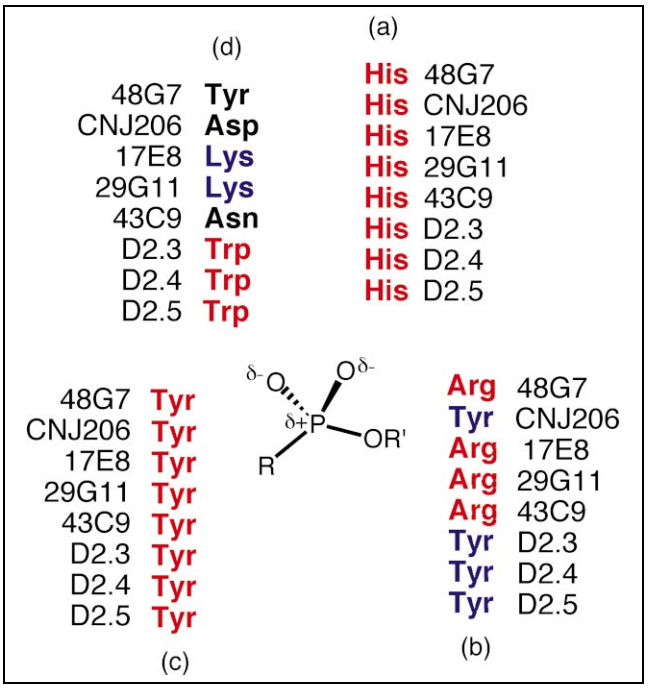


Fig. 2. Residues that line the phosphonate binding site. Site (a) corresponds to the residue at position 35H in all antibodies. Site (b) corresponds to the residue at position 96L in all antibodies. Site (c) corresponds to a residue in the vicinity of position 100H. Site (d) corresponds to a residue in the vicinity of position 33H or 95H. See Fig. 1 for the crystallographically determined positions of all residues. The most common residues at each site are colored red and blue.

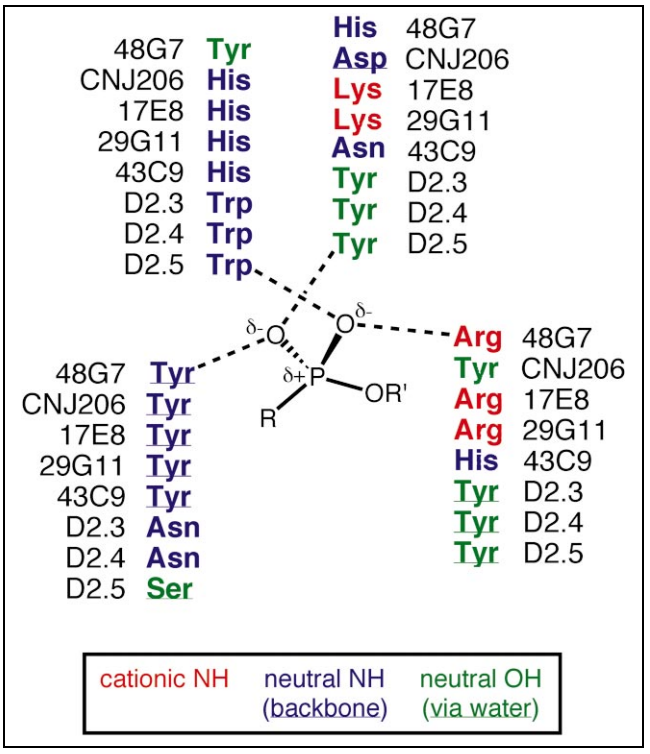


Fig. 3. Antibody residues that donate hydrogen bonds to oxygen atoms of the haptens. The residues are grouped into four groups (different from those in Fig. 2) based on their spatial relationships with the pro-*R* and pro-*S* oxygen atoms of the phosphonate haptens. See Fig. 1 for crystallographically determined positions of all residues. The residues are color-coded based on the type of hydrogen bond donor that they present to the hapten.

relative to the hapten oxygens rather than in sequence (as in Fig. 2). There is clearly greater similarity in the type of hydrogen bond donor presented to the hapten than in the specific identity of the residue that provides it, and in several cases the actual hydrogen bond donor is an oriented water molecule.

Recently, X-ray structures of two hydrolytic antibodies from another family (6D9 and 7C8) have cast some doubt on the generality of the phosphonate binding 'theme' [56–60]. These crystal structures (see Fig. 4) show that the benzyl phosphonate hapten used to elicit 6D9 and 7C8 (Table 1) does not bind in the same type of binding pocket used to bind the other aryl and benzyl phosphonate haptens. In addition, the hapten binds in different orientations in 6D9 and 7C8. These observations suggest that the binding site theme is limited to antibodies derived from relatively unsubstituted aryl and benzyl phosphonate haptens. The additional functionality present in the hapten used to elicit 6D9 and 7C8 (Table 1) comprises as much of its structure as does the benzyl phosphonate substructure, and therefore the antibody repertoire is presented with many additional recognition elements upon immunization

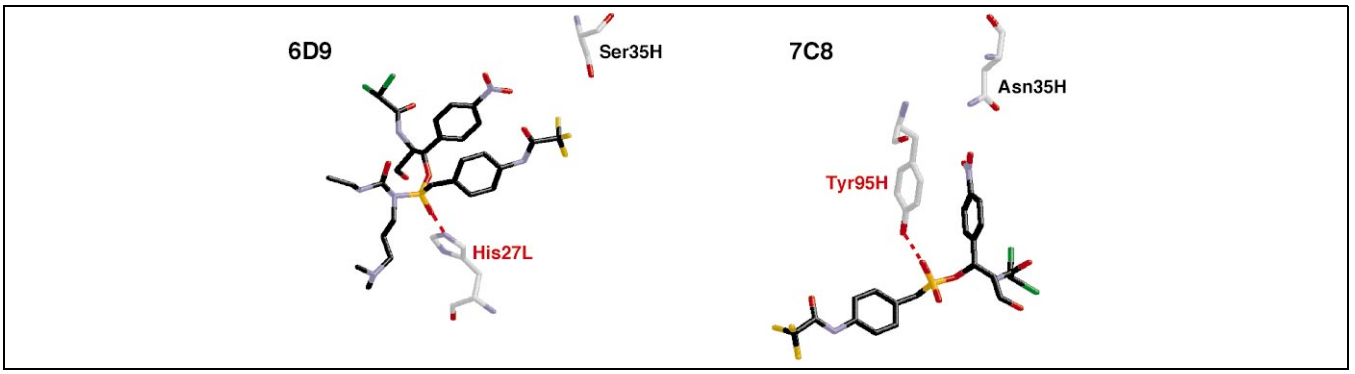


Fig. 4. Crystallographically determined binding sites of hydrolytic antibodies 6D9 and 7C8. Bound hapten (Table 1) is shown for 7C8, and a modified hapten, in which one phosphonate oxygen is replaced by a substituted nitrogen, is shown for 6D9.

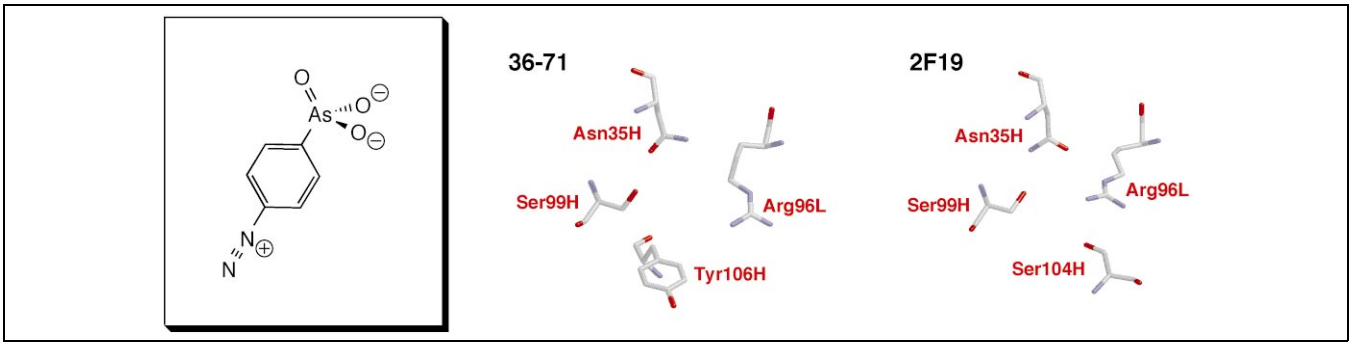


Fig. 5. Structures of the putative hapten binding sites of antibodies 36-71 and 2F19.

with this hapten. In other words, the 6D9 and 7C8 hapten appears to sport multiple epitopes, analogous to the situation usually observed for much larger antigens such as proteins. Nonetheless, the similarities between many of the hydrolytic antibodies are striking when the hapten has an exposed tetrahedral anion.

While the naive repertoire of antibodies possesses a few very similar binding sites suitable for recognition of phosphonate groups [21,25], these binding sites are not limited to phosphonate recognition.

A database search (Tantillo, D.J. and Houk, K.N., unpublished results) has revealed that several antibodies raised against *para*-azophenylarsonate-derivatized (Fig. 5) carrier proteins have very similar variable region sequences – and related germline precursors – to those of the phosphonate binders [30,63–65]. For example, light and heavy chain variable region sequences of the anti-arsonate antibodies 123E6, 124E1, 93G7, 91A3, 36-71 and 2F19 exhibit 67–80% identity to those of hydrolytic antibody 17E8. While the complementarity determining regions (CDRs) of the anti-arsonate antibodies differ from those of 17E8 at many sites, these differences are often conservative. In particular, hydrogen bond donor functionalities are maintained in these regions as would be expected for stabilization of the negative charge which should be distributed similarly over the phosphonate and arsonate groups. Several X-ray crystal structures of anti-arsonate antibodies have also been reported, albeit without haptens bound. The structures of the proposed hapten binding sites of antibodies 36-71 and 2F19 are shown in Fig. 5 [63–65]. These binding sites are extremely similar to those of hydrolytic antibodies (Fig. 1), containing, for example, the highly conserved Arg96L and replacing His35H of the phosphonate binders with the similar hydrogen bond donor Asn35H (see Fig. 2) [26].

Several anti-DNA antibodies [66] also have sequences similar to those of hydrolytic antibodies. In particular, the light chain variable regions of anti-DNA antibodies DP7 and DP11 are extremely similar to those of 48G7 (nearly 90% identity! ~60% identity to the 48G7 heavy chain variable region). Again many differences appear to be conservative, but without three-dimensional structures of these antibodies, binding site similarities remain speculative at best [30,51,67,68].

It should perhaps be expected that arsonate haptens and nucleic acids will elicit antibodies similar to those elicited in response to phosphonate haptens. Arsonate and phosphonate groups are obviously geometrically and electronically similar. It is also likely that anti-nucleic acid antibodies recognize the phosphodiester groups of the polyphosphate backbone which resemble the phosphonate groups present in the haptens, although DNA binding is most likely dominated by surface–surface interactions [67,68].

Recently, a crystal structure of antibody 21D8, an antibody decarboxylase, has also become available [21]. This

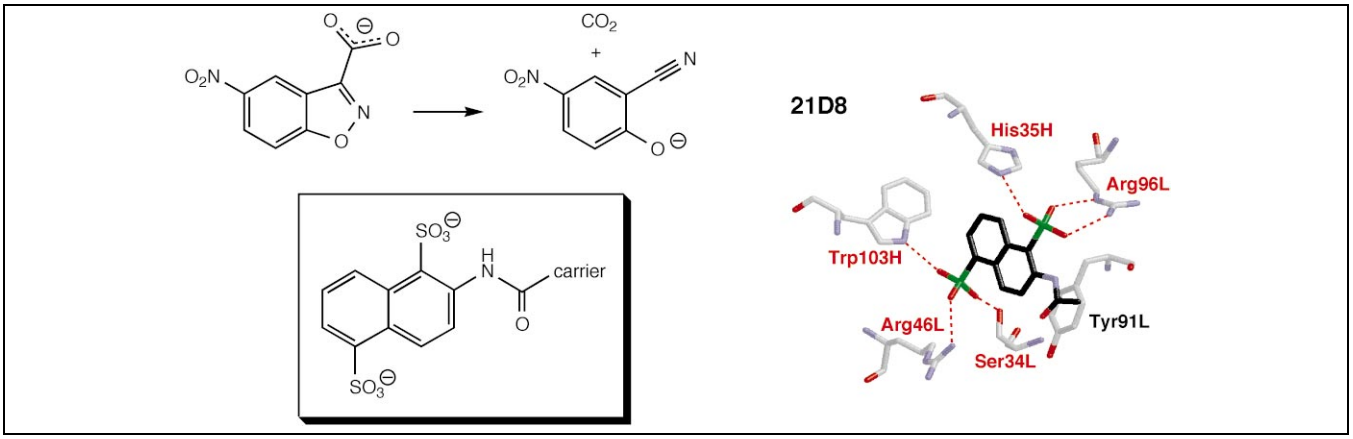


Fig. 6. The decarboxylation reaction catalyzed by antibody 21D8, the naphthalene disulfonate hapten used to elicit 21D8, and the 21D8 binding site.

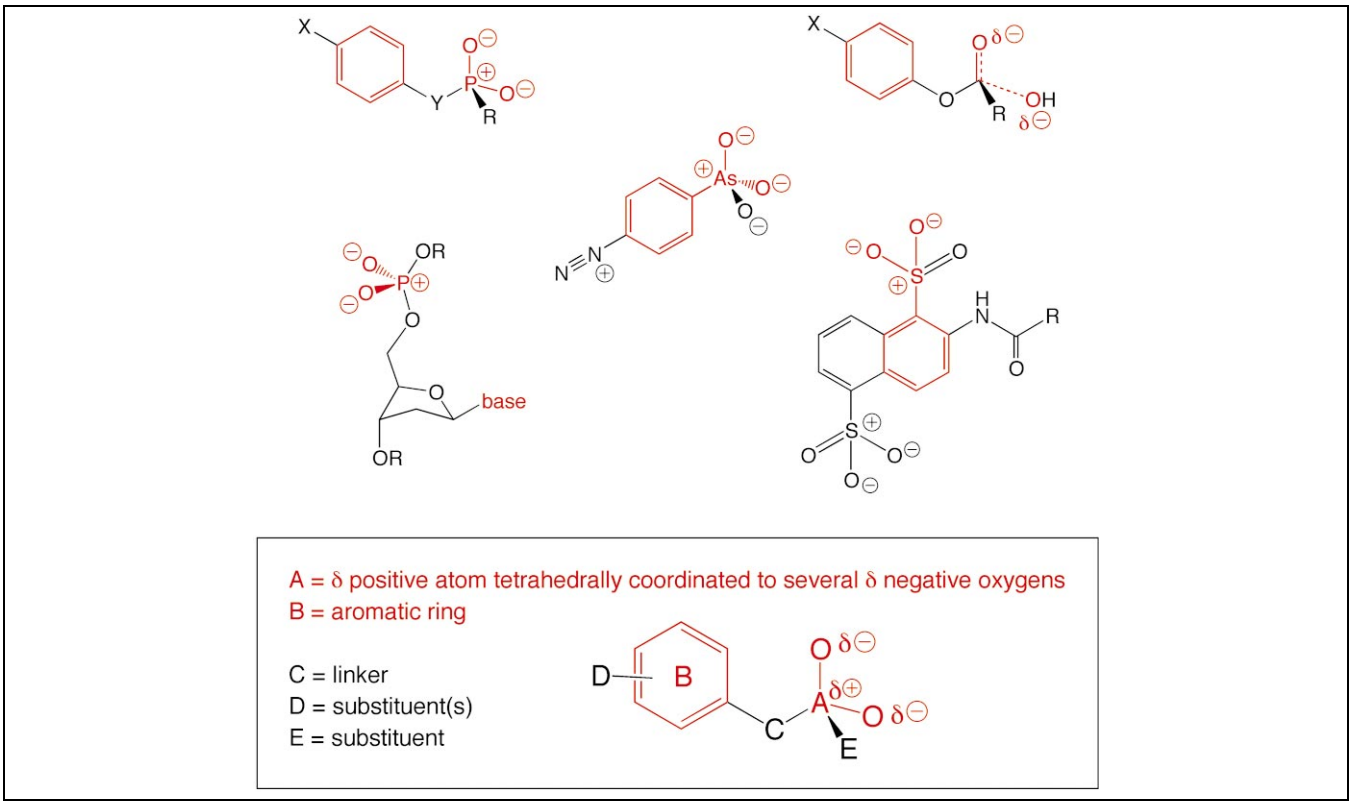


Fig. 7. Antibody ligands that share the TSE (shown in red and described at the bottom of the figure) and that are bound by the 'canonical array' described in the text: phosphonates and derivatives, transition states for hydroxide attack on aryl esters, *para*-azophenylarsonate, nucleotides, and a naphthalene disulfonate hapten.

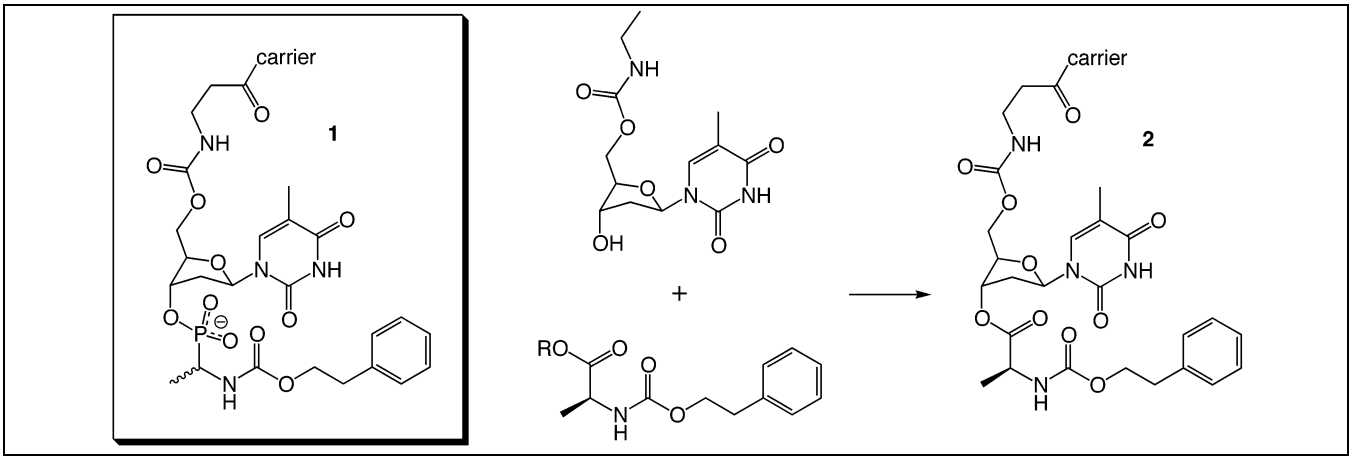
antibody catalyzes the decarboxylation of carboxybenzisoxazoles (Fig. 6), a reaction that clearly differs from ester hydrolysis. Antibody 21D8 was raised against a naphthalene disulfonate hapten (Fig. 6). The combining site of this antibody is extremely similar to that of the hydrolytic antibodies discussed above (compare Figs. 1 and 6) – especially that of 48G7 [21]. It is likely that this similarity is due to the fact that the haptens used to elicit both 21D8 and 48G7 contain tetrahedral anionic groups (phosphonates or sulfonates) connected to planar aromatic moieties. The similarity of 21D8 and 48G7 suggests that they may cross-react, and computational docking studies (Tantillo, D.J. and Houk, K.N., unpublished results) indicate that 21D8 should be able to bind the transition state for hydrolysis of *para*-nitrophenyl esters [69] and provide catalysis.

2.2. *Transition state epitopes (TSEs) and canonical binding arrays*

The similarities in both sequence and structure between this diverse group of antibodies are perhaps best explained by the presence of generic germline binders for tetrahedral anions connected to aromatic rings (Fig. 7). These functional groups together comprise an example of what we refer to as a TSE: the key recognition elements present in the transition state (and TSAs) for an antibody-catalyzed reaction. We define the binding motif shared by these germline antibodies – several key hydrogen bond donating residues oriented to specifically stabilize tetrahedrally displayed anionic heteroatoms coupled to a nearby hydrophobic pocket – as a 'canonical binding array' in the spirit of the canonical combinations of V gene segments of Manser and coworkers [70] and the canonical CDR classes of Chothia and Lesk [71]. A correlation has been observed previously between the combination of canonical classes used to bind an antigen and the type of antigen (protein, polysaccharide, nucleic acid, hapten, etc.) bound [72,73], and this correlation is now extended to amino acid side-chain functionalities as well. Interestingly, the canonical array concept was foreshadowed by Wu and Kabat who noted over 30 years ago that 'a mixture of structurally different antibody molecules (i.e. belonging to different immunoglobulin classes or subclasses) could have the same binding affinity and therefore probably have very similar or even identical combining sites' [74].

2.3. *Biological epitope mimicry*

The function of antibody binding sites is to bind fragments of common biomolecules such as proteins, carbohydrates, and nucleic acids that are signatures of foreign organisms. Generation of antibody catalysts may therefore be most effective when transition states and their analogs



Scheme 1.

resemble naturally occurring biological molecules. In this scenario, mature binding sites that are complementary to transition states for non-biological reactions are derived from germline binding sites that are complementary to biological epitopes. Phosphate and phosphodiester groups of nucleic acids are biological epitopes which are mimicked [75,76] by phosphonate, sulfonate, and arsonate haptens. Interestingly, some of the most efficient antibody catalysts known were raised against phosphonate TSA **1** (Scheme 1) and promote the transesterification reaction shown in Scheme 1 to produce the corresponding ester **2** with $(k_{cat}/K_m)/k_{uncat}$ values of approximately 10^8 and effective molarities (k_{cat}/k_{uncat}) of approximately 10^5 [77–79]. The similarity of hapten **1** to thymine dinucleotide is obvious.

Evidence exists for other cases of biological epitope mimicry as well. The three-dimensional structures of two antibodies, 39-A11 and 1E9, that catalyze Diels–Alder reactions have recently been determined to high resolution [14,80]. Stevens and Schultz [80] have noted the similarity in structure between 39-A11 and two other structurally related antibodies, DB3 and TE33, and Houk, Hilvert, and Wilson [14] have discussed the relationships between 39-A11, DB3, and 1E9 in detail. Antibody TE33 binds the 15-residue cholera toxin peptide [81,82], which contains a type II β-turn when bound, and DB3 binds steroids such as progesterone [80,83–85]. The β-turn structure of cholera toxin has been shown to bind in a pocket similar to those that bind progesterone and the bicyclic ring systems of TSA haptens **3** and **4** used to elicit 39-A11 and 1E9, respectively (Chart 1). The valine–proline–glycine sequence involved in the β-turn and the hydrocarbon rings of the haptens and steroids have all led to antibody hosts which contain deep hydrophobic pockets. Since type II β-turns often contain hydrophobic proline and glycine residues for geometric reasons [86], a deep hydrophobic pocket would seem ideal as a generic binding site for these structures. It is therefore plausible that generic β-turn binders in the naive repertoire have led to TE33, DB3, 39-A11, and 1E9, and the connections between the germline precursors to these antibodies have been noted [14,80]. This is an elegantly simple case of biological epitope mimicry in which a host for a biological hydrophobic array has led to catalysts for reactions that proceed through non-polar transition states.

2.4. Affinity maturation and the development of specificity

The fact that the binding sites of antibodies 39-A11, 1E9, TE33, and DB3 are similar does not necessarily mean that they will cross-react. While DB3 successfully binds many different steroids [83–85], it does not accelerate the Diels–Alder reaction promoted by 1E9 to any appreciable extent [14]. Moreover, when screened against a

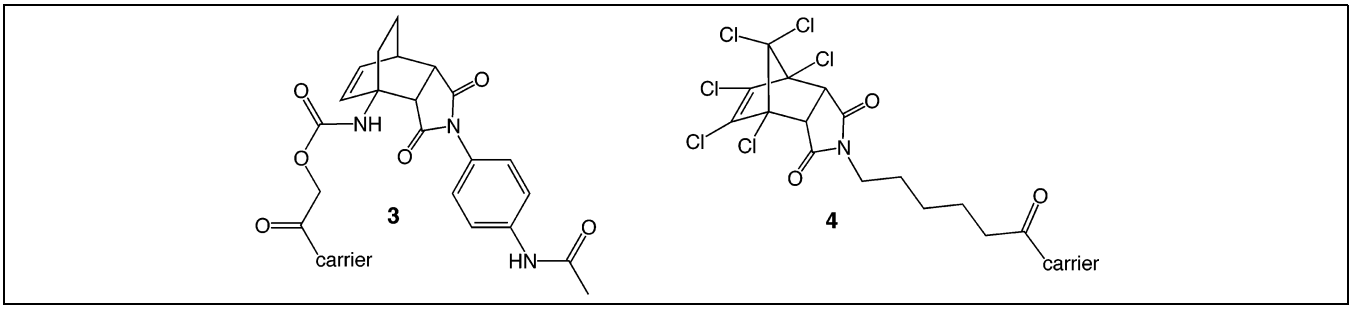


Chart 1.

'panel of 72 structurally diverse hapten–BSA conjugates', the germline precursor to 39-A11 displayed broad cross-reactivity, while no cross-reactivity was observed for 39-A11, suggesting that germline 'polyspecificity is tempered upon affinity maturation' [80]. These results emphasize that the alterations to antibody combining sites that occur upon maturation, while often structurally subtle, can have considerable consequences for cross-reactivity.

A similar loss of polyspecificity upon maturation has been reported for anti-arsonate antibodies [70]. It was observed that antibodies isolated from early in the primary immune response to *para*-azophenylarsonate conjugates bind single stranded DNA (the similarities between anti-arsonate, anti-DNA, and hydrolytic antibodies were discussed above). Antibodies isolated during the secondary immune response, however, lacked this polyspecificity.

An elegant study of hydrolytic antibody 48G7 by Schultz and Stevens [25–29], in which this antibody and its germline precursor were characterized both biochemically and structurally, provides great insights into the chemical details of affinity maturation. Mature 48G7 and its germline precursor differ by only nine mutations; these increase the affinity for hapten (a phosphonate TSA, Table 1) by a factor of 10^4 and the rate of hydrolysis by a factor of 10^2 [26,27]. None of the nine mutated residues actually contacts the hapten, but these mutations influence details of the combining site structure by causing subtle reorientations of combining site functionalities (Fig. 1).

In light of experiments such as these which show that affinity maturation proceeds with relatively few mutations, much of a mature antibody's ability to bind a substrate must be encoded by the germline. The affinity maturation process tends to tighten binding through relatively few and distant mutations [25–29,87], suggesting that the initial recognition events in the immune response need only to involve relatively loose binding. The ability of the immune response to produce antibody catalysts is most likely a result of the fact that TSAs (and transition states themselves) happen to fit into generic germline sites reasonably well.

If antibodies adopt multiple conformations [88–90], the diversity of the naive repertoire is greatly increased [91]. In fact, it has been observed in several cases that conformational changes upon binding in different antibodies lead to similar combining sites. This is an unusual and unexpected type of structural convergence.

In the case of hydrolytic antibodies D2.3, D2.4, and D2.5, crystal structure analysis did not reveal any significant conformational changes upon hapten binding [52–54]. However, subsequent kinetic experiments revealed that a pre-equilibrium exists for each of these antibodies that converts between active and inactive conformers. This equilibrium is shifted towards the active conformer in the presence of hapten [55], and it is the active conformer of each that displays the canonical binding array. It was estimated that this conformational change leads to a 30–170-fold increase in affinity for hapten. This situation suggests that the pressure of hapten binding can select for flexible antibodies capable of obtaining productive conformations in addition to those that are rigidly preorganized for binding.

The convergence of hydrolytic catalyst structures from different immunizations with different haptens was discussed above [61,62]. In the case of antibody CNJ206, a drastically different antibody conformation was observed in the X-ray structures of free and hapten-bound antibody [30–33], an observation that is consistent with a conformational change upon binding [32]. Again the canonical array was not necessarily preorganized in solution, but was selected for in the presence of hapten.

A similar situation has been reported for antibodies to foot-and-mouth disease virus [92]. Crystal structures of two different antibodies – complexed to their peptide antigens and antigen-free – showed that conformational changes upon antigen binding lead to a situation in which 'the two Fab fragments are closer in structure in the complexes than in the unbound state' [92].

The extra diversity afforded the immune system by conformational flexibility is likely to be most significant in the naive repertoire. Based on their crystallographic studies, Schultz and Stevens suggested that the conformational flexibility of hydrolytic antibody 48G7 is considerably less than that of its germline precursor [25–29], and recent molecular dynamics calculations by Kollman and coworkers support this hypothesis [93].

The blind versatility of the immune system also often leads to combining site functionalities that can interact favorably with elements of transition states not captured by TSA haptens. Automated docking of quantum mechanical transition states into antibody structures has provided insights into catalysis by pinpointing specific noncovalent interactions between transition states and antibody combining sites [14–24]. In the case of hydrolytic antibodies 48G7, CNJ206, 17E8 and 29G11, this type of analysis (Tantillo, D.J. and Houk, K.N., unpublished results) revealed that one of two possible enantiomeric pathways for reaction (si and re attack of hydroxide ion on aryl esters) is often heavily favored in antibody combining sites despite their selection for tight binding to symmetrical haptens. This selectivity arises from the inherent asymmetry of binding sites constructed from enantiopure amino acid residues, rather than elements of asymmetry in the haptens.

Infidelity between haptens and the transition states that they are intended to mimic [69] might also be expected to lead to mature antibody combining sites that lack certain functional groups that could enhance catalysis, and this is exactly what is observed in many cases. However, functionality not engineered through hapten design has also been found to interact with elements of transition states. For example, the increase in catalysis upon affinity maturation of antibody 48G7 can be largely explained by the

interaction between the transition state and an additional hydrogen bond acceptor (Tyr33H) upon maturation (see Fig. 1) [25–29,69]. The presence of a hydrogen bond acceptor in a combining site elicited against a phosphonate hapten with no hydrogen bond donor functionality is surprising – in fact, this residue actually behaves as a hydrogen bond donor when 48G7 is bound to its phosphonate hapten. These observations suggest that by displaying residues that may switch between donor and acceptor roles, the immune system again acquires increased diversity at no genetic cost. One might also consider the presence of active site nucleophilic residues, as found for antibodies 43C9 [51] and 7C8 [60], to be similarly serendipitous.

3. Summary

The available experimental and theoretical evidence suggests that there is a direct connection between TSEs and canonical binding arrays. Although a connection between hapten structure and mature antibody combining sites is not at all surprising – it is the very assumption upon which the proposal of antibody catalysis was predicated [3] – the connection between relatively small hapten substructures and particular combining site residues is more intriguing and of greater utility for catalyst engineering. According to our model, the origins of this correspondence can be traced to one or more of the following:

1. the presence of generic germline binders for particular types of functionality
2. biological epitope mimicry
3. sampling of multiple antibody conformations

These principles provide new avenues for design by chemists of 'input' that can be translated and expressed by nature in architectures compatible with transition state stabilization.

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