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Pyridoxal-5′-Phosphate-Dependent Catalytic Antibodies

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Abstract

Cofactors—i.e., metal ions and coenzymes—extend the catalytic scope of enzymes and might have been among the first biological catalysts. They may be expected to efficiently extend the catalytic potential of antibodies. Monoclonal antibodies (MAbs) against N^{α}-phosphopyridoxyl-L-lysine were screened for 1) binding of 5′-phosphopyridoxyl amino acids, 2) binding of the planar Schiff base of pyridoxal-5′-phosphate (PLP) and amino acids, the first intermediate of all PLP-dependent reactions, and 3) catalysis of the PLP-dependent α, β-elimination reaction with β-chloro-D/L-alanine. Antibody 15A9 fulfilled all criteria and was also found to catalyze the cofactor-dependent transamination reaction of hydrophobic D-amino acids and oxo acids ($k'_{cat} = 0.42\ min^{-1}$ with D-alanine at 25°C). No other reactions with either D- or L-amino acids were detected. PLP markedly contributes to catalytic efficacy—it is a 10^4 times more efficient acceptor of the amino group than pyruvate. The antibody ensures reaction specificity, stereospecificity, and substrate specificity, and further accelerates the transamination reaction ($k'_{cat(Ab)}/k'_{cat(PLP)} = 5 \times 10^3$). The successive screening steps simulate the selection criteria that might have been operative in the evolution of protein-assisted pyridoxal catalysis.

Index Entries: Catalytic antibodies; pyridoxal-5′-phosphate; transamination.

Pyridoxal-5′-phosphate (PLP) is one of the most versatile prosthetic groups of enzymes. PLP-dependent enzymes catalyze a great variety of reactions that synthesize, degrade, or interconvert amino acids *(1)*. The reactions include transamination, racemization, α and β decarboxylation, aldol cleavage, and β and γ elimination and replacement reactions. The PLP-dependent enzymes are of multiple evolutionary origin, belonging to at least five independent evolutionary lineages of homologous proteins *(2,3)*. Among the five independent lineages of PLP-dependent enzymes,

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the α/γ superfamily is by far the largest. The member enzymes of this superfamily catalyze many highly diverse reactions of amino acids, although they originate from a common ancestor and possess a similar fold of the polypeptide chain. We generated PLP-dependent catalytic antibodies in an effort to experimentally simulate the molecular evolution of PLP-dependent enzymes. The incorporation of cofactors has been proposed as a possible strategy to expand the catalytic scope of antibodies *(4)*.

Without exception, the PLP-dependent enzymes that act on amino-acid substrates share important mechanistic features, despite the multitude of different reactions that they catalyze. All PLP-dependent enzymes bind the coenzyme covalently through an imine linkage to the ε-amino group of an active-site lysine residue ("internal" aldimine). Invariably, the first step in their reaction with an amino-acid substrate is a transamination replacing the ε-amino group with the α-amino group of the incoming substrate amino acid. The ensuing "external" aldimine intermediate **3** forms an extended planar system of conjugated double bonds (*see* Fig. 1). The positively charged pyridine ring acts as an electron sink, withdrawing electrons from Cα and weakening the bonds to its substituents. The pathways of the many different reactions that are catalyzed by PLP-dependent enzymes diverge only after the common external aldimine intermediate. Depending on which bond is cleaved and on the subsequent covalency changes, one specific reaction is realized. All reactions are also catalyzed by PLP alone—the apoenzyme moiety is responsible for reaction and substrate specificity as well as part of the catalytic efficiency.

It seems almost unavoidable to assume that PLP existed before any of the corresponding apoenzymes developed. Not a single non-PLP-dependent protein has been found that seems to be evolutionarily related to a PLP-dependent enzyme. Perhaps there existed a "cofactor-world" with metal ions and coenzymes acting as biological catalysts before specialized proteins were recruited for this function. In a plausible hypothetical scenario for the evolution of PLP-dependent enzymes, PLP reacted with a protein that had to fulfill the following criteria in order to serve as a primordial apoenzyme: a lysine residue with adjacent binding sites for PLP and amino-acid substrate, and a geometry of the two binding sites that allowed formation of the planar aldimine adduct. Subsequent development of a catalytic apparatus and its optimization for reaction and substrate specificity and catalytic efficiency led to the modern PLP-dependent enzymes *(5)*.

Based on this interpretation of the evolutionary data, we explored—as substitutes for a hypothetical primordial PLP-dependent enzyme—MAbs that were capable of binding a planar PLP-amino-acid adduct. Antibodies elicited against N^{α}-(5′-phosphopyridoxyl)-L-lysine (*see* Fig. 2) may be assumed to comply almost ideally with the criterion for an ancestor PLP-enzyme—i.e., the existence of a binding site for a covalent coenzyme-amino-acid adduct. However, the hapten phosphopyridoxyl lysine

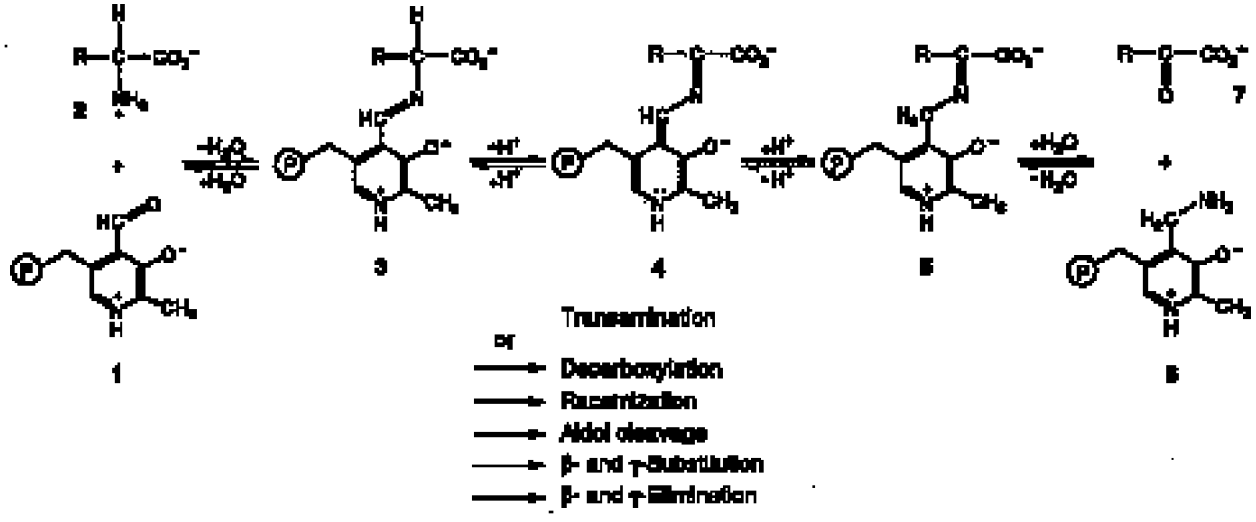

Fig. 1. PLP-dependent transformation of amino acids. **1**, PLP; **2**, amino acid substrate; **3**, aldimine intermediate; **4**, quinonoid intermediate; **5**, ketimine intermediate; **6**, pyridoxamine-5′-phosphate; **7**, keto acid product. In the enzymic reactions, the aldimine intermediate **3** is formed by transamination (see text) rather than de novo. The pathway of transamination is shown completely; the pathways of the various other PLP-dependent reactions diverge after the aldimine intermediate **3**.

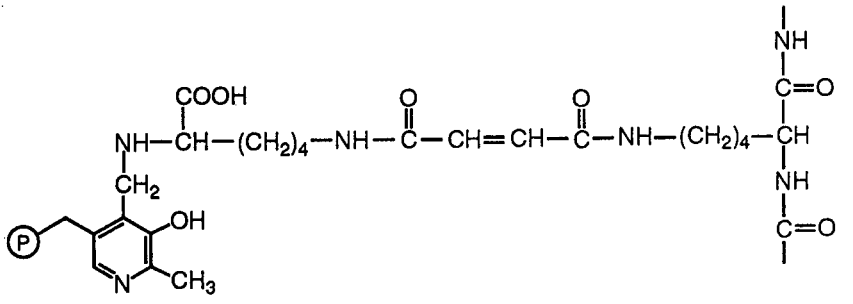

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Fig. 2. Structure of the antigen. N^{α}-(5′-Phosphopyridoxyl)-L-lysine, produced by reduction of the PLP-lysine aldimine with sodium borohydride, was coupled to maleylated carrier protein. For details of the experimental procedure, *see* refs. *6* and *7*.

is—in contrast to the covalent coenzyme-substrate adducts—nonplanar owing to the reduction of the C4′-N^{α} double bond. As a consequence, the antibodies elicited with phospopyridoxyl lysine may not be capable of accommodating the planar coenzyme-substrate adduct—a feature which is essential for the catalytic effect of PLP. This deficiency of the antigen used for immunization was compensated by the screening protocol. The multitude of possible transformation products of amino acids is a major problem in the design of a screening procedure for PLP-dependent catalytic antibodies. Because of this difficulty, we have devised a protocol that screens for the occurrence of two successive crucial reaction steps rather than for a final product.

In a first step, the hapten-binding IgG-antibodies were screened for binding of the planar Schiff base formed from PLP and D- or L-norleucine by a competition enzyme-linked immunosorbant assay (for experimental details, *see* ref. *7*). In this ELISA, the antibodies were tested to determine whether binding to immobilized antigen (with nonplanar phosphopyridoxal-L-lysine as hapten) was inhibited by PLP plus D- or L-norleucine. Binders of the planar coenzyme-substrate aldimine adduct were assumed to be inhibited more strongly by the conjoint effect of PLP plus amino acid than by PLP or the amino acid alone. Antibodies 13B10, 8H4, 15A9, 11C2, and 14G1 showed indeed that their binding to the antigen was inhibited more strongly in the presence of PLP plus D- or L-norleucine than in the presence of PLP or the amino acid alone (*see* Fig. 3). The inhibition of antibody–antigen binding by the Schiff base **3** formed from PLP plus glycine indicates the existence of a binding site for the amino-acid moiety of the hapten, and the difference in inhibition by PLP plus norleucine and PLP plus glycine reflects the contribution of the amino-acid side chain to the binding of the Schiff base. The inhibition profiles of antibodies 5G12 and 6E9 illustrate the binding properties of the great majority of the antibodies, which did not show a significant difference in the inhibition by PLP

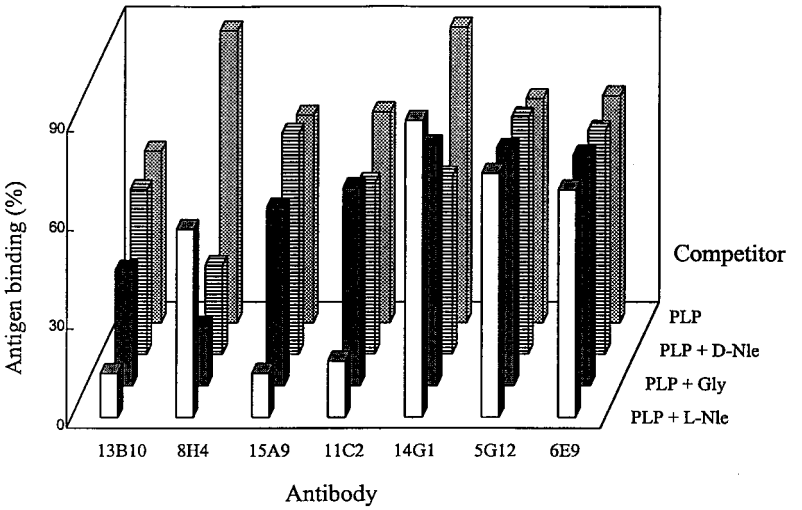

Fig. 3. Competition ELISA of antibodies for aldimine binding. The Schiff base **3** was formed from PLP in the presence of D- or L-norleucine or glycine. The assay measures the binding of the antibodies to the antigen **8** (*see* Fig. 2) in the absence and presence of PLP **1** or of PLP and amino acid **2**, which react nonenzymically to form the Schiff base **3**. For antibodies 13B10, 15A9, 11C2, 6E9, and 5G12, the concentration of PLP was 100 μ*M*; for 8H4 and 14G1, it was 1 and 2 m*M*, respectively. The amino-acid concentration was 25 m*M*. These conditions ensured that at least 80% of the cofactor in the incubation mixture was present as Schiff base. In all cases, inhibition of antibody-antigen binding by the amino acids alone was negligible.

and by PLP plus amino acids. Apparently, these antibodies cannot accommodate the planar aldimine adduct in their binding site. The antibodies differ from PLP enzymes by their lack of a lysine residue at the coenzyme-binding site. Because of the merely noncovalent binding of PLP to the antibodies, the external aldimine intermediate has to be formed de novo rather than by transamination (*see* Fig. 1). Experiments with nonenzymic model systems (H. P. Tobler, H. Gehring, and P. Christen, unpublished data; ref. *8*) and the residual activity of mutant PLP-dependent enzymes without active-site lysine residue *(9,10)* have indicated that formation of the coenzyme-substrate aldimine by transamination rather than de novo formation from PLP and amino acid is not essential for catalysis. The ubiquitous occurrence of the coenzyme-binding lysine residue might reflect a historic trait rather than a mechanistic necessity *(2,5)*.

In the second screening step, the aldimine-binding antibodies were screened for a catalytic effect—i.e., the cleavage of the Cα-H bond of the substrate moiety. In the molecular evolution of PLP-dependent enzymes, the analogous step after acquiring the capacity of aldimine binding may be assumed to have been the development of a catalytic apparatus facilitating the cleavage of one of the bonds between Cα and its substituents. The easily measured α,β-elimination of β-chloro-D/L-alanine served to test for Cα deprotonation, which underlies the majority of PLP-dependent reac-

Table 1
Kinetic Parameters for the Reactions Catalyzed by Antibody 15A9[a]

Reaction	Substrate(s)	k'_{cat} (min^{-1})	K'_m (mM)
α,β-Elimination[b]	β-Chloro-D-alanine	50	10
Transamination	D-Alanine + PLP	0.42	25
Transamination	Pyruvate + PMP	0.1	19
Transamination[c]	D-Norleucine + PLP	0.07	25

[a]The K'_m values refer to the amino acids and pyruvate. The concentration of the cofactor was 100 and 200 μ*M* in the reactions with β-chloroalanine and the other substrates, respectively. The measurements were performed at pH 7.0 for α,β-elimination and at pH 7.5 for transamination, both at 25°C. The rates were corrected for the rates observed in the absence of antibody. PMP is used as abbreviation for pyridoxamine-5′-phosphate. For experimental details, *see* ref. *6*.

[b]β-Chloro-L-alanine did not undergo elimination.

[c]Slower transamination was detected with N^{ε}-acetyl-D-lysine, D-lysine, D-tryptophan, D-tyrosine, and D-histidine as well as in the case of PMP plus 2-oxocapronate. No reaction was detectable with L-amino acids.

tions of amino acids. Owing to its good leaving group in the β position, this substrate analog is decomposed to chloride, ammonia, and readily detectable pyruvate in an α,β-elimination reaction that is initiated by deprotonation of Cα *(11)*. β-Chloroalanine thus allows a convenient and almost universal screening for deprotonation at Cα, which is an integral step in most PLP-dependent reactions of amino acids *(1)*. Antibody 13B10 was found to catalyze the α,β-elimination of β-chloro-L-alanine, which is consistent with its enantiomeric binding specificity. In contrast, antibody 15A9, which preferably binds the aldimine with L-amino acids (*see* Fig. 3), catalyzed exclusively the reaction of β-chloro-D-alanine. Apparently, the Cα-H bond of the L-amino-acid substrate is directed toward an inert surface region of the antibody. The more catalytically active antibody 15A9 was chosen for further exploration.

Three reactions were found to be catalyzed by antibody 15A9: formation of aldimine, deprotonation at Cα as reflected by α,β-elimination of β-chloroalanine, and transamination with hydrophobic D-amino acids (*see* Table 1). Catalysis of aldimine formation might reflect a favorable relative orientation of bound PLP and amino acid. α,β-Elimination of β-chloroalanine and transamination share one important feature: the crucial reaction steps are proton transfers (*see* Fig. 1). Apparently, in antibody 13B10 and 15A9 acid-base groups are positioned in proximity of Cα and Cα/C4′, respectively. Alternatively, water molecules might have access to these atoms and mediate the proton transfers. With antibody 15A9, transamination is two orders of magnitude slower than α,β-elimination, suggesting that reprotonation at C4′ is rate-limiting. Antibody 15A9 was also found to catalyze the stereoselective exchange of the α-protons of glycine *(12)*.

Table 2
Rate Acceleration by PLP and Catalytic Antibody 15A9 (Ab)[a]

Reaction	Reactive species	Relative rate constants
α,β-Elimination	β-Chloro-D-alanine	1
	Aldimine [β-chloro-D-alanine-PLP]	10^4
	[β-Chloro-D-alanine-PLP] · Ab	2×10^7
Transamination	Aldimine [D-alanine-PLP]	1
	[D-Alanine-PLP] · Ab	5×10^3
	D-Norleucine plus pyruvate	1
	D-Alanine plus PLP	10^4

[a]For both α,β-elimination and transamination, the first-order rate constants of the reactions of the indicated species are compared. All rates were measured at 25°C with the exception of those of D-norleucine and pyruvate. The two entries at the bottom of the table serve to estimate the contribution of PLP to catalysis of the transamination reaction; the second-order rate of the production of alanine from pyruvate and an amino acid is compared with the second-order rate of the reaction of PLP with an amino acid. For experimental details, *see* ref. *6*.

Antibody 15A9 is the only antibody found to catalyze the transformation of a natural amino acid. The antibody is remarkably reaction-specific; transamination is the only observable reaction. The antibody accelerates the transamination reaction of PLP and an amino acid, and also in the reverse direction with PMP and a keto acid as substrates. The orientation of the Cα-substituents relative to the plane of the resonance system of imine and coenzyme together with the presence (and absence) of catalytically effective protein side chains serving as general acid–base groups or modulating the electron repartition in the coenzyme-substrate adduct are thought to determine the reaction specificity in PLP-dependent enzymes *(13–15)*. In contrast to the reaction specificity, the substrate specificity of 15A9 is less strictly defined—apparently, hydrophobic amino acids and keto acids in the reverse reaction with PMP are generally accepted as substrates.

Nonproteinaceous cofactors endow many enzymes with chemical functions that their protein parts alone cannot provide. The present results show that the combination of a binding-specific antibody with a reactive cofactor—even if dissociable—seems a promising approach to extend the range of reactions that can be catalyzed by antibodies. Cofactor and antibody effectively complement each other (*see* Table 2). As in enzymes, the protein enhances the catalytic efficacy of the cofactor and ensures reaction specificity, stereospecificity, and substrate specificity. In the earliest attempt to generate catalytically active antibodies, a polyclonal antiserum against the reduced Schiff base formed from PLP and 3′-amino-L-tyrosine was prepared. The antibodies were found to bind the amino acid PLP and their aldimine adduct. In comparison to PLP alone, they brought about a five

times enhanced rate of α-proton exchange of L-tyrosine *(16)*. A MAb, generated against the reduced aldimine of pyridoxal and 4′-nitro-L-phenylalanine, accelerated aldimine formation between 5′-deoxypyridoxal and 4′-nitro-D-phenylalanine. However, the antibody did not promote any further reactions characteristic of pyridoxal catalysis *(17)*.

The success of the approach described here seems to be a result of the screening protocol. Its selection criteria were the formation of the planar resonance system of the external aldmine and catalysis of deprotonation at Cα. The successive screening steps plausibly simulate the functional selection pressures that probably have been operative in the molecular evolution of protein-assisted pyridoxal catalysis. The similarity is also evident in the development of specificity. In the evolution of PLP-dependent enzymes, specialization for reaction specificity clearly preceded that for substrate specificity *(2,18)*. The analogy reflects the interplay of chance and necessity, which is at work in both cases.

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Discussion

Schowen: Do you get both phases of the reaction? Do you get the regeneration—the ping and the pong?

Christen: Yes. The antibody catalyzes the reaction between pyridoxal phosphate and pyrvate.

Schowen: And do you know yet if it is stereospecific? Do you get the L-amino acids?

Christen: It is specific for D-amino acids, which is unexpected, because the hapten was an L-amino acid, and the good binders are L-amino acids. But the only reaction we could observe was with D-amino acids, in particular with the alanine. Our interpretation is that, with L-amino acids, the Cα-hydrogen bond points toward some inert part of the antibody. With like L-alanine, the CH bond acts as a proton donor to a group of the antibody, or it is just a base in the solvent that is mediating the deprotonation.

Paul: Could you comment on the transition state-like features of the hapten vs the ground-state features? Which one in your opinion might contribute more towards the generation of catalysis?

Christen: I think that our hapten is not a transition-state analog. It is an analog of a reaction intermediate, which has a finite lifetime and can be observed spectroscopically. A transition-state is something much more transitory.

Paul: I was struck by the evolutionary scheme you showed. I think you said that catalysis came first and substrate binding came later in evolution. Assuming that catalysis is more difficult than simple binding, do you consider your scheme to be an exception to the normal evolutionary rules that we have learned?

Christen: No, I think this is the truth. The notion that catalysis isn't more difficult but is just more important in evolution is probably correct. It could be both.

Sela: You're really saying the chemistry came first, not the exact structure of the compound as it is today.

Christen: It's conceivable that over the course of evolution, a substrate binding site was developed first, in this case the tyrosine-binding site, and then there was divergence of the sequence along with specialization for catalysis of different reactions, transamination, or decarboxylation, and so on. But this is not what happened. First, there was actually specialization of the primordial enzyme for catalysis with a particular reaction specificity—say alpha-decarboxylation or transamination. Then, in the last step of specialization and divergence, there evolved the development of substrate specificity.

Gololobov: At a certain stage of the reaction you may lose the stereoisomer configuration because you deprotonate the Cα atom. Since you said the reaction is D-amino-acid specific, what is the configuration of product? Is it still D or do you also get the L form?

Christen: The product is achiral. We can't say.

Green: I think being able to put in cofactors is a very important direction. Almost all of the transition-state analog-based catalytic antibodies do not use any cofactors, whereas enzymes certainly do. A few comments and questions. Since you used the reduced compound, maybe it is not so surprising that the antibody happens to choose a chirality that is specific for the D compound. Concerning evolution, have you thought about complementation—that is, putting this antibody into some species that lacks the enzyme and trying to see whether it could evolve a superior-rate constant, as has been done antibody with a chorismatic mutase in yeast? Have you thought about immunogens that are improved intermediate analogs, or as you said, transition analogs—for example, instead of the carbon–nitrogen single bond, maybe a carbon–carbon double bond or a nitrogen–nitrogen double bond to get the planar geometry?

Christen: Regarding the first question, metabolic screening is a possibility, but there are major hurdles learned from unencouraging experiences in another case. We thought it is better to stick to what we have and first determine its structure. Our efforts are now focused to get the structure of this antibody. On the basis of this structure—which we hope we will get—we will see how we can improve this antibody.

Neuberger: You said binding evolved before catalysis, because you got the transaminases in one family and the decarboxylases in a sibling family—they're cousins. I don't see how you can know that a transaminase didn't evolve all the transaminases, and then one of the transaminase evolved to the decarboxylases. It seems the only reason you say that they are cousins rather than children is because you cannot see close relationship between the decarboxylases and one individual of the transaminase family. But that does not seem to me sufficient to say that the two catalysts evolved parallel to each other rather than in a sequential order.

Christen: There might be a misunderstanding. I said the second step was the specialization for substrate specificity. Of course, there had to be binding of amino acids—there had to be a recognition site, for an α-decarboxylase and the α-amino group. Amino acids certainly also had to bind to this unspecific ancestor enzyme. Your direct question, as I understood it, was how we grouped the transaminases and the decarboxylases. All three transaminases are structurally more similar with each other than they are with any of the decarboxylases.

Neuberger: But what if one of the transaminases has evolved further to become a decarboxylase?

Christen: The decarboxylases could be the children of a very ancient transaminase; that is possible. We just have the modern structures, of course, and we find that all decarboxylases belong to one subfamily and all transaminases to another subfamily.

Schowen: On the question of transition-state vs intermediate analogs, I am ready to be corrected, but I think in all patents claiming to generate catalytic antibodies, the antigens have been intermediate analogs, not transition-state analogs.

Tramontano: I would go one step beyond that. I propose that all so-called transition-state analogs are simply electrostatic molecules engulfed in some deep pocket in the antibody, and that the electrostatic interactions within the pocket are used for catalysis. Have you considered whether doing the same experiment with a neutral molecule such as the one Buddy Green suggested will give you a different result?

Christen: We had this idea that the special two-step screening is important, which is why we followed this route. Your suggestion is certainly a possibility.

Sela: Do you have the primary structure of this abzyme?

Christen: We have the primary structure, but we do not have a three-dimensional structure.

Sela: Have you done some modeling? Is the antibody close to a transaminase or a decarboxylase? I understand very well your position on why the chemistry came first before the protein in this example of catalysis. You make the appropriate point about proper orientation of the orbitals, and some chemistry by the antibody is clearly taking place.

Paul: In view of all of the data, it seems appropriate to remain skeptical about the basic model of transition-state analog immunization and the origins of the catalytic activity. While the structure of the immunogen is clearly partly ground-state-like and partly transition state-like, the key issue remains—when you immunize with a so-called transition-state analog, is the catalytic activity purely accidental, is it due to development of substrate-binding capability combined with some innate tendency toward catalysis, or is there some true advantage to mimicking the transition-state-like features? It is valid to question the basic premise regardless of the electostatic or orbital steering mechanism of antibody–antigen interactions.