proteins from three important classes: binding proteins, immunoglobulins, and enzymes.

Design of therapeutic CAs would, of course, necessitate the selection of the most pharmaceutically effective, nonallergenic hydrolytic enzyme possible but such therapeutics could be administered at substoichiometric levels at potentially lower dosages than normally required for a similar drug. Our results are highly encouraging and represent steps towards therapeutically viable selectivity; further enhancement is an important goal.

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[26] Experiments were conducted by using a mixed substrate solution of target enzyme HLADH and decoy enzyme (CA or BG) in the same experiment to give a true measure of competitive selectivity for target over decoy.
[27] The fragility of the decoy clearly influences the level of collateral damage; while the very robust protein bovine carbonic anhydrase is virtually undegraded, more fragile proteins will obviously require greater selectivity. Bovine β-galactosidase was chosen as an additional more fragile decoy than bovine carbonic anhydrase to allow a measure of selectivity. We are currently examining a range of decoys of differing fragility; clearly selectivity will also be decoy dependent.
[28] Reduced degradation of BG is more apparent at longer reaction times and, in addition to being due to targeting-enhanced selectivity for HLADH, may also be the result of inhibition by low-molecular-weight peptides formed by protein degradation. The inhibitory effect of such protein fragment peptides is under investigation.
[29] During this study the following ratios of CA to target were employed: CA/avidin, 1:13; CA/con-A, 1:20; CA/antibiotin-IgG, 1:2; CA/HLADH, 1:4.

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Binding and Catalysis: A Thermodynamic Study on a Catalytic Antibody System

Herschel Wade[a, c] and Thomas S. Scanlan*[a, b]

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The idea that enzymes catalyze chemical reactions by binding transition states tightly has provided an important basis for generating molecules with catalytic activity.[1a] The diverse

[a] *Dr. T. S. Scanlan, Dr. H. Wade*
Department of Pharmaceutical Chemistry
University of California San Francisco
San Francisco, California 94143 (USA)
E-mail: hwade@mail.med.upenn.edu, scanlan@cgl.ucsf.edu

[b] *Dr. T. S. Scanlan*
Current address:
Departments of Pharmaceutical Chemistry
and Cellular and Molecular Pharmacology
University of California San Francisco
San Francisco, California 94143 – 2280 (USA)
Fax: (415) 502-7220

[c] *Dr. H. Wade*
Current address:
Department of Biochemisty and Biophysics
University of Pennsylvania Philadelphia
Pennsylvania 19104 (USA)

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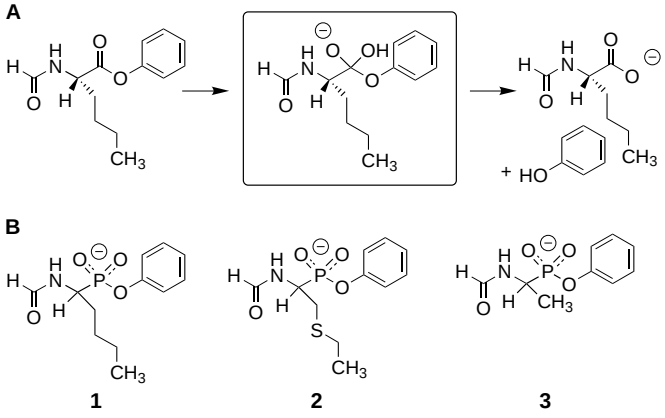
activities of antibodies and other molecules programmed with transition-state analogues to bind transition-state-like entities tightly has provided proof of this concept. However, in most cases the activities of these catalysts fall short of their natural counterparts, which perform analogous reactions. For example, the use of phosphonate antigens has generated antibodies with hydrolytic properties, but their activities are low even when compared to mutant hydrolases that have much of their catalytic machinery removed.[1b,c] Thus, the connection between the tight binding of a transition-state analogue and potent catalytic activity must be more complex than generally appreciated.

Here we examine the relationship between the binding of transition-state (TS) analogues and the stabilization of the TS by the hydrolytic antibody 17E8. 17E8 catalyzes the hydrolysis of the phenyl esters of *n*-formyl amino acids (Scheme 1).[2] The activity of 17E8 against several alternative substrates directly correlates with the binding affinity of the corresponding phosphonate analogues, which suggests that the phosphonates are good mimics of the catalytic transition state and can be used as probes of TS – 17E8 interactions.[2]

Table 1. *Thermodynamic parameters associated with the binding of* ***1****,* ***2****, and* ***3*** *to antibody 17E8.[a]*

	[1]	[2]	[3]
$K_a \times 10^{-5}$ [M^{-1}]	6 ± 1	3 ± 1	0.34 ± 0.08
ΔH°_{bind} [kcal mol^{-1}]	-4.0 ± 0.2	-2.9 ± 0.2	-1.7 ± 0.2
ΔG°_{bind} [kcal mol^{-1}]	-7.9 ± 0.1	-7.5 ± 0.2	-6.2 ± 0.1
$T\Delta S^\circ_{bind}$ [kcal mol^{-1}]	3.9 ± 0.3	4.7 ± 0.6	4.4 ± 0.3
ΔCp°_{bind} [cal (mol·K)$^{-1}$]	-118 ± 8	-65 ± 9	-53 ± 5

[a] K_a, association constant; ΔG°_{bind}, free energy change on binding; ΔH°_{bind}, enthalpy change on binding; ΔS°_{bind}, entropy change on binding; ΔCp°_{bind}, change in heat capacity on binding. The calorimetric experiments were performed at 24.5 ± 1.0 °C in a solution containing phosphate (50 mM) and NaCl (150 mM) at pH 7.2. Racemic mixtures of the phosphonates were used. The ΔG°_{bind} and ΔS°_{bind} values were calculated from the equation, $\Delta G^\circ_{bind} = -RT\ln K_a = \Delta H^\circ_{bind} - T\Delta S^\circ_{bind}$. The errors associated with the parameters ΔG°_{bind} and ΔS°_{bind} (standard state = 1.0 M) were obtained through the propagation of errors associated with the ΔG°_{bind} and ΔH°_{bind} values. The ΔCp°_{bind} values were obtained from the temperature dependence of ΔH°_{bind} by using the equation: $\Delta H^\circ_{bind} = \Delta H_o + \Delta Cp^\circ_{bind}(T - T_o)$, where ΔH_o is the binding enthalpy at an arbitrary reference temperature T_o. The following correlation coefficients were obtained from the fits: **1**, $R = 0.993$; **2**, $R = 0.972$; **3**, $R = 0.989$. These values were obtained from experiments carried out under the conditions given above.



Scheme 1. *A) Reaction catalyzed by 17E8. The putative tetrahedral intermediate formed during the reaction is shown in the box. B) Phosphonates used in the calorimetry experiments.*

Table 1 shows thermodynamic data for phosphonate binding to 17E8. The binding of **1** – **3** is governed by small favorable enthalpie changes, in each case with significant favorable entropic contributions (Table 1). The $T\Delta S^\circ_{bind}$ value contributes more favorably to ΔG°_{bind} than does the ΔH°_{bind} value for **2** and **3**, whereas both components contribute equally to the free energy change with **1**. The ΔH°_{bind} values are somewhat smaller than expected based on calorimetric studies of many small-molecule – protein binding reactions, where the values obtained are usually of the order of -10 kcal mol^{-1}.[3] The ΔS°_{bind} values are also unexpected as the entropy is usually adversely affected by the cost of bringing two molecules together, with ΔS°_{bind} values typically around $+10 - 20$ cal mol^{-1} K^{-1}.[3, 4] Negative ΔCp°_{bind} values are obtained from the temperature dependence of the ΔH°_{bind} energy change, which suggests that the hydrophobic effect plays an important role in binding, despite the anionic character of the phosphonates (Figure S1, see the Supporting Information).[5] Furthermore, the relative ΔCp°_{bind} values are also consistent with the hydrophobic effect as the hydrophobic character of the side-chains decreases in the order **1** > **2** > **3**.

The uptake of a proton is not observed at pH 7.0, but is apparent as the pH value is increased to pH 9.2 (Figure S2 in the Supporting Information), which suggests that a salt bridge is critical for phosphonate binding. The importance of a salt bridge in 17E8 hydrolysis has been proposed on the basis of the pH dependence of 17E8 catalysis.[2] The structure of the 17E8·**1** complex reveals that Lys97H forms a salt bridge with the phosphonate (Figure 1), which suggests that this interaction assists in both the electrostatic stabilization of the anionic transition state and the binding of the phosphonate.[2] The $T\Delta S^\circ_{bind}$ values for **1** and **3** indicate that the favorable $T\Delta S^\circ_{bind}$ contribution remains essentially unaltered upon removal of the antibody – side-chain interactions, which suggests that the electrostatic interactions between the anionic portions of the TS analogue and the 17E8 active site make a substantial contribution to the positive $T\Delta S^\circ_{bind}$ term, a thermodynamic hallmark of electrostatic interactions.[3, 5]

Our results suggest that although electrostatic interactions contribute favorably to the $T\Delta S^\circ_{bind}$ term of the free energy of binding between 17E8 and the phosphonate oxyanions, they may not contribute favorably to the observed ΔH°_{bind} values. The binding of **3** with one carbon atom in a side chain is characterized by a small ΔH°_{bind} value (-1.7 kcal mol^{-1}), which includes interactions involving the remaining side-chain carbon atom, the phenyl group, and the anionic part of the phosphonate. It is not unreasonable to expect the interactions involving the phenol group and the antibody to be similar to the side-chain-pocket interactions, which makes it apparent that the interactions involving the anionic moiety probably make a negligible or an unfavorable enthalpic contribution to binding. Moreover, the complete removal of the side chain normally

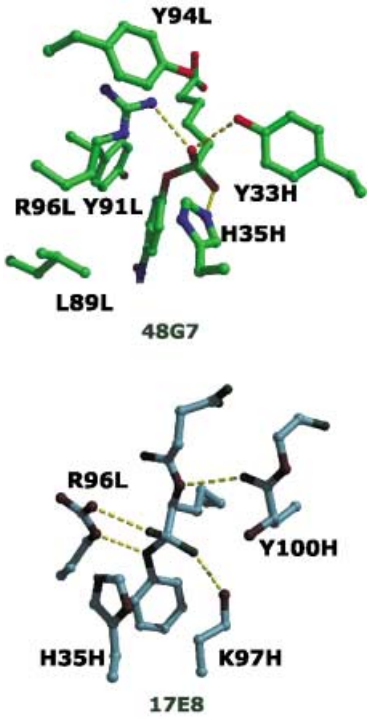


Figure 1. *Important active-site interactions of two hydrolytic antibodies, 48G7 and 17E8, with their corresponding phosphonate substrate transition-state analogues. Hydrogen bonds between active-site residues and the phosphonates are indicated by dashed yellow lines. The letters L and H refer to the light and heavy chains of the antibody, respectively.*

involved in binding and of the binding interactions with the phenol moiety results in unfavorable increases in the ΔG°_{bind} value of more than 5 kcal mol^{-1} (data not shown), which indicates that the anionic portion of the phosphonate does not provide a strong anchor for binding. These results are corroborated by several theoretical studies on antibody – antigen systems that also show that electrostatic interactions can contribute unfavorably to ΔG°_{bind} and ΔH°_{bind} values.[6]

The structural analyses of hydrolytic antibody hapten complexes provide evidence that their active sites contain preorganized polar environments that would be expected to lower the enthalpic and entropic requirements of activation relative to those for the uncatalyzed reactions (Figure 1).[1b, 7] These active sites are reminiscent of those in hydrolytic enzymes, which are preorganized and serve to stabilize the developing oxyanion in the transition state.[8] Indeed, the strategy of using phosphonates to elicit hydrolytic antibodies is based on the assumption that an active-site environment that stabilizes this oxyanion will be beneficial for catalysis.

The extent to which phosphonates are good mimics of the ester hydrolysis transition state is limited and the thermodynamic values associated with phosphonate binding to 17E8 may not reflect ideal properties for catalysis at the active site. However, the observed values, which include the smaller than typical favorable enthalpic, and larger than typical favorable entropic contributions to the ΔG°_{bind} value, are consistent with what is known about the thermodynamic characteristics of hydrophobic and electrostatic interactions.[3, 5] Thus, there appear to be thermodynamic differences between the binding of phosphonates to hydrolytic antibodies and the active-site interactions needed for efficient catalysis.

Stabilization of transition states often involves electrostatic and other polar interactions.[6, 7] The desolvation of charged groups on ligands and proteins is often not equally compensated by the coulombic attraction between the interacting groups, so electrostatic complementarity does not always contribute favorably to ΔG°_{bind} and ΔH°_{bind} values.[9] Therefore, candidate protein catalysts obtained from screens based purely on transition-state analogue binding will not necessarily contain active sites with strong electric fields that can balance the energetics of a transition state. For example, in a 17E8 phage display study, frequently occurring clones containing the Arg96L to Trp96L mutation showed increased binding to **1**, although none of these clones proved to be active catalysts.[10] Like Lys97H, Arg96L interacts with the phosphonate through salt bridges and most likely contributes to the oxyanion hole.[2] In addition, a structural comparison of hydrolytic antibody active sites suggests that the number of positive charges correlates to the efficiency of catalysis, whereas phosphonate affinity is not as sensitive to such changes.[6] Such positive fields are not likely to increase phosphonate binding affinity because of the higher desolvation penalty that may become associated with the more highly charged active site.[9, 11] The fact that there are more solutions for binding and fewer for catalysis may be another indication of the thermodynamic differences between binding sites and catalytic sites (Figure 1).[1c, 6] Furthermore, it has also been suggested that the creation of such a highly charged site is likely to compromise protein stability and that this strain is compensated by tertiary interactions in natural enzymes.[11] The complex connection between transition-state analogue binding and catalysis should become clearer with thermodynamic analyses on additional catalytic systems.

Experimental Section

General: All molecular biology grade buffers used were purchased from commercial suppliers (Aldrich, Sigma, and Fisher) and were used without further purification. The phosphonate ligands (Scheme 1) used in the studies were synthesized as described previously.[2]

Antibody Preparation: 17E8 was isolated from ascites fluid and purified by protein A affinity chromatography according to the method described by Guo et. al., with several modifications.[2] After loading ascites fluid/binding buffer (1.5 M Glycine, 3 M NaCl, pH 9.5) mixture onto protein A matrix, the column with washed with several column volumes of phosphate-buffered saline (PBS). The column was then washed with sodium acetate (0.1 M, pH 5.0). The antibody was eluted with glycine (0.1 M, pH 2.6). The fractions were immediately neutralized with tris(hydroxymethyl)aminomethane · HCl (1.0 M, pH 9.0). The fractions containing protein were pooled and dialyzed against PBS.

Isothermal titration calorimetry: The free energy and enthalpy of inhibitor binding was measured with an Omega titration microcalorimeter (MicroCal, Inc.). The protein concentrations used to

obtained the binding isotherms ranged from 0.015 μM to 0.040 μM. The ligand concentrations used were as follows: **1**, 0.5 mM; **2**, 0.5 mM; **3**, 1.0 mM. All buffers used in the calorimetry experiments were degassed prior to use. The parameter c, where $c = K[M_T]_O$, K is the association constant, and $[M_T]_O$ is the total protein concentration, ranged from 20 – 50 in all experiments performed. The ligand solutions were prepared with the degassed buffers and then filtered through a 0.2 μm filter. The protein was exchanged into the desired degassed buffer solution by gel filtration on PD-10 columns (Pharmacia) that were pre-equilibrated with the desired buffer and filtered through a 0.2 μm filter before use.

Data analysis: The titration isotherm data were analyzed by using the program ORIGIN v2.9 (MicroCal, Inc). The data were fit to a single-site binding model by using the Wiseman isotherm.[12] The variations in the parameters obtained from the titration data (temperature dependencies, pH dependencies, etc.) were fit to the equations given in the text by using the KALIEDAGRAPH (Synergy Software) plotting program. Parameters obtained from the fits (including errors) are given in the figure legends as are the correlation coefficients for the fits to the equations.

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