Transition State of the Base-Promoted Ring-Opening of Isoxazoles. Theoretical Prediction of Catalytic Functionalities and Design of Haptens for Antibody Production

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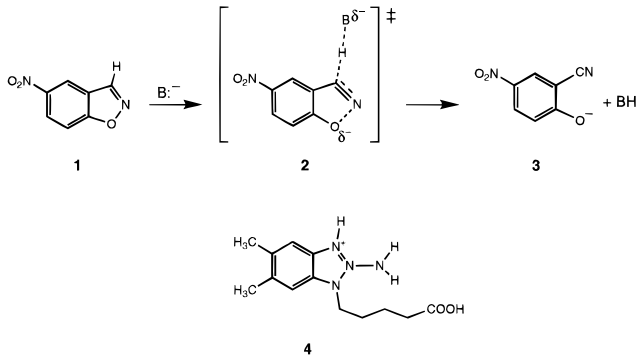
Abstract: In previous research, Hilvert and co-workers developed an antibody which catalyzes the decomposition of a nitrobenzisoxazole with a rate $>10^8$ times faster than the acetate-catalyzed reaction in water. Quantum mechanical calculations were carried out on a model system, the reaction of isoxazole with formate. The orientation of the carboxylate group has a significant effect on the rate. Complexation of the formate base by one water retards the reaction by approximately 5 kcal/mol; hence desolvation of the catalytic base could account for as much as four orders of magnitude in reaction rate. It was also determined that hydrogen-bonding to the forming oxide could potentially lead to greater rate acceleration. The gas phase activation barriers predict that water is the most effective general acid, lowering the activation energy by 9.5 kcal/mol. Methanol and formic acid are also effective, lowering the activation energy by 7.5 and 7.8 kcal/mol, respectively. Our calculations suggest that the combined effects of proper base orientation and acid catalysis could lead to an additional factor of 10^5-10^6 increase in rate acceleration. Based on these results, various new haptens were proposed. Each was quantitatively assessed for similarity with the located transition states to predict their potential as successful haptens.

Introduction

The rate of the base-promoted elimination reaction shown in Scheme 1 is subject to large accelerations in nonpolar solvents.[1] Hilvert and co-workers designed the hapten, **4**, to stimulate the immune system to produce antibodies to catalyze this reaction.[2] They envisioned that some antibodies to **4** would have binding sites with a carboxylate located in a hydrophobic cavity. In analogy to Kemp's findings,[1] in which a large rate acceleration was observed when the acetate-catalyzed opening of nitrobenzisoxazole was moved from aqueous to acetonitrile solvent, this should produce a rapid reaction. Of the antibodies generated against this hapten, two were found to be very effective catalysts. For example, antibody 34E4 catalyzes the reaction with $>10^3$ turnovers per active site and a rate acceleration (k_{cat}/K_m vs k_{OAc^-}) of greater than 10^8 when compared to the acetate-promoted reaction in aqueous solution ($k_{cat}/K_m = 5.5 \times 10^3$ M^{-1} s^{-1} for antibody 34E4, and $k_{OAc^-} = 5.26 \times 10^{-5}$ M^{-1} s^{-1}).[2] This is one of the larger accelerations observed yet with catalytic antibodies.[3] However, the antibody-catalyzed rate is still about 10^5 less than is observed in the most proficient enzymes,[4] those which have been perfected to achieve rate-determining diffusion of substrate on or off the enzyme. Thus, when compared to enzymatic catalysis, there is still considerable opportunity for improvement of this system.

Scheme 1



This study was undertaken in order to provide knowledge about the transition state of the reaction, to gain insight into the origins of catalysis of reaction 1, and to determine whether theoretical methods could be used to predict hapten structures which more closely resemble transition state **2** than hapten **4**. We have located the transition structure for the base-promoted opening of a model for nitrobenzisoxazole, **1**. In addition, we have explored the role of the correct positioning of the catalytic base, how desolvation influences the catalytic rate, and how side chains other than carboxylates could enhance k_{cat}. Based on transition state analysis and hapten-side chain interaction, we have also considered several alternative haptens for the production of more potent antibody catalysts for the isoxazole opening reaction. We aim for catalytic efficiency comparable to that of enzymes.

Computational Methods

Reactant, transition state, and product structures were located with the GAUSSIAN 92[5] and GAUSSIAN 94[6] programs. Transition structures were optimized at the RHF/6-31G* level and tested with

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(1) (a) Casey, M. L.; Kemp, D. S.; Kenneth, G. P.; Cox, D. D. *J. Org. Chem.* **1973**, *38*, 2294. (b) Kemp, D. S.; Casey, M. L. *J. Am. Chem. Soc.* **1973**, *95*, 6670. (c) Kemp, D. S.; Cox, D. D.; Paul, K. G. *J. Am. Chem. Soc.* **1975**, *97*, 7312.
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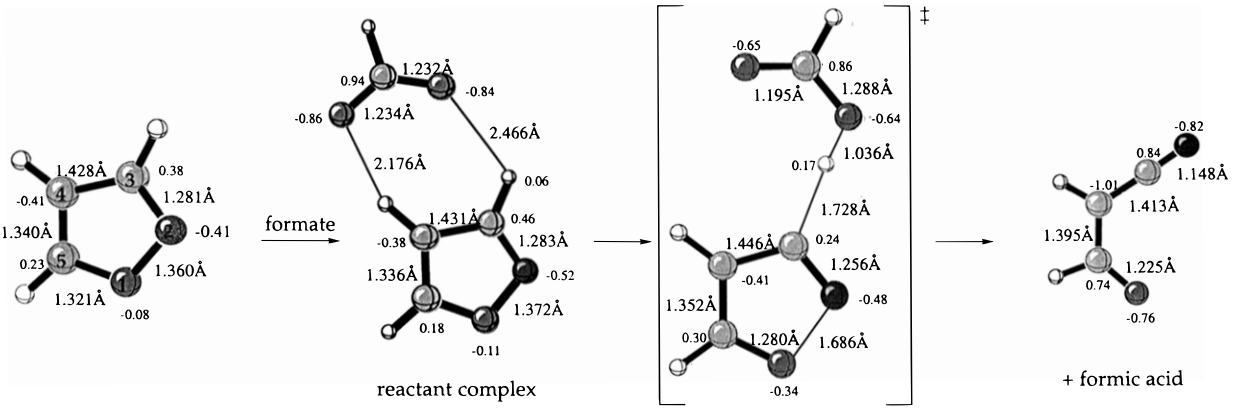
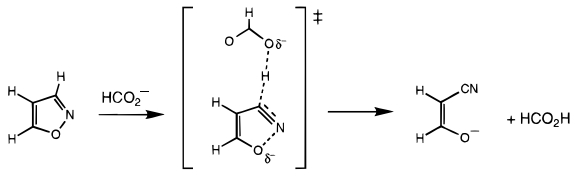


Figure 1. RHF/6-31G* optimized structures for the formate-catalyzed opening of isoxazole. CHELPG charges are calculated from RHF/6-31+G* single points on the RHF/6-31G* optimized structures.

Scheme 2



harmonic frequency analysis. Energy calculations at the MP2 level of theory were done by single point calculations with the 6-31+G* basis set on RHF/6-31G* optimized geometries. Calculations on the interaction of haptens with stabilizing groups were performed with the semiempirical AM1 method.[7]

Results and Discussion

Isoxazole Deprotonation Transition State. Transition state calculations were carried out on the model reaction of isoxazole (replacing nitrobenzisoxazole) as the substrate and formate representing the carboxylate side chain in the antibody or acetate in the solution reaction (Scheme 2). Figure 1 shows the structures of reactants, transition state, and products for the gas phase reaction of isoxazole with formate. The transition state is relatively late in terms of proton transfer, with the proton nearly fully transferred to formate. Fragmentation of the isoxazole ring lags behind: the isoxazole N−O bond is stretched from 1.360 to 1.686 Å in the transition state, while the C−N bond has contracted from 1.281 to 1.256 Å, only 15% of the extent of contraction in the product. The other bond lengths are intermediate between the reactant and product lengths, except for C_3-C_4, which is longer in the transition state than in either reactant or product. The general shape of the isoxazole ring is retained, as shown by the C−C−N and the C−N−O angles, which change little between reactant and transition state. The activation energy predicted at the 6-31G* level is 17.0 kcal/mol, and a single-point calculation with electron correlation corrections at the MP2 level gives a predicted value of 11.7 kcal/mol. The overall reaction is predicted to be exothermic by 20.1 kcal/mol (MP2/6-31G*//RHF/6-31G*). Experience indicates that this level of theory generally gives activation energies which are within a few kcal/mol of the experimental values. Relative activation energies for closely related systems are more accurate. However, solvation has a large effect on activation energies. Earlier studies[8] have shown that the anion formed by deprotonation of isoxazole is unstable, opening spontaneously to cyanoenolate, so the alternative stepwise process was not investigated.

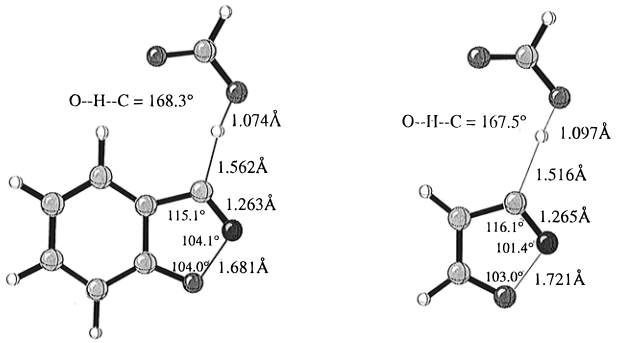


Figure 2. Optimized transition states for the formate-catalyzed openings of benzisoxazole and isoxazole (AM1).

As a check on the validity of our model as compared to the actual system, we compared the transition state of this reaction to the transition state for benzisoxazole opening, both computed at the semiempirical AM1 level. As shown in Figure 2, the

(5) *Gaussian 92*, Revision A; Frisch, M. J.; Trucks, G. W.; Head-Gordon, M.; Gill, P. M. W.; Wong, M. W.; Foresman, J. B.; Johnson, B. G.; Schlegel, H. B.; Robb, M. A.; Replogle, E. S.; Gomperts, R.; Andres, J. L.; Raghavachari, A. K.; Binkley, J. S.; Gonzalez, C.; Martin, R. L.; Fox, D. J.; Defrees, D. J.; Baker, J.; Stewart, J. J. P.; Pople, J. A. Gaussian, Inc.: Pittsburgh, PA, 1992.

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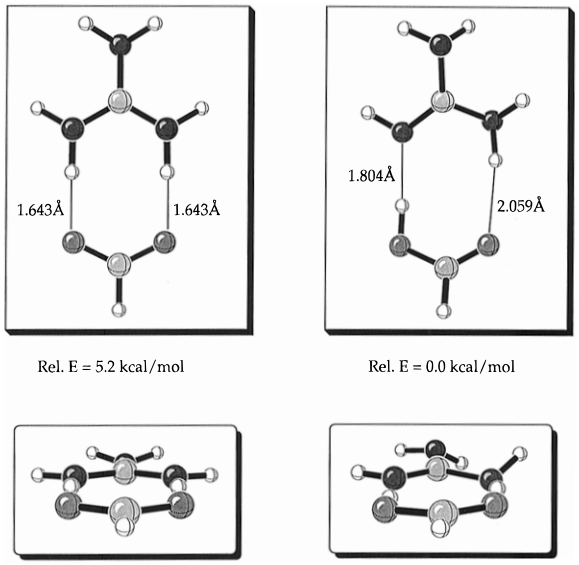
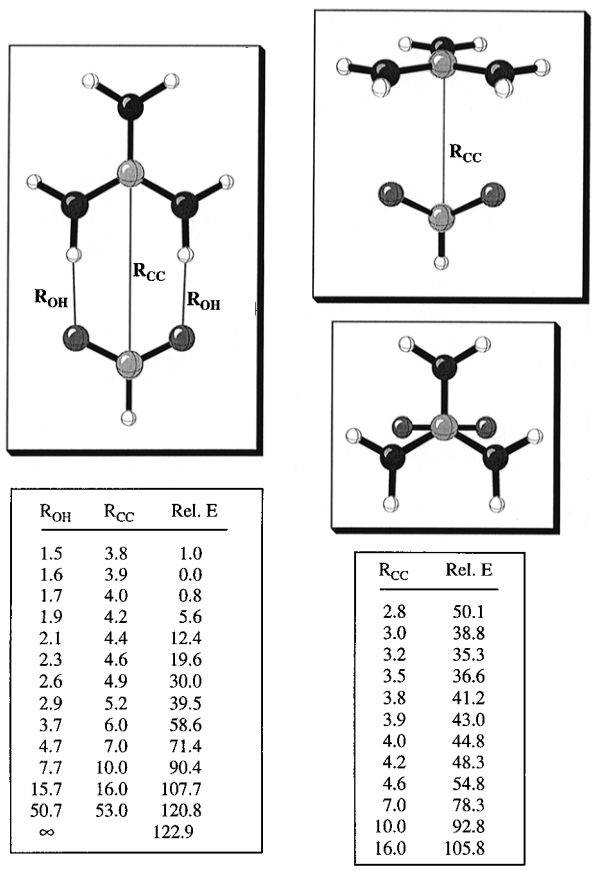


Figure 3. RHF/6-31G* optimized complexes between guanidium and formate in the double hydrogen-bonded species and the neutral complex. Both structures are nonplanar (C_1 symmetry) by a slight pyramidalization of the amino nitrogen atom (bottom views).

AM1 transition state is quite similar to the ab initio transition state for the model reaction, and the two transition states are almost identical, with greater proton transfer to the formate and a slightly shorter N−O breaking bond in the benzisoxazole reactant. As with the isoxazole reactant, the transition state for the benzisoxazole opening retains the reactant-like, rather than product-like, geometry.

Influence of Carboxylate Position on Activation Energies. The observed catalysis shown by antibody 34E4 could come from having a carboxylate positioned optimally in the binding pocket to deprotonate benzisoxazole **2**. To test this idea, we calculated the energy required to move the carboxylate from its optimized position in the transition state. We began by examining the interaction between carboxylate and a model of the hapten, **4**, to determine where the hapten was most likely to induce a carboxylate for maximum binding. The guanidinium ion was used to represent the guanidinium portion of the hapten, and formate represents the carboxylate side chain. Calculations maintaining C_{2v} symmetry at the RHF/6-31G* level show the ion pair forming a doubly-hydrogen-bonded species, with O−H distances of 1.643 Å (Figure 3). This conformation has been shown to exist between arginine and aspartate or glutamate residues in many proteins[9] such as the enzyme lactate dehydrogenase.[10] Upon relaxation of the C_{2v} symmetry requirement, a single proton transfer gives a neutral doubly-hydrogen-bonded species, which is 5.2 kcal/mol lower in energy. These results are in agreement with previous calculations by Radom et al.,[11] who predict the proton transfer to be a barrierless process in the gas phase. Even in a moderate dielectric medium, the ion-pair form should be favored. The possibility of a perpendicular approach by the formate to the guanidinium ion was also investigated. Figure 4 shows that this approach would be approximately 43 kcal/mol higher in energy than the parallel



R_{OH}	R_{CC}	Rel. E
1.5	3.8	1.0
1.6	3.9	0.0
1.7	4.0	0.8
1.9	4.2	5.6
2.1	4.4	12.4
2.3	4.6	19.6
2.6	4.9	30.0
2.9	5.2	39.5
3.7	6.0	58.6
4.7	7.0	71.4
7.7	10.0	90.4
15.7	16.0	107.7
50.7	53.0	120.8
∞		122.9

R_{CC}	Rel. E
2.8	50.1
3.0	38.8
3.2	35.3
3.5	36.6
3.8	41.2
3.9	43.0
4.0	44.8
4.2	48.3
4.6	54.8
7.0	78.3
10.0	92.8
16.0	105.8

Figure 4. Comparison of relative energies of guanidium−formate ion pair in hydrogen-bonding and perpendicular geometries. The hydrogen-bonded complex was optimized at RHF/6-31G* and taken as the reference minimum. All other calculations are RHF/6-31G* single points. Energy values in kcal/mol, distances in Å.

approach when the C−C distances are identical. There is a local minimum at about 3.3 Å, but relaxation of geometry constraints causes this to collapse to the planar doubly-hydrogen-bonded species. Attempts to locate planar single hydrogen-bonded ion pairs were not successful, and optimizations always give the minimum shown in Figure 4.

In the RHF/6-31G* optimized transition state for the formate-promoted elimination, the formate is positioned with the carbonyl oxygen pointed away from the nitrogen of the isoxazole ring. This avoids electrostatic repulsion between the carbonyl oxygen and the ring nitrogen atom. However, the orientation expected to be elicited by the hapten, **4**, would have the carbonyl oxygen near the nitrogen atom on the 2-amino substituent, assuming the carboxyl group forms the energetically favored double-hydrogen-bonded conformation shown in Figure 5. Consequently, the carboxylate elicited by the hapten may not be in an ideal position for deprotonation.[12] To estimate the energy of moving the carboxylate group from its ideal location for deprotonation, the formate was displaced by 20° from its optimized position in the plane and out of the plane of the isoxazole ring (see Chart 1). MP2 single point calculations on 6-31G* optimized geometries show that distorting the catalytic base from its optimized position (complex B of Figure 5) by 20° raises the transition state energy, relative to the ideal transition state, by 1.7 (out-of-plane) to 2.2 (in-plane) kcal/mol, while displacing the carboxylate to the hapten-induced conformation A (as shown in Figure 5) would increase the energy by 3.7 kcal/mol. These calculations indicate that correct alignment of the catalytic base is very important, and a hapten design which

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(11) Agranat, I.; Riggs, N. V.; Radom, L. *J. Chem. Soc., Chem. Commun.* **1991**, 80.

(12) Our postulate is based on the geometry optimization. The crystal structure of this antibody could confirm the position of this carboxyl group.

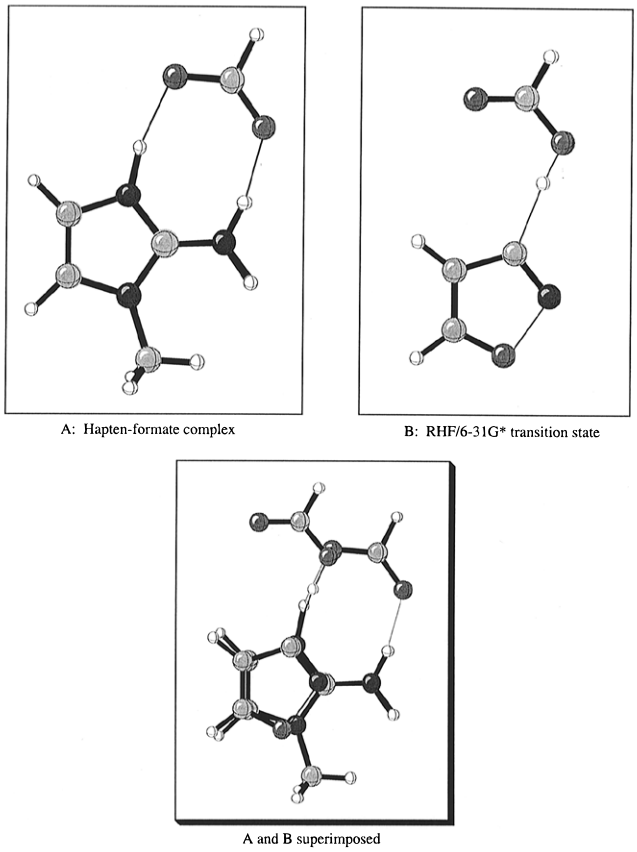
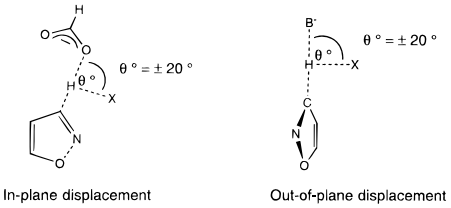


Figure 5. A comparison of the guanidium−formate complex and the formate-catalyzed isoxazole opening reaction (RHF/6-31G*).

Chart 1



leads to a carboxylate in the binding site in the orientation B rather than A could give increased antibody catalysis (Figure 5). An additional lowering of the activation energy by 3.7 kcal/mol, possible through correct orientation of the catalytic base, would give an acceleration factor of 6×10^2. The calculations are for the gas phase, and the energy differences will be reduced in the presence of polar groups which raise the local dielectric constant. Nevertheless, the effect on rate of proper orientation should be substantial.

Influence of Desolvation on Rates. Earlier studies by Kemp[1] and by Hilvert et al.[13] on the decarboxylation of various benzisoxazole-3-carboxylates and elimination of substituted benzisoxazoles have shown the importance of medium effects on the reaction rate. For example, the acetate-induced elimination of 5,7-dinitrobenzisoxazole occurs 3×10^7 faster in acetonitrile as compared to water.[1c] A similar rate enhancement was observed in the reaction catalyzed by antibody 34E4, leading to the postulate that the antibody has the carboxylate base situated in an aprotic binding site. Protic solvents stabilize the base through hydrogen-bonding and slow the rate of reaction. To provide a simple model of such solvation effects, we have

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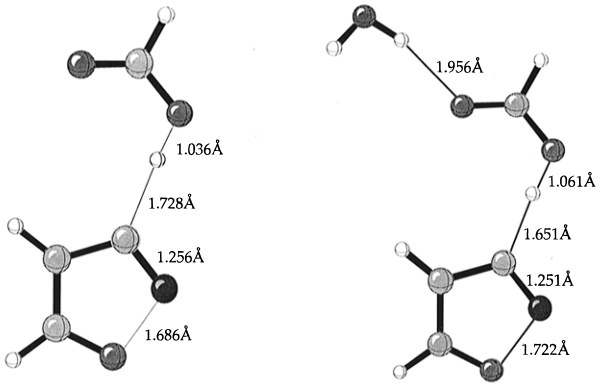


	E_a (formate only)	E_a (plus water)
6-31G*	17.0	24.2
MP2	11.7	17.1

Figure 6. Comparison of the RHF/6-31G* optimized transition states for formate-catalyzed isoxazole opening and the same reaction with water complexing the formate base. MP2 values are single point calculations on 6-31G* optimized geometries.

located the transition state of the elimination with a water molecule complexing the formate base. This is compared to the unsolvated reaction in Figure 6. At the RHF/6-31G* level, the calculated activation barrier for the water-complexed reaction is 7.2 kcal/mol higher than the uncomplexed reaction. With MP2 correlation, the difference in activation energies becomes 5.4 kcal/mol in favor of the unsolvated reaction, translating to a rate difference of approximately 4 orders of magnitude. There is less proton transfer but more bond reorganization in the water-complexed reaction. This base-solvating effect is expected to be greater for the reaction in aqueous solution, since the acetate ion will be solvated by a shell of solvent, rather than just one water molecule.

Additional Sources of Catalysis. Catalysis of the reaction by antibody 34E4 is likely to occur through desolvation of the base through the active site microenvironment and proper positioning of the general base for deprotonation of the substrate. We have analyzed the transition state of the formate-catalyzed opening of isoxazole to investigate possible alternative sources of catalysis. One possibility is general acid catalysis, or electrostatic stabilization of the developing negative charge at oxygen of the breaking N−O bond. For the related decarboxylation of benzisoxazole-3-carboxylates, Kemp looked carefully, but in vain, for general acid catalysis in solution by variation of buffer concentrations. He estimated the pK_a of protonated benzisoxazole as −4.7. Consequently, there is no chance that it will be protonated except in strong acid. The product *o*-cyanophenol has a pK_a of 6.9 and will be partially protonated at neutral pH. The 2-cyano-4-nitrophenolate ion is more stable (pK_a = 4.1) and will not be protonated under the conditions of the reaction. Thus, general acid catalysis is not possible for the nitrobenzisoxazole elimination at physiological pH. Jencks[14] described the requirements for general acid−base catalysis in aqueous solution. His "libido rule" states that concerted acid−base catalysis can occur (a) at sites undergoing a large change in pK_a during the reaction and (b) when the change in pK_a makes proton transfer favorable. The failure of Kemp to observe general acid catalysis, even with appropriate buffer concentration, may indicate that the pK_a of the isoxazole oxygen has not increased sufficiently in the transition state. Jencks specifically excluded special transition state stabilization by hydrogen-

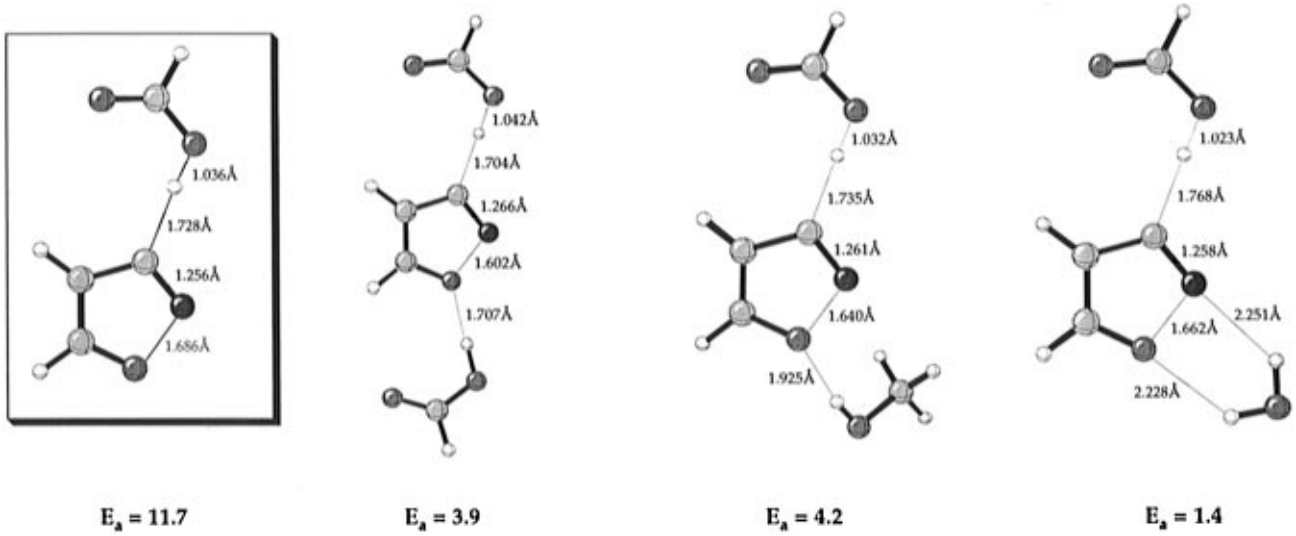


Figure 7. RHF/6-31G* optimized transition states and MP2/6-31+G* activation energies for the formic acid, methanol, and water-catalyzed reactions. Energies in kcal/mol.

bonding,[14] while Swain, Kuhn, and Schowen proposed the possibility of special hydrogen-bonding due to polarizability of the transition state.[15] This controversy has resurfaced recently under the new name, "low-barrier hydrogen bonds".[16] An antibody binding site has the possibility of orienting a catalytic group at a location where it is particularly effective in the transition state. In a nonpolar cavity, relatively strong hydrogen bonds are possible, since a charged group is not stabilized by hydrogen bonds to solvent. We explored the possibility that a Lewis acid strategically located to provide transition state stabilization could give additional rate enhancement.

There is good reason to believe that hydrogen-bonding to oxygen will result in rate acceleration: negative charge build-up occurs on the oxygen atom in going from the reactant to the transition state. The CHELPG[17] charges are −0.08 for the reactant oxygen atom and −0.34 in the transition state. Hence hydrogen-bonding should effectively lower the activation barrier and increase the reaction rate.

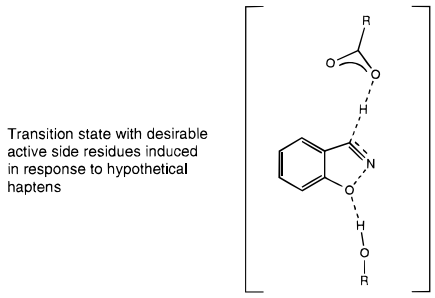
To assess the potential for such catalysis, we located the transition states stabilized by several types of hydrogen-bond donors commonly found in proteins. A molecule of formic acid represents glutamic or aspartic acids at low pH or in a nonpolar environment, while methanol is used to mimic the alcohol side chains of serine or threonine. We also performed calculations with a water molecule to account for possible catalysis by a water molecule in the binding site.

Figure 7 shows the transition state geometries and activation energies calculated at the highest level used here. Activation energies are calculated from the energy difference between the optimized transition states and the optimized reactant complexes. Formic acid, methanol, and water all serve as powerful catalysts, stabilizing the transition states shown by 8−10 kcal/mol more than they stabilize the isoxazole reactants. All three hydrogen-bonding species tend to shift the transition state earlier, with less advanced NO cleavage and less CN shortening.

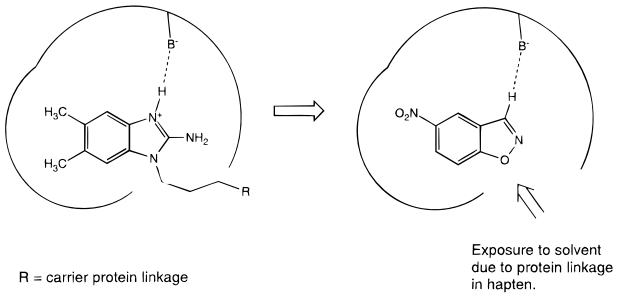
Formic acid forms the strongest hydrogen bond to the isoxazole oxygen atom in the transition state, with a hydrogen bond energy of 22.1 kcal/mol, versus 13.2 kcal/mol for methanol and 13.7 kcal/mol for water (MP2/6-31+G*//RHF/6-31G*). This is reflected in the O−H distance of 1.707 Å for formic acid, as compared to 1.925 Å for methanol and 2.228 Å for the O−H and 2.251 Å for the N−H hydrogen bonds in the water complex (Figure 7). The very high hydrogen-bonding energies are reasonable for the gas phase but will be lowered substantially in aqueous solution. The relevance of these strong "low-barrier hydrogen bonds" to enzymatic catalysis is a subject of current debate.[18]

Because of this strong binding to the transition state, formic acid was expected to be overwhelmingly the most effective general acid catalyst. This was not found to be the case when the counterbalancing hydrogen-bonding to the isoxazole reactant is included. Although formic acid can best stabilize the developing negative charge on the oxygen atom in the transition state, it also forms the strongest hydrogen bond with the isoxazole reactant, 14.3 kcal/mol, versus only 5.7 kcal/mol for methanol and 4.2 kcal/mol for water (MP2/6-31+G*//RHF/6-31G*). The gas phase activation barriers predict water as the most effective general acid, lowering the uncatalyzed activation energy by 9.5 kcal/mol. Methanol and formic acid are less

Chart 2



Scheme 3



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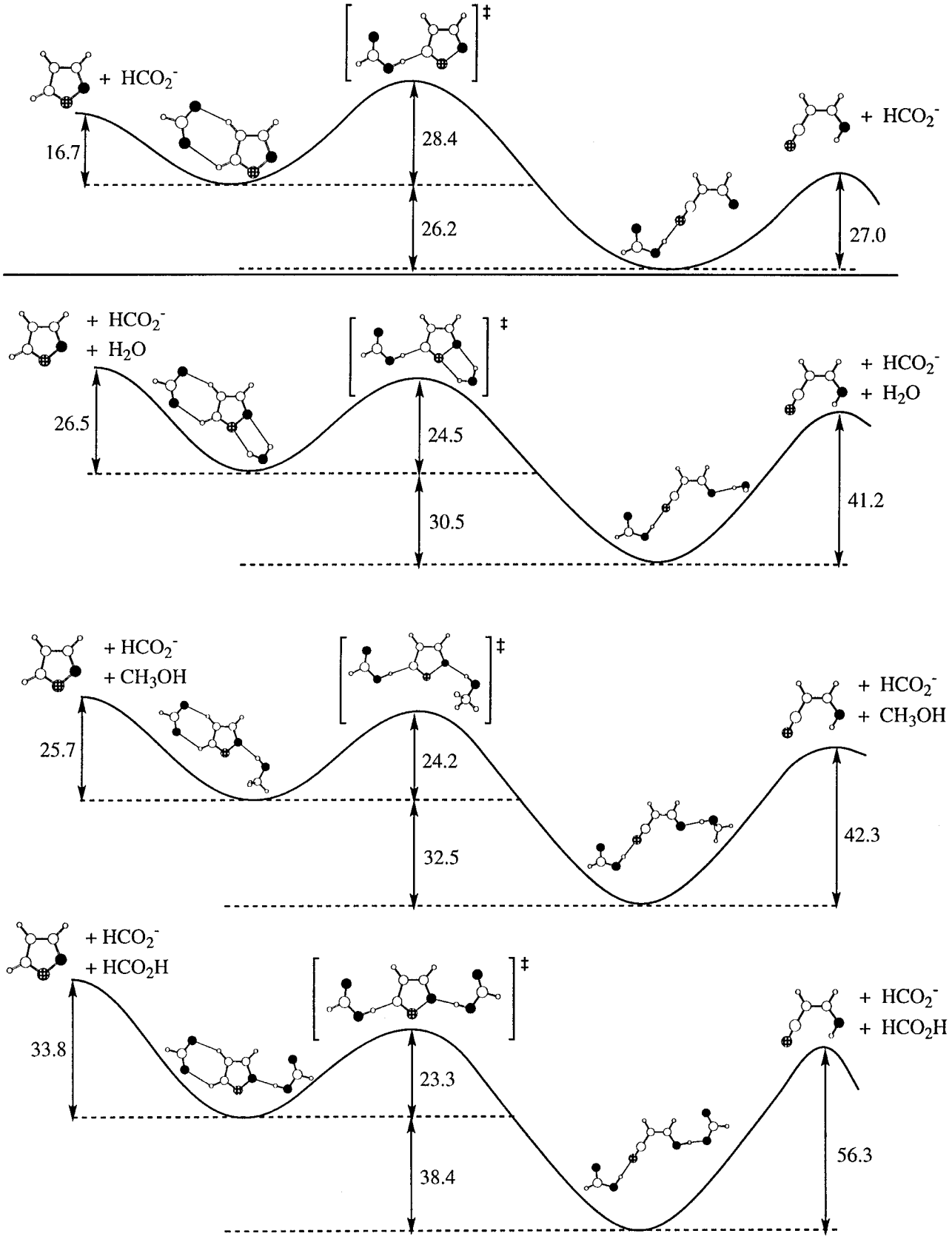

Figure 8. Summary of complexation energies, activation energies and reaction energies (MP2/6-31+G*//RHF/6-31G*). All values in kcal/mol.

effective, lowering the activation energy by 7.5 and 7.8 kcal/mol, respectively. Water is the best catalyst, because the transition state is a doubly hydrogen-bonded structure, involving the two protons of water and both atoms of the breaking N−O bond, whereas the reactant molecule has only a single hydrogen bond to the oxygen atom.[19] It is possible that in the active site environment of antibody 34E4, the oxygen atom of the benzisoxazole substrate is exposed to solvent as a result of the carrier protein linkage and thus stabilized by a solvent water molecule (Scheme 3).

Figure 8 shows the energetics of each of these processes on an energy scale more relevant to antibody catalysis in solution.

(19) The bidentate conformation between water and isoxazole, where water hydrogen bonds to both atoms of the N−O bond in the reactant, could not be located. However, this conformation was found as a local minimum in the reactant complex.

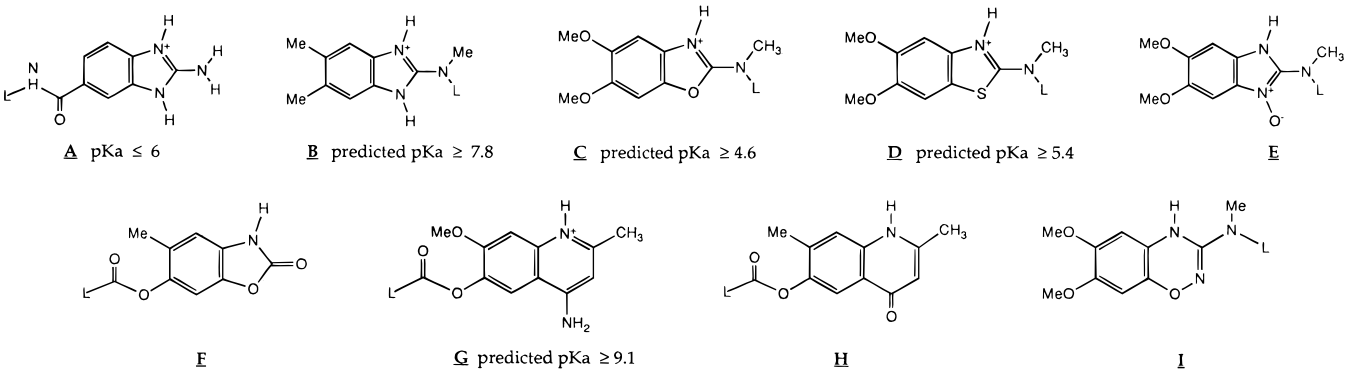

Figure 9. Proposed haptens based on transition state analysis.

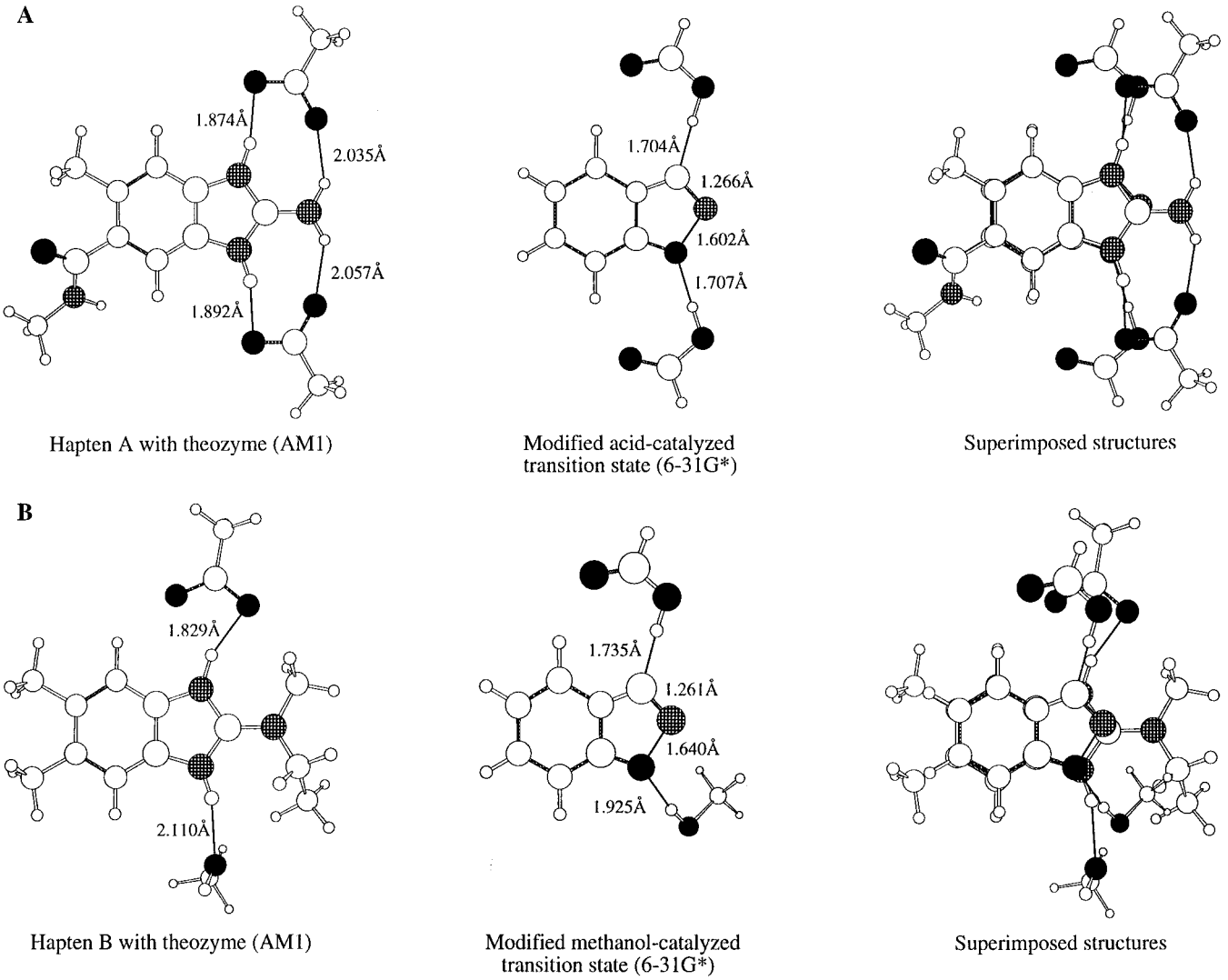

Figure 10. (A) Hapten A complexed by two acetate molecules. (AM1) (B) Hapten B complexed by acetate and methanol. (AM1)

In the gas phase, the conversion of isoxazole to hydroxyacrylonitrile is exothermic by 15.9 kcal/mol. The diagram represents the binding of isoxazole in a catalytic site by the carboxylate base plus hydrogen-bond donor, when present. The transition state and product energies in the presence of catalytic groups are shown. This is followed by energies of the product and catalytic groups after separation.

To mimic more accurately the energetics of an antibody-catalyzed reaction, the diagram shown here should be corrected by the energy required to desolvate the substrate isoxazole as well as the catalytic groups which are likely partially solvated in the unoccupied binding site. In addition, there is binding energy afforded by interaction of the substrate with groups in the binding site other than the catalytic groups. Using Still's GB/SA solvation model,[20] the aqueous solvation energies of isoxazole and the product hydroxyacrylonitrile can be estimated as 2.8 and 6.6 kcal/mol, respectively. These calculations predict that significant rate enhancement can result from increased hydrogen bonding during the conversion of bound substrate to bound product. There is also strong hydrogen bonding to the product, which may result in product inhibition of catalysis.

Combined with the previously discussed effect of correct orientation of the deprotonating carboxylate base, our calculations predict that the reaction rate might be increased by an

(20) Still, W. C.; Tempczyk, A.; Hawley, R. C.; Hendrickson, T. *J. Am. Chem. Soc.* **1990**, *112*, 6127.

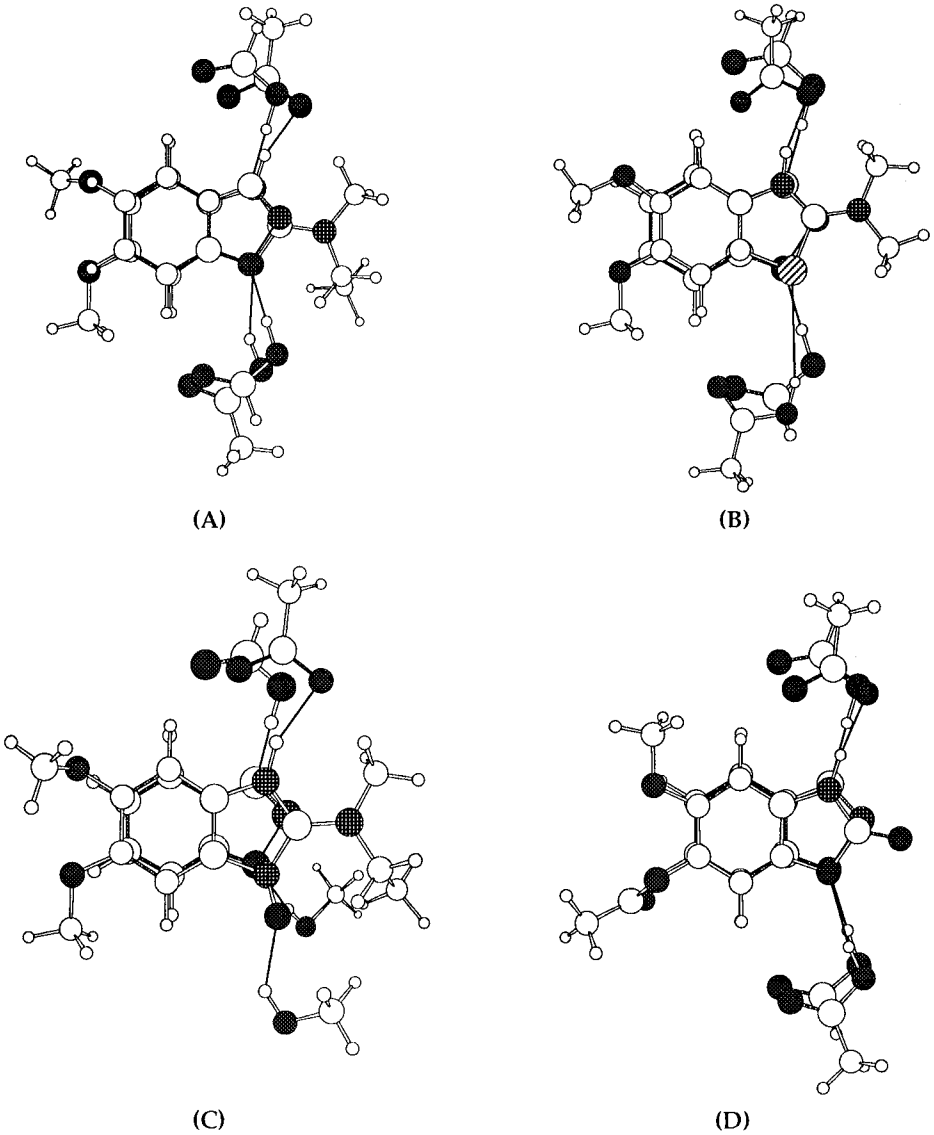

Figure 11. Haptens complexed by acetate and acetic acid or methanol, then superimposed over the corresponding acid-catalyzed or methanol-catalyzed transition state: (A) Hapten C complexed by acetate and acetic acid. (B) Hapten D complexed by methanol and acetate. (C) Hapten E complexed by methanol and acetate. (D) Hapten F complexed by acetate and acetic acid.

additional factor of 10^5-10^6. This would give a k_{cat}/K_m of about 10^8 M^{-1} s^{-1}, a rate competitive with diffusion in water, that is, equal to optimal enzymatic rates.

Evaluation of Potential Haptens. Maximum catalysis should be achievable by correctly orienting the carboxylate base and inducing a hydroxyl group to provide a strong hydrogen bond in the transition state (Chart 2). If information about the binding site of the known catalytic antibodies can be obtained, then modification of the antibody binding pocket through chemical methods[21] or by site-directing mutagenesis[22] could be undertaken to introduce these functionalities. Alternatively, the hapten used by Hilvert and co-workers so successfully might be modified to increase the probability of eliciting the desired set of interactions directly during the evolution of the immune response in vivo. Some of the alternative hapten structures which we have examined are shown in Figure 9.

One approach involves exploration of different coupling strategies. For example, an unalkylated imidazolium ring would provide two NH groups for eliciting an acid−base pair capable of bifunctional catalysis. Our calculations also suggest that the 2-amino group of hapten **4** is not an ideal design feature, since it likely promotes a doubly-hydrogen-bonded carboxylate in the binding site. Haptens like **A** and **B** (Figure 9) address these issues in different ways.

Compound **A** retains the 2-aminobenzimidazolium moiety of hapten **4** but is linked to carrier proteins via its benzene ring. In experiments in progress, a good immune response to this molecule was obtained. Yet few stable hybridomas were achieved. Further fusions will therefore be necessary to test whether catalysts can be produced. The relatively low pK_a of hapten **A** (≈6.1 in 33% aqueous EtOH[23]) precludes significant protonation and may limit the chances of inducing a carboxylate base in the binding site.

Hapten **B** is sufficiently basic (p$K_a \geq 7.8$[23,24]) to be protonated under physiological conditions. Consequently, both the desired

(21) (a) Pollack, S. J.; Nakayama, G. R.; Schultz, P. G. *Science* **1988**, *242*, 1038. (b) Pollack, S. J.; Schultz, P. G. *J. Am. Chem. Soc.* **1989**, *111*, 1929.

(22) (a) Stewart, J. D.; Roberts, V. A.; Thomas, N. R.; Getzoff, E. D.; Benkovic, S. J. *Biochemistry* **1994**, *33*, 1994. (b) Roberts, V. A.; Stewart, J.; Benkovic, S. J.; Getzoff, E. D. *J. Mol. Biol.* **1994**, *235*, 1098. (c) Aitken, D. J.; Alijah, R.; Onyiriuka, S. O.; Suckling, C. J.; Wood, H. C. S.; Zhu, L. *J. Chem. Soc., Perkins Trans. 1*, **1993**, 597. (d) Jackson, D. Y.; Prudent, J. R.; Baldwin, E. P.; Schultz, P. G. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 58. (e) Glockhuber, R.; Stadlmuller, J.; Pluckthun, A. *Biochemistry* **1991**, *30*, 3049. (f) Baldwin, E.; Schultz, P. G. *Science* **1989**, *245*, 1104.

(23) Perrin, D. D. *J. Chem. Soc.* **1965**, 5590.

(24) Dean, J. A. *Lange's Handbook of Chemistry*; McGraw-Hill, Inc.: San Francisco, 1992; pp 8.19−8.71.

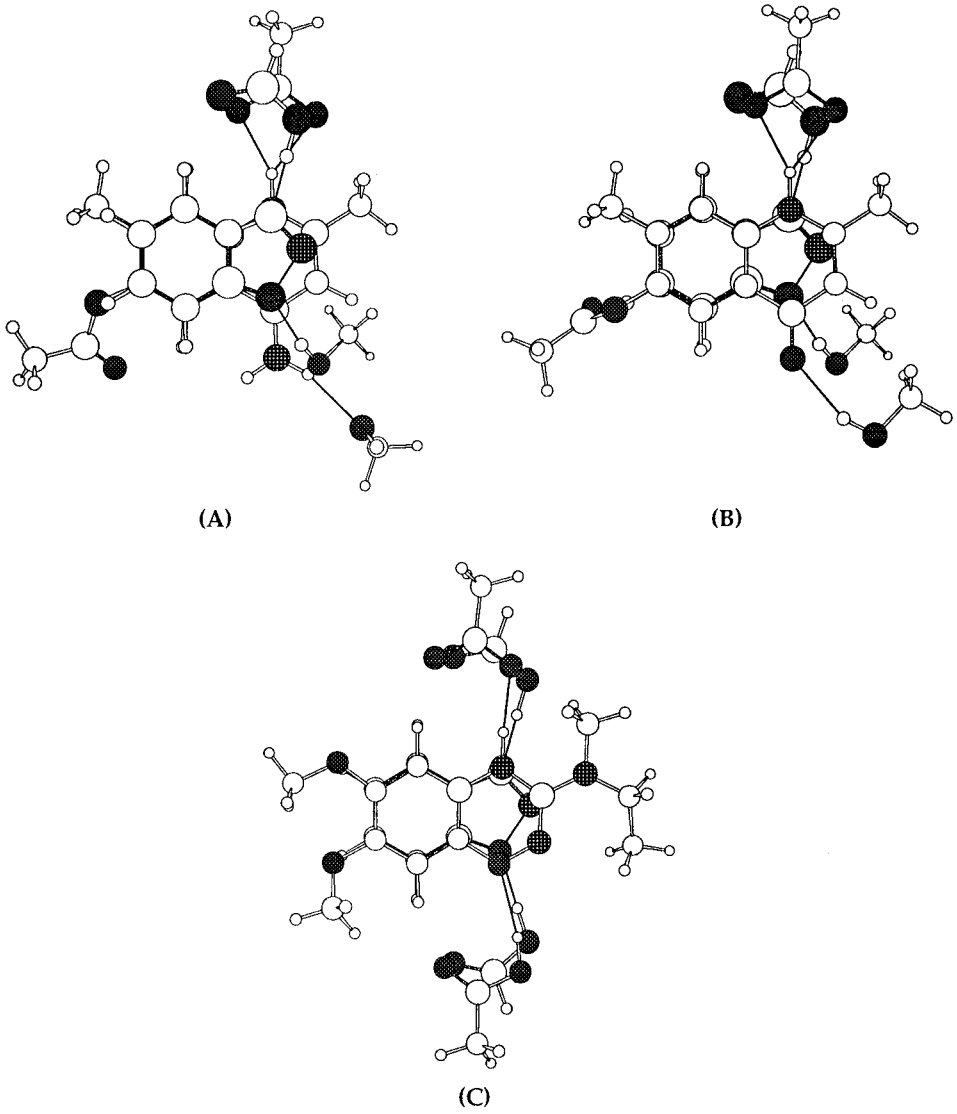

Figure 12. Haptens complexed by acetate and acetic acid or methanol, then superimposed over the corresponding acid-catalyzed or methanol-catalyzed transition state: (A) Hapten G complexed by acetate and methanol. (B) Hapten H complexed by acetate and methanol. (C) Hapten I complexed by acetate and acetic acid.

carboxylate base and a hydrogen bonding group, such as a serine or a tyrosine, could conceivably be induced in response to the two imidazolium NH groups. Replacement of the 2-amino group with a dimethylamino moiety will, moreover, favor a catalytically optimal carboxylate orientation by preventing bidentate coordination with the exocyclic amine. Alternative attachment points for the linker might also be profitably explored; linkage through the benzene ring, for example, might ensure that the isoxazole is buried in the hydrophobic binding site.

The benzoxazole and benzothiazole (haptens **C** and **D**) would be attractive haptens if protonated. The thiazole C−S bond (1.65 Å) mimics the elongated N−O bond (1.686 Å) in the transition state. Both of these haptens have relatively low predicted pK_a values, however.

The *N*-oxide of hapten **E** will enhance the possibility of eliciting a proton donor as a catalyst, and N−1 will be partially positively charged, providing the potential for inducing a carboxylate.

Although neutral haptens may have a relatively low probability of eliciting a carboxylate base, neutral haptens would allow the efficacy of other bases, such as histidine, to be investigated.[25] Hapten **F**, a benzoxazolinone compound, retains the necessary amide proton for eliciting a hydrogen-bond acceptor, while the carbonyl oxygen should force the elicited base to adopt its energetically-favored orientation. Furthermore, both oxygens atoms can elicit well-positioned hydrogen-bonding groups.

Haptens **G** to **I** replace the five-membered ring congruent with the reactant with a six-membered ring. This change in geometry may not adversely affect the ability of the hapten to elicit the desired functional groups.

Estimations of Hapten Similarity to Transition State. Here we undertake quantitative assessment of the degree of similarity of each hapten to the elimination transition state.

For each hapten, we optimized the position of an acetate and of a hydrogen-bond donor group. Depending on the hapten, the hydrogen-bond donor is an acetic acid if the 1-position has a heteroatom hydrogen-bond acceptor (haptens **C**, **D**, **F**, and **I**), or otherwise a methanol acting as either hydrogen-bond donor or acceptor. Due to the sizes of these systems, we used semiempirical AM1[7] for these optimizations. All hapten

(25) **Note Added in Proof**: Unpublished experiments (Kikuchi, K.; Thorn, S.; Hilvert, D.) show that borine serum albumin also catalyzes the decomposition of substituted benzisoxazoles. Chemical modification and pH-rate profile suggest that a lysine is the catalytic base.

Table 1. Complexation Energies (AM1) of Haptens **B**−**I** with Various Stabilizing Groups[a]

hapten	complexation E	hapten	complexation E
A	−155.4	**F**	−29.5
B	−101.8	**G**	−99.6
C	−103.6	**H**	−35.2
D	−92.6	**I**	−13.9
E	−34.2		

[a] Values in kcal/mol.

complexes were optimized for 100-200 cycles, until the energy changed by less than 1 kcal/mol and the geometry of the complex exhibits little change. The interaction energies are given in Table 1.

Hapten **B** was optimized with an acetate as a general base and a molecule of methanol as a general acid. As seen in Figure 10B, the orientation of the acetate is similar to the formate of the methanol-catalyzed transition state, but the methanols are not in the same location or orientation. Hapten **C** (Figure 11) is an improvement. The positions of the acetate and acetic acid in the complex are almost identical to the formate and formic acid groups in the transition state complex.[26] Hapten **D** (Figure 11), though similar in design to hapten **C**, was not as promising. The sulfur atom is less effective as a hydrogen-bond acceptor, shown by the relatively long interaction distance of 3.3 Å between sulfur and proton of acetic acid. It experiences the least stabilization (93 vs 100-104 kcal/mol) of the charged haptens upon interaction with the formate/formic acid dyad.

The neutral haptens give binding energies of 14-35 kcal/mol. The *N*-oxide of hapten *E* is a good hydrogen-bond acceptor, indicated by a relatively short hydrogen-bond distance of 2.1 Å (Figure 11). However, the location of this methanol deviates from the methanol in the optimized transition state complex. With hapten **F**, the location of the elicited acetic acid and acetate base are virtually identical to the similar groups in the acid-catalyzed transition state (Figure 11). In addition, the two oxygen atoms of hapten **F** may also interact with the guanine side chain of an arginine, providing the possibility for the doubly-hydrogen-bonded interaction observed in the water-catalyzed transition state. The probability of inducing a carboxylate may be quite low, however, due to the absence of charge.

Pyridine hapten **G** (pK_a of ≥ 9.1[27]) will be in the protonated form under physiological conditions. The acetate base forms a bifurcated hydrogen bond to the pyridinium proton, and the position of the carboxylate coincides almost perfectly with the formate group in the methanol-catalyzed transition state (Figure 12). The location and orientation of the elicited methanol group does not, however, match well with the methanol in the transition state complex. The acetate base in the hapten **H** complex also exhibits the bifurcated hydrogen-bonding pattern (Figure 12). The hydrogen-bonded methanol is displaced in a similar position and orientation as the methanol-catalyzed transition state. Hapten **I** also has both the base and acid in the proper location and orientation (Figure 12). However, the interaction energies are lowest of those calculated for any of these haptens.

Considering geometrical features and energies of interaction, compounds **A**−**H** all have features which recommend them as potential haptens. Overall, compounds related to **B**, **E**, and **F** are particularly intriguing, due to ideal positioning expected for induced groups and hydrogen-bonding complementarity.

Summary. Quantum mechanical calculations have provided insights into the nature of the benzisoxazole elimination transition state. The role of carboxylate orientation and general acid catalysis have been predicted, and haptens have been proposed and evaluated based upon this information.

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(26) The optimization was carried out with the N3 NH constrained to avoid proton transfer to the acetate group. The same is true for haptens **D** and **G**.

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