This, plus the presence of the longer Cu–N distances in addition to the shorter Cu—C distances,[18] gives 1 the longest average Cu–ligand distance. On addition of 1 equiv of BuLi the coordination number decreases to 2. This, together with replacement of a relatively long Cu—N≡C distance by a shorter Cu—C(butyl) distance, gives a 0.07 Å decrease in the average bond length. Addition of a second equivalent of BuLi leads to loss of the remaining cyanide, with a small increase in distance as expected on the basis of the difference in size for sp vs sp^3 hybridized C. The average Cu—C distance in 3 is marginally longer than the 1.92–1.94 Å distance found in isolated CuR_2^- units.[9] This may be due to weak secondary interactions between the Cu and the solvent and/or the Li cations, or it may simply reflect the uncertainty in the measurements.

The present data unambiguously show that most of the Cu atoms (>90%) in solution 3 do *not* contain coordinated cyanide, consistent with recent NMR studies.[5] The absence of EXAFS-detectable Cu···N scattering does not, of course, exclude the possibility that a species such as $Bu_2Cu(CN)Li_2$ may be present in 3 in catalytic amounts and be responsible for the higher reactivity of $R_2CuLi·LiCN$ compared to $R_2CuLi·LiX$ (X = halide).

Supplementary Material Available: Details of sample preparation (1 page). Ordering information is given on any current masthead page.

(18) In crystallographically characterized Cu cyanides, typical bond lengths are Cu–C ≈ 1.82 Å (1.90 Å) and Cu–N = 1.91 Å (1.97 Å) for 2-coordinate (3-coordinate) complexes.

A Catalytic Antibody for Imide Hydrolysis Featuring a Bifunctional Transition-State Mimic

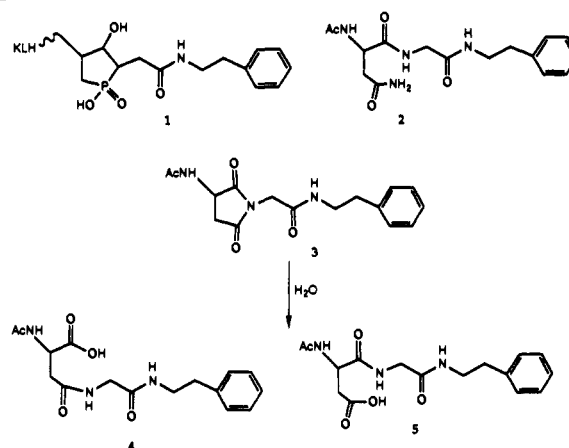Louis J. Liotta, Patricia A. Benkovic, Grover P. Miller, and Stephen J. Benkovic*

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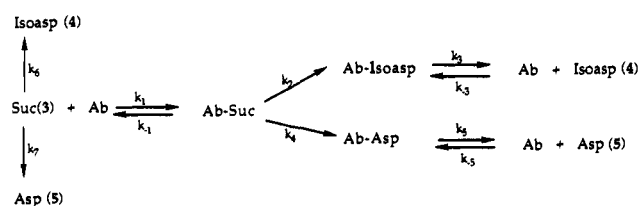
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Monoclonal antibodies raised against tetrahedral transition-state analogs have consistently been shown to catalyze acyl transfer reactions.[1] Antibodies generated against 1, a cyclic phosphinate containing two tetrahedral transition-state mimics, have previously been reported to catalyze the deamination of *N*-acetyl-asparaginylglycine phenethylamide 2 presumedly through generation of the succinimide intermediate 3.[2] Since the final product ratio of isoaspartate 4 to aspartate 5 was affected by several of these antibodies, we inferred that they also may catalyze the hydrolysis of 3 (Scheme I). Herein, we report an antibody generated against 1 that is capable of catalyzing the hydrolysis of succinimide 3 by accelerating cleavage at both amide carbonyls. Insight into the functional relationship between the two possible tetrahedral recognition sites on the antibody and the consequences for the regiochemistry of the hydrolysis has been provided by the evaluation of the kinetic parameters for both the D and L isomers of 3 as substrates.

The hapten synthesis and generation of antibodies have previously been reported.[2] Enantiomerically pure succinimides 3 were synthesized from the corresponding asparagines. An initial screen to determine the ratio of the isoaspartate 4 to aspartate 5 products

(1) Lerner, R. A.; Benkovic, S. J.; Schultz, P. G. *Science* 1991, 252, 659–667.
(2) Gibbs, R. A.; Taylor, S.; Benkovic, S. J. *Science* 1992, 258, 803–805.

Scheme I

Scheme II

Table I. Kinetic Constants[a] for D- and L-Succinimide 3 Hydrolysis by Antibody RG2-23C7

	D-succinimide	L-succinimide
k_{-1}/k_1	0.24 μM	0.83 μM
k_2(isoasp)	0.52 min^{-1}	3.5 min^{-1}
k_4(asp)	0.56 min^{-1}	0.12 min^{-1}
k_3/k_{-3}(isoasp)	0.24 μM	0.25 μM
k_5/k_{-5}(asp)	0.14 μM	0.10 μM
k_2/k_6	70.3	486
k_4/k_7	287	61.5

[a] ±10%.

was performed by HPLC.[3] An antibody designated RG2-23C7 among a grouping of 30 antibodies produced the largest deviation from background in the ratio of 4 to 5. Ratios of 10.9 for the L-succinimide and 1.5 for the D-succinimide were found as compared to 3.7 for the background reaction. This antibody was then subjected to further kinetic analysis.

The full reaction course was followed by HPLC, and the data were fit to Scheme II using computer simulations of the reaction time course (KINSIM).[4] The product dissociation constants for the D- and L-aspartate 5 and the D- and L-isoaspartate 4 (k_5/k_{-5} and k_3/k_{-3}, respectively) were determined by fluorescence titrations.[5] The succinimide dissociation constant (k_{-1}/k_1) and the catalytic rate constants (k_2 and k_4) were then determined from computer-generated fits to the HPLC data. The kinetic constants for the hydrolysis of both the L- and D-succinimides thus obtained are given in Table I. Since the fluorescence changes observed during the titrations were small, the strong product inhibition was verified by conducting the hydrolysis of 3 in the presence of an initial 1 mM concentration of either 4 or 5 and showing that the product data was fit by the corresponding simulations.

Evaluation of the kinetic constants indicates that RG2-23C7 binds the D isomer about 4 times better than it binds the L isomer. Since the antibodies were produced in response to a racemic mixture, this result implies that the true hapten was the D isomer of phosphinate 1. It has previously been shown that catalytic
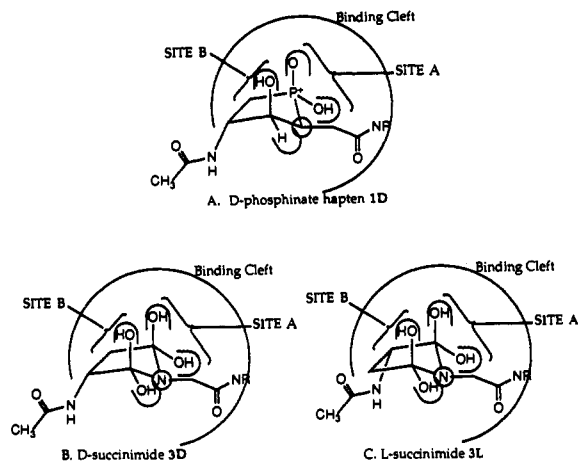
(3) HPLC was performed on a Vydac C18, 0.46 × 25 cm column (cat. no. 201TP54) using a gradient from 5:95 to 40:60 CH3CN/0.1% TFA.
(4) Barshop, B. A.; Wrenn, R. F.; Frienden, C. *Anal. Biochem.* 1983, 130, 134–145. Anderson, K.; Sikorski, J. A.; Johnson, K. A. *Biochemistry* 1988, 27, 7395–7406.
(5) Taira, K.; Benkovic, S. J. *J. Med. Chem.* 1988, 31, 129–137.

Figure 1. Binding of D- and L-succinimide into the catalytic pocket of antibody generated to bifunctional phosphinate 1.

antibodies produced in response to racemic mixtures usually show enantiomeric preferences.[6] Once the D isomer is bound, the energetics of hydrolysis at both carbonyls are equivalent since $k_2 \approx k_4$. In the case of the L isomer, however, hydrolysis to the isoaspartate product 4 is 30 times more likely than hydrolysis to the aspartate product 5.

One possible explanation for these results is illustrated in Figure 1. Provided the D isomer of 1 is the antigen leading to RG2-23C7 (Figure 1A), then the antibody binding site should stabilize both tetrahedral intermediates of the D-succinimide 3D leading to 4 and 5 (Figure 1B). Since the phosphinate more closely resembles the hydrolysis intermediate than the secondary alcohol, one presumes that site A in Figure 1 might be more active than site B. Thus the aspartate product 5 would be the preferred product. However, this preference is offset by the intrinsically 4-fold greater rate of isoaspartate formation owing to the electronic effect of the α-N-acetyl substituent leading to similar values for k_2 and k_4.[7] On the other hand, when the L-succinimide 3L is fit into this same pocket it must be flipped 180° in order to accommodate the N-acetyl group (Figure 1C). Since the α-amide of 1 serves to link the hapten to the carrier protein, it is likely that the N-acetyl protrudes from the binding site owing to the insensitivity of many catalytic antibodies to changes in the linkage region of their haptens.[8] The carbonyl adjacent to the N-acetyl now occupies site A (the more active of the two sites). The 30-fold faster catalytic rate for the formation of isoaspartate 4 (k_2) as compared to aspartate 5 (k_4) reflects the electronic effects of the N-acetyl augmenting the better catalytic site. The ratio of k_2 for the L isomer to k_4 for the D isomer of 3 (rate constants for attack at the carbonyl center associated with the phosphinate mimic) is 6-fold, principally reflecting the 4-fold difference arising from the electronic effect of the α-N-acetyl group.

Although other interpretations are plausible, the variations in k_2 and k_4 between the L- and D-succinimides and their increased magnitudes relative to k_6 and k_7 (Table I) are in accord with both tetrahedral mimics acting to generate catalytically active binding pockets. For the conversion of the L-succinimide to the isoaspartate product, the value of $k_2/(k_{-1}/k_1)$ is 7×10^5 M^{-1} s^{-1} as compared to values of ca. 10^7 M^{-1} s^{-1} for diffusion-controlled enzymatic processes, the upper limit on enzyme-catalyzed turnovers. Thus

this antibody is within a factor of 10^2 of maximal efficiency.

Supplementary Material Available: Details of the synthesis of 3L and 3D, listings of physical and spectral data for 3–5, and kinetic analyses and plots for the hydrolyses of the succinimides (5 pages). Ordering information is given on any current masthead page.

Model Compounds Can Mimic Spectroscopic Properties of Bovine Rhodopsin

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Visual pigments (rhodopsins) consist of 11-*cis*-retinal chromophore bound to a protein (opsin) via a protonated Schiff base linkage with a lysine ϵ-amino group.[1-4] The absorption maxima of the various rhodopsins are characterized by a wide range of wavelengths (440–620 nm) despite the fact that all of them consist of a similar chromophore. The red shift observed in these pigments relative to a model retinal protonated Schiff base (RSBH⁺) in a methanol solution which absorbs at 440 nm was defined as the opsin shift (OS).[5] The mechanism through which the protein regulates the absorption maxima has been studied extensively, and various models have been suggested.[6,7]

One of the striking spectroscopic characteristics of bovine rhodopsin (λ_{max} = 498 nm) is its C=N stretching frequency (1656 cm^{-1}), which resembles that of retinal protonated Schiff base in methanol solution.[8] It was shown previously that weakening the interaction between the positively charged Schiff base linkage and its counteranion in model compounds leads to a lower C=N frequency accompanying the observed red shift.[9] Thus, one of the major difficulties in explaining the red shift observed in bovine rhodopsin (498 nm) by only separating its positively charged Schiff base linkage from its counteranion is the above explained contradiction of the high C=N stretching frequency observed for bovine rhodopsin despite its red-shifted absorption.

In the present study, we demonstrate experimentally by model compound studies that it is possible to red shift the absorption maximum of a retinal protonated Schiff base by weakening the

(6) Pollack, S. J.; Hsiun, P.; Schultz, P. G. *J. Am. Chem. Soc.* **1989**, *111*, 5961–5962. Janda, K. D.; Benkovic, S. J.; Lerner, R. A. *Science* **1989**, *244*, 437–440. Janda, K. D.; Ashley, J. A.; Jones, T. M.; McLeod, D. A.; Schloeder, D. M.; Weinhouse, M. I. *J. Am. Chem. Soc.* **1990**, *112*, 8886–8888. Wirsching, P.; Ashley, J. A.; Benkovic, S. J.; Lerner, R. A. *Science* **1991**, *252*, 680–685.

(7) Sondheimer, E.; Holley, R. W. *J. Am. Chem. Soc.* **1954**, *76*, 2467–2470.

(8) Janda, K. D.; Benkovic, S. J.; McLeod, D. A.; Schloeder, D. M.; Lerner, R. A. *Tetrahedron* **1991**, *47*, 2503–2506. Tawfik, D. S.; Zemel, R. R.; Yellin, R. A.; Green, B. S.; Eshlar, Z. *Biochemistry* **1990**, *29*, 9916–9921. Pollack, S. J.; Hsiun, P.; Schultz, P. G. *J. Am. Chem. Soc.* **1989**, *111*, 5961–5962.

(1) Ottolenghi, M. *Adv. Photochem.* **1980**, *12*, 97.
(2) Packer, L. *Methods Enzymol., Biomembr.* **1981**, Part I, 81.
(3) Ottolenghi, M.; Sheves, M. *J. Membr. Biol.* **1989**, *112*, 193.
(4) Birge, R. *Biochim. Biophys. Acta* **1990**, *1016*, 293.
(5) Nakanishi, K.; Balogh-Nair, V.; Arnaboldi, M.; Tsujimoto, K.; Honig, B. *J. Am. Chem. Soc.* **1980**, *102*, 7945.
(6) (a) Kropf, A.; Hubbard, A. *Ann. N.Y. Acad. Sci.* **1958**, *74*, 266. (b) Irving, C.; Byers, G.; Leermakers, P. *Biochemistry* **1970**, *9*, 858. (c) Blatz, P.; Mohler, J. *Biochemistry* **1972**, *11*, 3240. (d) Ebrey, T.; Honig, B. *Q. Rev. Biophys.* **1975**, *8*, 129 and references cited therein. (e) Warshel, A. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 2558. (f) Ishikawa, K.; Yoshidara, T.; Suzuki, H. *J. Phys. Soc. Jpn.* **1980**, *49*, 1505. (g) Arnaboldi, M.; Motto, M.; Tsujimoto, K.; Balogh-Nair, V.; Nakanishi, K. *J. Am. Chem. Soc.* **1979**, *101*, 7082. (h) Honig, B.; Dinur, U.; Nakanishi, K.; Balogh-Nair, V.; Gawinowicz, M.; Arnaboldi, M.; Motto, M. *J. Am. Chem. Soc.* **1979**, *101*, 7084.
(7) (a) Birge, R.; Murray, L.; Pierce, B.; Akita, H.; Balogh-Nair, V.; Findsen, L.; Nakanishi, K. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 4117. (b) Birge, R.; Einterz, C.; Knapp, H.; Murray, L. *Biophys. J.* **1988**, *55*, 367.
(8) (a) Narva, D.; Callender, R. *Photochem. Photobiol.* **1980**, *32*, 273. (b) Bagley, K.; Dollinger, G.; Eisenstein, L.; Singh, A.; Zimanyi, L. *Proc. Natl. Acad. Sci. U.S.A* **1982**, *79*, 4972. (c) Palings, I.; Pardoen, J.; van den Berg, E.; Winkel, C.; Lugtenburg, J.; Mathies, R. *Biochemistry* **1987**, *26*, 2544.
(9) (a) Baasov, T.; Sheves, M. *Biochemistry* **1986**, *25*, 5249. (b) Baasov, T.; Sheves, M. *Biochemistry* **1987**, *26*, 3210.