W. G. Sippell, *Ann. Hum. Biol.* **15**, 103 (1988).
8. W. A. Marshall, *Arch. Dis. Child.* **46**, 414 (1971); J. K. H. Wales and R. D. G. Milner, *ibid.* **62**, 166 (1987); J. M. Wit, D. M. Teunissen, J. J. J. Waelkens, W. J. Gerver, *Acta Paediatr. Scand. Suppl.* **337**, 40 (1987).
9. The sample infants were characterized by birth weights ≥2500 g, 38 to 42 weeks gestational age, uncomplicated pregnancy and delivery, 1- and 5-min Apgar scores between 8 and 10, and no subsequently diagnosed medical problems during the course of the study.
10. N. Cameron, in *Human Growth: A Comprehensive Treatise*, F. Falkner and J. M. Tanner, Eds. (Plenum, New York, ed. 2, 1986), vol. 3, pp. 3–46.
11. The infant measuring board was specially designed after the Harpenden-Holtain infant length board (*10*), equipped with a fixed headboard and mobile footboard. One observer fixes the infant's head as the second applies gentle pressure to the body to ensure that the legs are straight and the ankles are at right angles. The footboard is brought into firm contact with the subject's feet. A final check on the proper alignment of the head and body is made before length assessment. Ninety percent of the measurements were taken within 3 hours of the same time on each visit to control for diurnal variation. Replicate examinations were conducted within 1 hour, and the observer and recorder were the same for each replicate.
12. The technical error of measurement (tem) is the square root of the sum of squared differences between replicates divided by twice the number of paired observations (*10*).
13. G. A. Harrison, G. Brush, A. Almedom, T. Jewell, *Ann. Hum. Biol.* **17**, 407 (1990). The tem of measurements here is slightly less than that of field studies previously reported: R. Martorell, J.-P. Habicht, C. Yarbrough, G. Guzman, R. E. Klein, *Am. J. Phys. Anthropol.* **43**, 347 (1975); C. C. Gordon, W. C. Chumlea, A. F. Roche, in *Anthropometric Standardization Reference Manual*, T. B. Lohman, A. F. Roche, R. Martorell, Eds. (Human Kinetics, Champaign, IL, 1988), pp. 3–9. The technical errors in the present study are not biased by the design or any other known factor except the particular care given to measurement technique by both the anthropometrist (M.L.) and the assistant. The conditions of this study were unusual in that all measurements were taken in the home with mothers as assistants, conditions substantially different from those of previous studies in clinical environments. The mothers of the three children measured daily are all professional anthropometrists. In addition, 250 measurements were taken with an independent rater to verify measurement reliability.
14. J. D. Veldhuis and M. L. Johnson, *Methods Enzymol.* **210**, 539 (1992). This method fits the actual serial growth data by the use of a weighted, nonlinear least-squares procedure of parameter estimation. The analytical method is designed to compare the actual experimental data to a series of models with increasing numbers of pulses. From an initial model with no pulses, subsequent models are generated through the addition of pulses at points of greatest decrement in variance, until no statistically significant improvement of fit is gained from pulse addition.
15. M. Hermanussen, K. Geiger-Benoit, W. G. Sippell, *Acta Paediatr. Scand. Suppl.* **75**, 601 (1987). A comparison was made between the growth episodes, the stasis intervals, and all illnesses of the subjects. There was no positive association between growth stasis and illness (Pearson chi-square).
16. M. Lampl, unpublished data.
17. N. Mauras *et al.*, *J. Clin. Endocrinol. Metab.* **64**, 596 (1987); K. Albertsson-Wikland and S. Rosberg, *ibid.* **67**, 493 (1988); P. M. Martha *et al.*, *ibid.* **69**, 563 (1989).
18. The maximum sample size available for this comparison consists of weekly data from 14 infants between 3 and 9 months of age, assessed as standard deviation scores for age and sex.
19. D. Lloyd and S. W. Edwards, in *Cell Cycle Clocks*, L. Edmunds, Ed. (Plenum, New York, 1986), pp. 27–46.
20. E. A. Wang *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2220 (1990); R. Bortell, L. M. Barone, M. S. Tassinari, J. B. Lian, G. S. Stein, *J. Cell. Biochem.* **44**, 81 (1990); B. A. A. Scheven and N. J. Hamilton, *Acta Endocrinol. Copenhagen* **124**, 602 (1991).
21. E. B. Hunziker and R. K. Schenk, *J. Physiol. London* **414**, 55 (1989); G. J. Breur, B. A. VanEnkevort, C. E. Farnum, N. J. Wilsman, *J. Orthop. Res.* **9**, 348 (1991); S. Stevenson, E. B. Hunziker, W. Herrmann, R. K. Schenk, *ibid.* **8**, 132 (1990).
22. R. A. Weinberg, *Science* **254**, 1138 (1991).
23. J. B. Ghiara *et al.*, *Cell* **65**, 163 (1991); H. Matsushime, M. F. Roussel, R. A. Ashmun, C. J. Sherr, *ibid.*, p. 701; T. Motokura, *Nature* **350**, 512 (1991); U. Surana *et al.*, *Cell* **65**, 145 (1991); T. Chittenden, D. M. Livingston, W. G. Kaelin, Jr., *ibid.*, p. 1073; S. P. Chellappan, S. Hiebert, M. Mudryj, J. M. Horowitz, J. R. Nevins, *ibid.*, p. 1053; L. R. Bandara and N. B. La Thangue, *Nature* **351**, 494 (1991).
24. We thank the parents who participated in this study, L. Hileman for measuring assistance, R. N. Emde and M. Reite for their support of earlier versions of this work, and K. Ryan for technical assistance with the manuscript (M.L.). Supported by the Developmental Psychobiology Research Group, the Grant Foundation, and the Wenner-Gren Foundation (M.L.); National Institute of Child Health and Human Development Research Career Development Award KO4HD00634, National Institutes of Health (J.D.V.); National Science Foundation Science Center for Biological Timing (J.D.V. and M.L.J.); Diabetes and Endocrine Research Center grant DK 38942; and the University of Virginia Pratt Foundation and Educational Enhancement Fund (J.D.V. and M.L.J.).

16 March 1992; accepted 4 August 1992

Antibody-Catalyzed Rearrangement of the Peptide Bond

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The generation of antibodies from a bifunctional cyclic phosphinate transition-state analog provided agents capable of efficiently catalyzing both steps of the overall conversion of a substrate containing an asparaginyl-glycyl sequence through a succinimide intermediate to the products aspartyl-glycyl and the rearranged isoaspartyl-glycyl sequence. This reaction provides a potential means in addition to amide cleavage for the deactivation of protein or peptide biological functions in vivo.

A major goal in the field of catalytic antibodies is the inactivation of proteins or peptides. There are two principal means by which this may be achieved. One is through the cleavage of the amide bond by hydrolytic or oxidative methods, and a second is through the modification of side-chain residues or through the rearrangement of the main peptidic chain (*1*). Examples of the latter include the deamidation of Asn or Gln residues and the related β-aspartyl shift mechanism. One can generally prevent these processes from rapidly occurring by having the protein chains adopt conformations that exclude the favorable reactive distances and bond angles required for these processes (*2*, *3*). The deamidation of Asn residues has been implicated in protein denaturation (*4*), in the initiation of proteolytic processes leading to protein degradation in vivo (*5*), and in the loss of enzymic activity (*6*).

We were particularly interested in the possibility that properly selected antibodies might restrain the side-chain amide carbonyl and the $n + 1$ amide nitrogen in an alignment and distance favorable for reaction to form a succinimide intermediate. That in turn would hydrolyze to the Asp deamidation and the isoaspartyl (IsoAsp) β-shift mechanism products (Fig. 1). In addition to providing a favorable ground-state conformation, the antibody binding site must also complement the metastable tetrahedral intermediates **1** and **3** (Fig. 1) and their associated transition states, which presumably are involved in the two-step reaction sequence. Differential ground- and transition-state complementarity are necessary for efficient catalysis to occur.

We chose the *N*-acetylasparginylglycyl (*N*-phenethyl)amide, **4**, as representative of the Asn-Gly linkage in proteins and the cyclic phosphinate, **5**, to mimic the intermediate species **1** to **3**. **5** possesses two tetrahedral moieties—the phosphinate and secondary alcohol—so that it mimics both transition state **2** and **3**. The synthesis of **4** and **5** are outlined in Fig. 2. The synthesis of **5** begins with the previously described chloride **6** (*7*) and leads to a racemic product ultimately formed from trans addition of azide to the racemic precursor epoxide. The final product **5** exhibits a single ^{31}P resonance at 49.8 ppm, consistent with values reported for other cyclic phosphinates (*8*, *9*). An immunogenic conjugate was prepared from **5** through its linkage to a carrier protein (keyhole limpet hemocyanin). Monoclonal antibodies were obtained by standard protocols (*10*) and purified to

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>90% homogeneity as judged by SDS–gel electrophoresis (*11*); we screened 30 antibodies both for catalytic activity and altered IsoAsp-Asp product ratios at pH 9.0 by monitoring ammonia formation with a coupled glutamate dehydrogenase enzyme assay or high-performance liquid chromatography (HPLC) separation of the Asp-Gly **7** or IsoAsp-Gly **8** products (Fig. 3).

As noted above, the overall reaction is a two-step process and is rate-limited by the initial step of cyclization to form the succinimide. Two antibodies, 2E4 and 24C3, had significant activity above the background rate, as measured by either the coupled glutamate dehydrogenase assay for ammonia liberation or by HPLC determination of both peptide products formed from the D-isomer as a function of time. With HPLC, there is no apparent significant buildup of the succinimide intermediate. The steady-state Michaelis-Menten parameters (k_{cat}, catalytic rate constant, and K_m, Michaelis constant) and the ratio of k_{cat}/k_0 (where k_0 is the spontaneous rate) measured at pH 8.95 are presented in Table 1. The catalysis is effectively blocked by the addition of an equivalent amount of the phosphinate **5**, which suggests that inhibition is stoichiometric at these concentrations in accord with an inhibition constant (K_i) ≤ 0.1 μM^{-1} for dissociation of the hapten. The half-life for the spontaneous cyclization reaction under our conditions is ~4.7 days, which the antibody reduces to ~1.6 hours. The rate acceleration is characteristic of rotational restrictions imposed on an intramolecular process (*12*). Although the binding of the substrate, expressed as K_m, is in the submillimolar range, it can probably be lowered by the addition of one or two amino residues to the NH_2- and COOH-terminal ends of the hapten, thereby increasing the ratio of k_{cat} to K_m and simultaneously enhancing the specificity of the antibody toward a protein target sequence.

The results of the HPLC assay of the products formed from either the L- or D-isomers of the substrate, **4**, are shown in Table 2. There are a number of salient features.

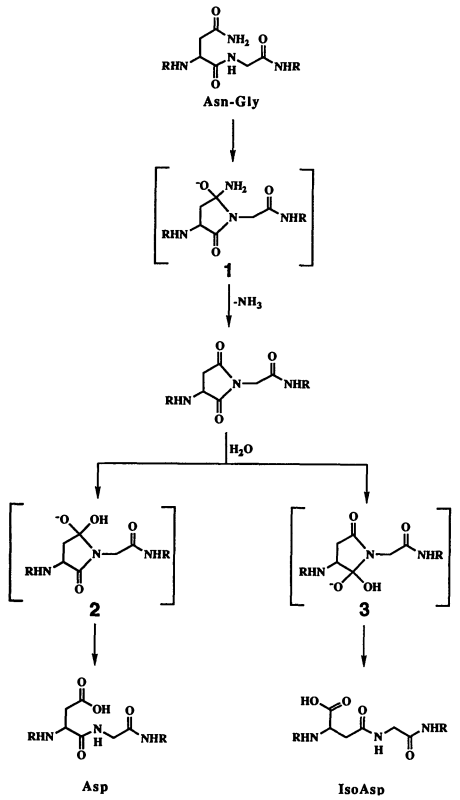

Fig. 1. The conversion of an Asn-Gly sequence to Asp-Gly and IsoAsp-Gly products. Presumed tetrahedral intermediates formed along the reaction pathway are shown.

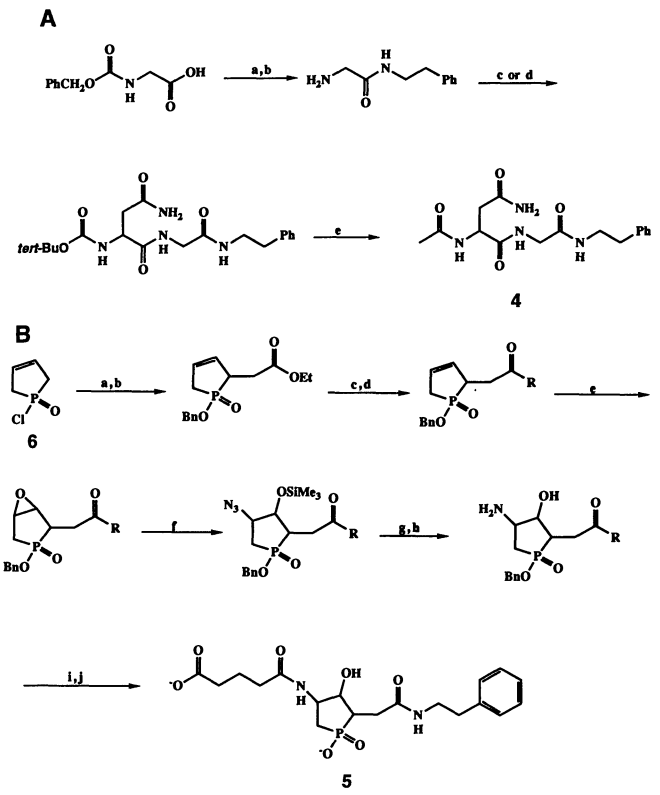

Fig. 2. Synthesis of intermediate species **4** and **5**. (**A**) Synthesis of **4**. Reagents and conditions: a, $PhCH_2$-CH_2NH_2, EDC, CH_2Cl_2, room temperature (rt) (62% yield); b, $Pd(OH)_2$-C/H_2, ethanol, rt (100% yield); c, Boc-L-Asn-ONp, DMF, rt (63% yield); d, Boc-D-Asn-OSu, DMF, rt (56% yield); e, trifluoroacetic acid, CH_2Cl_2, rt, 1 hour; CH_3CO-ONp, DMF, rt (15% yield of L-isomer; 37% yield of D-isomer). EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; Boc, *tert*-butoxy-carbonyl; DMF, dimethylformamide; Np, *p*-nitrophenyl; Su, succinimide, Bu, butyl; and Ph, phenyl. (**B**) Synthesis of **5**. Reagents and conditions: a, BnOH, triethylamine, CH_2Cl_2, 0°C → rt (81% yield); b, lithium diisopropylamide, Me_2NCH_2-CH_2NMe_2, tetrahydrofuran, −78°C; then ICH_2CO_2Et (Et, ethyl; Me, methyl), −78°C to −10°C (21% yield); c, NaOH, acetonitrile-H_2O, rt; d, $PhCH_2CH_2NH_2$, triethylamine, EDC, 1-hydroxybenzotriazole, CH_2Cl_2, rt (50% yield for both steps); e, *m*-$ClC_6H_4CO_3H$, CH_2Cl_2, reflux (64% yield); f, Me_3SiN_3, Al (isopropoxide), CH_2Cl_2, rt (40% yield); g, Ph_3P, tetrahydrofuran, rt; h, NH_3, MeOH, rt (53% yield for both steps); i, $Bn_2O_2CCH_2CH_2CH_2CO_2H$, EDC, triethylamine, CH_2Cl_2, rt (55% yield); j, H_2, $Pd(OH)_2$/C, $NaHCO_3$, H_2O-EtOH, rt (~100% yield). Bn, $PhCH_2$; R, $PhCH_2CH_2NH$.

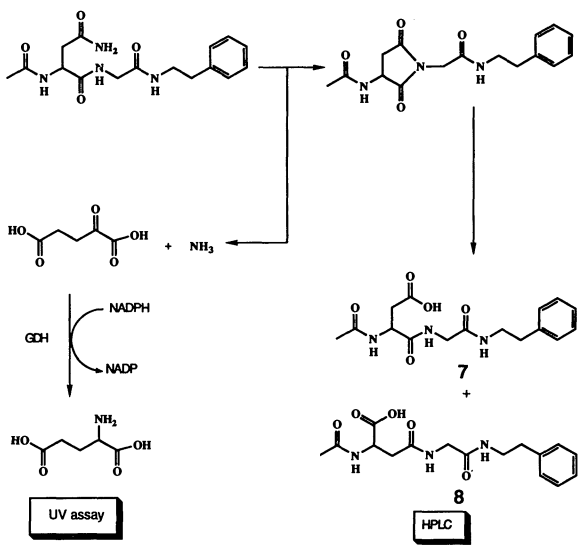

Fig. 3. Structures of the Asp-Gly and IsoAsp-Gly products (**7** and **8**) and methods of analysis. UV, ultraviolet; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced form of NADP; and GDH, glutamate dehydrogenase.

Table 1. Kinetic parameters for the cyclization of substrate **4** by monoclonal antibody 2E4. The rates were determined spectrophotometrically by a coupled enzyme assay with the use of glutamate dehydrogenase. Protein concentration was determined by measurement of the absorbance at 280 nm. A typical reaction mixture consisted of 100 mM bicine (pH 9.0), α-ketoglutarate (14.1 mM), adenosine 5′-diphosphate (1.4 mM), reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) (283 μM), and 2E4 antibody (14.9 μM). To 280 μl of this solution was added 17.5 μl of undiluted glutamate dehydrogenase (beef liver enzyme from Boehringer Mannheim in 50% glycerol-phosphate buffer, NH_3 < 10 μg/ml), which we gently mixed by withdrawing and then expelling the solution from a Pipetman (Gilford, Villiers-LeBel, France). A 50% dimethyl sulfoxide (DMSO)–water solution was added so that when the reaction was initiated by addition of the D-substrate (also in a 50% DMSO-water solution), the total amount of DMSO was 5% and the total volume was 330 μl. The solution was mixed again with the use of a Gilford Pipetman and transferred to quartz cuvettes and incubated for 10 to 15 min at 25°C before data acquisition. The pH of the solution after addition of all of the materials was 8.95. The reactions were monitored by the decrease in absorbance at 340 nm that resulted from the disappearance of NADPH [extinction coefficient (ε) = 6.22×10^{-3} μM^{-1} cm^{-1})]. The background rate was determined under identical conditions except that antibody was not added. The background rate was subtracted from the rates of the antibody-catalyzed reactions. The rate of the uncatalyzed reaction was determined under similar conditions.

K_m (μM^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M\ s^{-1}$)	k_{cat}/k_0
193 ± 41	(1.2 ± 0.1) × 10^{-4}	(6.3 ± 0.9) × 10^{-7}	70 ± 10

First, antibodies 39F3 and 14A8 act on either the L- or D-isomer, respectively, whereas, for example, 23C7 can process both isomers. Second, the group of 4OH4, 2E4, and 24C3 produces for the L-isomer an IsoAsp/Asp ratio that is less than the background reaction (IsoAsp/Asp = 3.5) but for the D-isomer produces a value for the ratio that is greater than the background reaction (behavior that is the converse of that for 23C7). These data are consistent with the presence of two classes of antibodies arising from the racemic hapten; classes of antibodies that process one of two enantiomers have been previously encountered in antibody-catalyzed lactone formation (*13*) and ester hydrolysis (*14*, *15*). One subtle but important further distinction is the ability of a given antibody to process both the L- and D-stereoisomers in a reciprocal fashion relative to the Iso/Asp = 3.5 background ratio. For example, 23C7 showed a pronounced Iso/Asp ratio of 16 for the L-isomer but a decreased Iso/Asp ratio of 1 for the D-isomer.

Table 2. Product distribution for RG2 antibody-catalyzed reactions of the D- or L-isomer of **4**. The product distribution was determined by HPLC (Waters 600E) with an analytical reverse-phase C_{18} column (VYDAC) with an acetonitrile-water (0.1% trifluoroacetic acid) gradient and a detector setting at 254 nM. A typical determination was as follows: to 238 μl of antibody in 100 mM bicine (pH 9.0) was added 12.5 μl of a 10.0 mM solution of substrate in DMSO. The antibody concentration was different for each antibody and was between 35 and 95 μM. The final concentration of substrate was 500 μM, and the DMSO content was 5%. The reaction was run at room temperature, and at various time intervals 50-μl samples were withdrawn and quenched with 1 μl of 14% perchloric acid (the solution immediately became cloudy upon addition of perchloric acid) to bring the pH to approximately 2. The quenched sample was immediately centrifuged for 2 min, and then 20 μl of this was injected into the HPLC. The reactions were followed for at least 100 hours, and during this time period three determinations were made. The product ratio remained constant throughout this time period. The product distribution of the uncatalyzed reaction was determined in a similar manner except the total volume was 1 ml. The uncatalyzed reaction was followed to 50% completion, and six determinations were made, during which time the product distribution ratio remained constant.

Antibody	IsoAsp/Asp product ratio	
	L-isomer	D-isomer
39F3	8.3	3.6*
14A8	3.4*	1.4
23C7	16.4	1.2
40H4	1.9	5.7
2E4	2.4	4.7
24C3	2.1	4.8

*Spontaneous reaction. The IsoAsp/Asp ratio for spontaneous reaction is 3.5.

We propose that either of the two enantiomeric haptens can generate an antibody binding site for the substrate that possesses in some cases a sufficiently large binding pocket to accommodate the *N*-acetyl group of either the L- or the D-isomer of the succinimide. One can achieve this by binding the phosphinate and side-chain phenethylamine groups deep in the hapten combining site so that the *N*-acetyl group is near the surface of the pocket or possibly protruding into solution. Processing of either the L- or D-isomer of the substrate by an antibody induced by a stereoisomer of **5** with a given configuration can produce an IsoAsp/Asp ratio greater or less than 3.5 as a consequence of the balance between steric hindrance, which retards hydrolytic attack at the buried carbonyl, and an electronic effect, which favors cleavage of the carbonyl α to the *N*-acetyl group. That a given antibody actually catalyzes imide hydrolysis has been shown by direct kinetic studies (*16*).

The generation of antibodies capable not only of accelerating the rate-limiting step of succinimide formation but also of directing the course of imide ring opening to various IsoAsp/Asp ratios represents an advance in the diversity of reaction types catalyzed by these agents (*17*). The use of a bifunctional transition-state analog permitted the generation of antibodies that catalyzed both steps of a multistep process that proceeds through a stable intermediate and thus effectively dealt with the problem of multiple transition states. The immunization with the racemate of the hapten also provided two classes of antibodies so that either the L- or D-isomer of the substrate could be processed to a final product ratio of choice. Finally, of particular importance is the experimental basis these catalysts provide for the further development of catalytic antibodies that target specific Asn-Gly sequences for the deactivation of protein function in vivo.

REFERENCES AND NOTES

1. H. T. Wright, *CRC Crit. Revs.* **26**, 1 (1991).
2. S. Clarke, *Int. J. Pept. Protein Res.* **30**, 808 (1987).
3. A. A. Kossiakoff, *Science* **240**, 191 (1988).
4. T. J. Ahern and A. M. Klibanov, *ibid.* **228**, 1280 (1985); S. Tomazic and A. M. Klibanov, *J. Biol. Chem.* **263**, 3092 (1988).
5. S. W. Rogers and M. Rechsteiner, *J. Biol. Chem.* **263**, 19850 (1988).
6. P. M. Yuan, J. M. Talent, R. W. Gracy, *Mech. Ageing Dev.* **17**, 151 (1981).
7. K. Modritzer, *Synth. React. Inorg. Met. Org. Chem.* **5**, 45 (1975).
8. L. D. Quin, *The Heterocyclic Chemistry of Phosphorus* (Wiley, New York, 1981).
9. In a preliminary study, an analog of **5** was prepared with R = OEt (Et, ethyl) instead of $NHCH_2CH_2Ph$ (Ph, phenyl). This compound was obtained as a single isomer with a ^{31}P nuclear magnetic resonance (NMR) signal at 40.6 ppm. The regiochemistry of this compound was verified by ^1H NMR decoupling experiments. However, two isomers (which can be separated by HPLC) were obtained when the synthesis was carried out with R = $NHCH_2CH_2Ph$. We believe that the other phosphinate is the undesired regioisomer of **5** resulting from azide attack on the other carbon of the intermediate epoxide [R. A. Gibbs and S. J. Benkovic, unpublished results].
10. H. L. Niman and J. H. Elder, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4524 (1980).
11. U. K. Laemmli, *Nature* **227**, 680 (1970).
12. M. I. Page and W. P. Jencks, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1678 (1971).
13. A. D. Napper, S. J. Benkovic, A. Tramontano, R. A. Lerner, *Science* **237**, 1041 (1987).
14. K. D. Janda, S. J. Benkovic, R. A. Lerner, *ibid.* **244**, 437 (1989).
15. S. J. Pollack, P. Hsiun, P. G. Schultz, *J. Am. Chem. Soc.* **111**, 5961 (1989).
16. R. A. Gibbs, S. Taylor, S. J. Benkovic, unpublished results.
17. R. A. Lerner, S. J. Benkovic, P. G. Schultz, *Science* **252**, 659 (1991).

18. We thank D. Schloder for her assistance in the preparation of the monoclonal antibodies. R.A.G. is recipient of NSF postdoctoral fellowship CHE8808377. S.T. is recipient of a Natural Sciences and Engineering Research Council (Canada) postdoctoral fellowship. We also thank K. D. Janda and R. A. Lerner for their many helpful discussions.

20 May 1992; accepted 5 August 1992