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Prodrug activation via catalytic antibodies

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ABSTRACT **Prodrug activation via antibodies was examined by using the antibiotic chloramphenicol as a model drug. Based on the conformational change between substrate and product, this antibody-catalyzed reaction was designed to prevent product inhibition, thus enhancing turnover. Antibodies elicited against a phosphonate transition-state analogue were found to catalyze hydrolysis of a nonbioactive chloramphenicol monoester as a prodrug at a significantly higher rate above the uncatalyzed background reaction to regenerate chloramphenicol as a parent molecule. The antibody-catalyzed prodrug activation was tested by the paper-disc diffusion method using *Bacillus subtilis* as an indicator strain. The antibody 6D9 catalyzes the reaction with multiple turnover to generate enough chloramphenicol to inhibit bacterial growth, as indicated by a clear inhibitory zone after incubation with monoester. Using the same method, no inhibition was detected by incubation of either the monoester or the antibody alone. This result reveals that only the antibody hydrolytically activates the monoester, which can be expected to be a suitable prodrug, as it is resistant to the action of bacterial hydrolytic enzymes. The approach in this study demonstrates the use of catalytic antibody technology in medicine and may be applicable to drugs with undesirable effects, particularly in the field of cancer therapy.**

In the short period of time that has elapsed since the initial reports of catalytic antibodies in 1986, a considerable number of different reactions have been catalyzed by using antibodies, and the advances in both catalytic efficiency and specificity have also been impressive (1). The research process will not only provide insight into the general potential of natural enzymes but may also afford catalysts to facilitate reactions not catalyzed by natural enzymes. One goal of studying catalytic antibodies is to generate tailor-made catalysts for applications in medicine (2, 3). Although many reviews of catalytic antibody technology have described the possibilities of future applications, there are no specific examples to date.

An example of a possible use with catalytic antibodies is related to the action of prodrugs. In the rational design of prodrugs, it is necessary to consider (*i*) what structural modifications of the parent molecule are necessary to reduce or eliminate the particular undesirable effects, and (*ii*) what enzymes are available *in vivo* to regenerate the parent molecule from the prodrug. However, the design of structurally related analogues of a parent molecule is limited by the enzyme's specificity, the type of reaction catalyzed, and the enzyme distribution and level (4). Using catalytic antibody technology for the design of prodrugs allows for effective structural modifications of the molecule in question. It is also a valuable aid in overcoming the problem of drug delivery by using bifunctional chimeric antibody technology (5–7), through which site-specific antibodies are combined with antibodies catalyzing reactions that cannot be accomplished by natural enzymes *in vivo*. We demonstrate here an example of prodrug activation via catalytic antibodies, which hydrolyze nonantibiotic chloramphenicol monoester derivatives to regenerate chloramphenicol **1** (as shown in Fig. 1).

Chloramphenicol **1** (8, 9) and its monoester derivatives were chosen as the parent drug and the prodrug, respectively, in the model system for this approach for three reasons. First, a relatively simple bacterial growth inhibition assay of chloramphenicol activity could be used. Second, the unique structure of these compounds, which incorporates a *p*-nitrophenyl group in the molecule, could be expected to be highly immunogenic (10). Third, the conformational change between the monoester as a substrate and chloramphenicol as a product could be expected to suppress product inhibition. Whereas chloramphenicol binds to the 50S subunit of bacterial ribosomes and inhibits the peptidyl transferase reaction (11), acetylation of the antibiotic prevents ribosome binding (12). Chloramphenicol possesses two hydroxyl groups that form a six-membered ring system via strong hydrogen bonding. This structure exists even in an aqueous solution (13, 14). Any chloramphenicol analogues that lack either this propanediol type substituent or the intramolecular hydrogen bonding are devoid of antibiotic activities, since binding to the bacterial ribosome is no longer possible (8). On the basis of NMR studies, Jardetzky (13) suggested that chloramphenicol bears a striking resemblance to a pyrimidine ribonucleotide in its size, orientation of individual moieties, and distribution of electronegative groups. Any alteration of the propanol moiety would destroy the similarity of the ribose ring. Therefore, we expected that antibodies generated against a haptenic phosphonate derivative, with a conformation that is not affected by intramolecular hydrogen bonding, should have a stronger binding affinity for the monoester than for chloramphenicol. As a result, the antibodies would be able to catalyze the hydrolysis with multiple turnovers to yield a sufficient amount of chloramphenicol for bacterial growth inhibition.

MATERIALS AND METHODS

Materials. Chloramphenicol—2,2-dichloro-*N*-[2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)ethyl]acetamide: $[\alpha]_D^{24}$ + 19° (c = 4.9; EtOH)—was purchased from Wako Pure Chemical (Osaka) and was used without further purification. Phosphonate **4a** was prepared from chloramphenicol **1** by treatment with methyl *N*-trifluoroacetyl-4-aminobenzylphosphonochloridate (15) in the presence of trimethylamine (CH_2Cl_2; 70% yield), followed by demethylation with NaI in methylethylketone under reflux conditions (68% yield). Phosphonate **5a** was prepared from 1-acetyl chloramphenicol by condensation with *N*-trifluoroacetyl-4-aminobenzylphosphonic acid using 1,3-dicyclohexylcarbodiimide as a coupling reagent in pyridine (24% yield), followed by gentle deprotection of the acetyl group in 0.1 M NaOH at −20°C for 5 min (78% yield). Each phosphonate was purified by reverse-phase (RP) high-

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Abbreviations: RP-HPLC, reverse-phase HPLC; KLH, keyhole limpet hemocyanin; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; TFA, trifluoroacetic acid.

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performance liquid chromatography (HPLC) on a C-18 column [elution; acetonitrile/H_2O/0.1% trifluoroacetic acid (TFA)]. Monoester **2** was prepared by reaction of chloramphenicol **1** with *N*-trifluoroacetyl-4-aminophenylacetic anhydride in the presence of triethylamine (78% yield) and was purified by silica gel column chromatography (AcOEt/*n*-hexane, 1:1; R_f, 0.35). For preparation of **3**, the primary alcohol at C-3 was protected with *t*-butyldimethylsilyl chloride in the first step (85% yield). Condensation of the silylether with *N*-trifluoroacetyl-4-aminophenylacetic acid, via bis(2-oxo-3-oxazolidinyl)phosphinic chloride, afforded the ester in CH_2Cl_2 (71% yield). Deprotection of the silylether under acidic conditions (AcOH/tetrahydrofuran/H_2O, 3:1:1) gave monoester **3** as the sole product (90% yield), which was purified by silica gel column chromatography (AcOEt/*n*-hexane, 1:1; R_f, 0.21). In contrast, deprotection with *n*-Bu_4NF resulted in spontaneous isomerization to give only **2** (62% yield). Monoesters **2** and **3** were confirmed to exist in a mixture of regioisomers (**2**/**3**, 4.6:1.0) at rapid equilibrium in the assay conditions (pH 8.0) by RP-HPLC assay on a C-18 column (elution, 50% acetonitrile/H_2O, 0.1% TFA; flow rate, 1.0 ml/min) of an aliquot of the assay solution (retention times: **2**, 15.0 min; **3**, 11.6 min). The structures of all new compounds were verified by spectroscopic (NMR, IR, and high-resolution mass) analyses. Both **4a** and **5a** reacted with glutaric anhydride to afford **4b** and **5b**, which were attached to carrier proteins—either keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA)—via the activated ester coupling method using *N*-hydroxysuccinimide.

Preparation and Purification of Monoclonal Antibodies. Four-week-old BALB/c mice were immunized with the KLH conjugates of **4b** and **5b** (i.p.; 50 μg per mouse in complete Freund's adjuvant), and a booster injection was administered once every 10 days for 1 month. Three days after the final injection (i.v.; 50 μg in phosphate-buffered saline), the spleen cells were fused with X63/Ag8653 myeloma cells according to standard protocols (16). Tissue culture supernatants in wells containing macroscopic colonies were assayed with the BSA conjugates by an ELISA. The class of initially produced antibodies specific for haptens was determined, and only colonies yielding IgG were subcloned. The antibodies produced by single clones were subsequently screened by a competitive inhibition enzyme immunoassay using 40 μM haptens **4a** and **5a** to identify and discard antibodies binding only to the linker portion (17). Finally, 23 and 12 monoclonal antibodies specific for haptens **4a** and **5a**, respectively, remained active. The corresponding hybridomas were propagated in mouse ascites, and the antibodies for the assay were purified via three steps. The antibodies from ascitic fluid were precipitated by dropwise addition of saturated ammonium sulfate at 4°C (pH 7.4) to achieve a final concentration of 45%, and the precipitates were dissolved and dialyzed against 10 mM phosphate buffer (pH 7.4). The concentrated antibodies were next purified by cation-exchange chromatography (Mono S, Pharmacia) and were eluted with a salt gradient (0–1.0 M NaCl/20 mM sodium acetate buffer, pH 5.0). The fractions (350–400 mM NaCl) with the antibodies were adjusted to pH 7.4 and were purified by affinity chromatography on a protein G-Sepharose column (Pharmacia) (15). The column was washed with 0.1 M sodium phosphate (pH 7.0) to remove nonadherent material. The antibodies were eluted with 0.05 M citric acid (pH 2.6) and fractions were immediately neutralized with 1.0 M sodium phosphate (pH 7.4). All fractions of the antibody were dialyzed into 20 mM sodium phosphate with 140 mM NaCl (pH 7.4) and were stored at 4°C. All preparations were typically 95% pure as judged by Coomassie blue staining after SDS/PAGE. Antibody concentration was determined by absorbance at 280 nm with extinction coefficient (ε, 0.1%) = 1.4 and a M_r of 150,000 for IgG. Immediately prior to antibody assay, the stock solutions were dialyzed into 50 mM Tris·HCl (pH 8.0).

Antibody Assays. The antibodies were then assayed for their ability to catalyze hydrolysis of monoesters. The reactions were initiated by adding 10 μl of various concentrations of a stock solution of substrate in dimethyl sulfoxide (DMSO) to 90 μl of antibody solution in Tris buffer. The reaction mixture consisted of 2 μM highly purified antibody and monoesters in 10% DMSO/50 mM Tris, pH 8.0, and was incubated at 30°C. Hydrolysis rates were measured by monitoring the production of chloramphenicol **1** via RP-HPLC on a C-18 column eluted with water/acetonitrile (50:50)/0.1% TFA at a flow rate of 1 ml/min, with UV detection at 278 nm. Initial rates were determined from the first 5–10% of the reaction for a given range of substrate concentration. The observed rates were corrected by using the background rate of hydrolysis in buffer. The background rate of the hydrolysis without antibodies was determined to be 7.11×10^{-5} min^{-1}. The kinetic constants were obtained from Lineweaver–Burk plots.

Inhibitory Assay of Bacterial Growth. The assay of growth inhibition of *Bacillus subtilis* ISW 1214 was performed by the paper-disc agar diffusion method (18). An overnight culture (100 μl) of *B. subtilis* ISW 1214 and melted top agarose (3.0 ml adjusted to pH 8.0 by addition of 1.0 M NaOH) were mixed and spread onto a 90-mm LB agar plate. After each sample of 10 μl was applied to the 6.0-mm paper discs resting on the plates, the plates were placed at 37°C for 18 h. The titer of the antibiotic, which is the concentration of chloramphenicol derived from the antibody catalysis, was calculated by using a standard curve of the diameters of the bacterial growth inhibitory zones. Inhibitory zones with diameters of 9.0, 12.0, 15.0, and 18.5 mm corresponded to chloramphenicol concentrations of 0.5, 1.0, 2.0, and 5.0 mM, respectively.

RESULTS AND DISCUSSION

As the monoester of chloramphenicol **1** exists in a mixture (**2** and **3**) of regioisomers at C-3 and C-1, as shown in Fig. 1 (19, 20), two haptens (**4** and **5**) were designed based on the concept of transition-state stabilization (Fig. 2) (21–23). Phosphonates (**4b** and **5b**) were synthesized and conjugated with KLH for use as antigens for production of antibodies. The immunizations and cell fusions were performed according to standard protocols (16). The resulting monoclonal antibodies were first screened for binding to the BSA conjugates and then for inhibition of binding of the BSA conjugates using haptens **4a** and **5a** by competitive inhibition enzyme immunoassay to identify and discard antibodies binding only to the linker portion (17). Immunization of haptens **4** and **5** produced 23 and 12 monoclonal antibodies, respectively, specific for the haptens. The corresponding hybridomas were propagated in mouse ascites, and antibodies for the assay were purified via salt precipitation, cation-exchange chromatography (Mono S; Pharmacia), and affinity chromatography (protein G).

A preliminary assay for the hydrolytic activity of purified antibodies was accomplished by HPLC detection of the chloramphenicol product. The reaction consisted of 2 μM highly purified antibody and 150 μM substrate in 10% DMSO/50 mM Tris, pH 8.0, and was incubated at 30°C. No hydrolytic activity was detected with any of the 23 antibodies generated against hapten **4**. On the other hand, 6 of the 12 antibodies generated against hapten **5** were found to catalyze the hydrolysis at a significant rate above the uncatalyzed background reaction. Interestingly, only haptenic phosphonate **5** efficiently induced catalytic antibodies, in spite of the fact that both haptens **4** and **5** possessed the same functional groups and molecular skeletons. Based on the concept that catalytic antibody activity can be regarded as a function of the interaction between the antibody combining site and the transition state in the hydrolysis reaction, this result may be related to recognition of the transition state in the antibodies

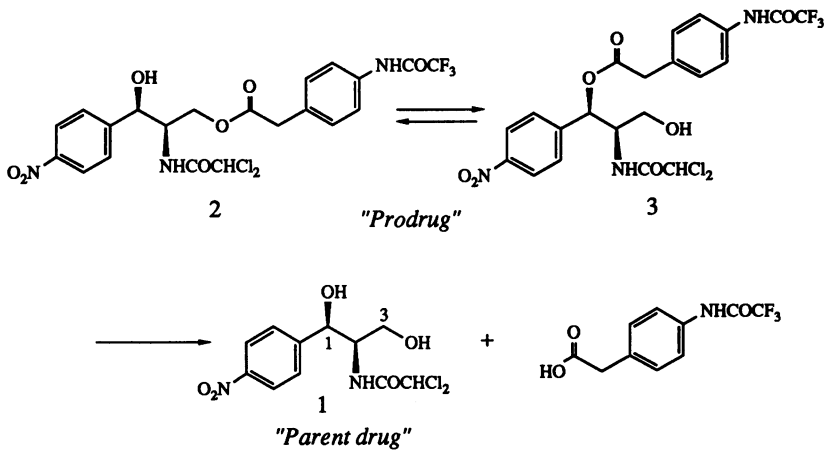

FIG. 1. Simplified model for prodrug activation via the antibody-catalyzed reaction.

elicited against **5**, which would be more strict than that against **4** due to the shorter distance between the two anchoring aryl groups. This information should be useful with regard to the future design of haptens for catalytic antibody generation.

One antibody, 6D9, was characterized in more detail. This antibody catalyzed prodrug hydrolysis in a manner consistent with Michaelis–Menten kinetics (24). A Lineweaver–Burk plot of the steady-state data afforded a k_{cat} of 0.133 min^{-1} and a K_m of 64 μM. Comparison of the k_{cat} value with the rate constant for the uncatalyzed reaction gives a 1.8×10^3-fold acceleration rate. The antibody-catalyzed reaction using 2 μM antibody 6D9 was completely inhibited by addition of 5 μM hapten **5a** but not by the same concentration of hapten **4a** (remaining activity, >90%), demonstrating that catalysis takes place against monoester **3** in the antibody combining site. A Dixon analysis with hapten **5a** afforded a K_i of 0.06 μM. In transition-state theory, the ratio k_{cat}/k_{uncat} may be predicted from K_m/K_i for a typical antibody-catalyzed reaction, provided that the hapten is an ideal transition-state analog. Kinetic values obtained for antibody 6D9 appear to be in close agreement with this hypothesis ($K_m/K_i = 1.1 \times 10^3$; $k_{cat}/k_{uncat} = 1.8 \times 10^3$). A hallmark of an efficient catalyst, like a natural enzyme, is rate enhancement and turnover—i.e., the ability of a single catalyst molecule to repetitively process substrate molecules. Several of the previously reported antibody-catalyzed reactions cannot be expected to display multiple turnovers due to their design even though they may display high stereoselectivity and/or enhanced initial rates (25–27). With catalytic antibodies, turnover is often found to be limited by severe product inhibition (ref. 28 and references therein). In our design for catalytic antibodies, the conformational change between a substrate and a product could be expected to suppress product inhibition. Therefore, inhibition of the antibody-catalyzed reaction by chloramphenicol **1** was examined by monitoring the production of *N*-trifluoroacetyl-4-aminophenylacetic acid by RP-HPLC, using C-18 column eluted with water/acetonitrile (45:55) at a flow rate of 1 ml/min with UV detection at 254 nm. The reaction was unaffected by addition of 10 mM chloramphenicol **1** (product) to the reaction mixture under experimental conditions identical to those described above (2 μM antibody), so the problem of product inhibition was circumvented in this case.

Chloramphenicol prodrug activation via antibody 6D9 was examined by a bacterial growth inhibition assay. For this assay, we chose *B. subtilis* as the bacterium, since it is known to be sensitive to the antibiotic and to secrete many kinds of hydrolytic enzymes into the medium (29). It is an essential requirement for prodrugs activated via antibodies to be resistant to the action of natural enzymes. Furthermore, this assay made it possible to test the turnover efficiency of antibody 6D9, since it should catalyze a reaction to generate enough chloramphenicol to inhibit bacterial growth.

Growth-inhibited zones were observed by using the paper-disc agar diffusion method (18), as shown in Fig. 3. The simultaneous application of a 100 mM solution of monoester **3** (10 μl) and a 20 μM solution of antibody 6D9 (10 μl) to the disc, and incubation for 18 h at 37°C, yielded a clear inhibitory zone of 12.0 mm, which corresponds in size to that of 1.0 mM chloramphenicol. On the other hand, no inhibitory zones due to the addition of either monoester **3** or antibody 6D9 alone were detected. This result reveals that only an antibody (6D9) hydrolytically activates monoester **3** to regenerate bioactive chloramphenicol **1** and that monoester **3** could not be degraded by bacterial hydrolytic enzymes secreted into the medium. It further emphasizes that this antibody (6D9) successfully achieves our initial goal for the use of antibodies

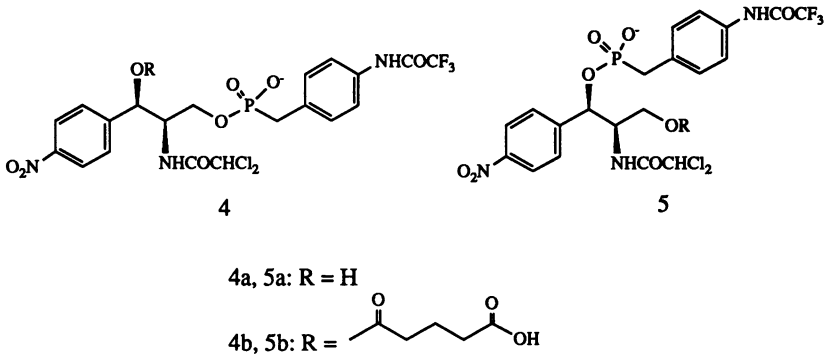

FIG. 2. Haptens **4a** and **5a** and the activated linkers **4b** and **5b**.

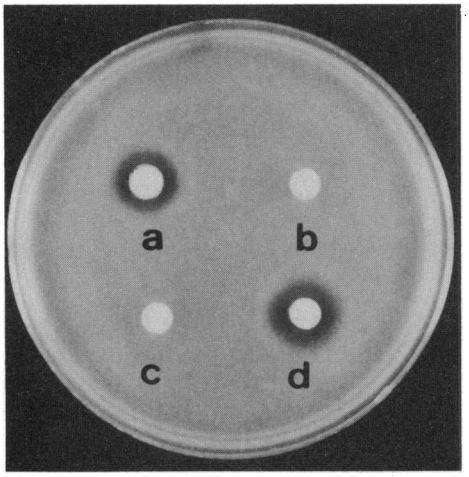

FIG. 3. Growth inhibition assay of *B. subtilis* ISW 1214 was performed by the paper-disc agar diffusion method (18): 100 mM substrate plus 20 μM antibody (a), 100 mM substrate alone (b), 20 μM antibody alone (c), 2.0 mM chloramphenicol (d). An overnight culture (100 μl) of *B. subtilis* ISW 1214 and melted top agarose (3.0 ml adjusted to pH 8.0 by addition of 1.0 M NaOH) were mixed and spread onto a 90-mm LB agar plate. After each sample of 10 μl was applied to the 6.0-mm paper discs resting on the plates, the plates were placed at 37°C for 18 h.

as catalysts for prodrug activation. Moreover, the size of the growth-inhibited zone, corresponding to that generated by 1.0 mM chloramphenicol, is due to the multiple turnover of this antibody-catalyzed reaction, which results from prevention of product inhibition. A growth inhibition assay using *Escherichia coli* strain XL1-Blue was also examined. Results the same as those described above were obtained, and an inhibitory zone of 11.5 mm was observed.

The approach in this study demonstrates usage of catalytic antibody technology in medicine and may be applicable to other important drugs with undesirable side effects. Given the enormous specificity inherent in antibody binding interactions and the fact that antibodies can be generated against virtually any substance, it may be possible to prepare a tailor-made catalyst to activate a specific prodrug. Clearly, more efficient catalysis—that is, enhancement of rate as well as turnover—will be required in further applications. Since recent advances in bacterial expression for antibodies, as well as combinatorial λ libraries in *E. coli*, make possible the generation of large libraries of antibody molecules (30–33), the combination of this antibody-catalyzed reaction methodology with bacterial expression in *E. coli* allows selection of antibodies with increased catalytic activity by simply screening large numbers of bacterial colonies. This methodology may be applied to screen for antibodies with increased catalytic efficiency.

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1. Lerner, R. A., Benkovic, S. J. & Schultz, P. G. (1991) *Science* **252,** 659–667.
2. Green, B. S. & Tawfik, D. S. (1989) *Trends Biotechnol.* **7,** 304–310.
3. Lerner, R. A. & Benkovic, S. J. (1988) *BioEssays* **7,** 107–112.
4. Banerjee, P. K. & Amidon, G. L. (1985) *Design of Prodrugs*, ed. Bundgaard, H. (Elsevier Science, Amsterdam), pp. 93–133.
5. Imura, Y., Stassen, J.-M., Kurakawa, T., Iwasa, S., Lijnen, H. R. & Collen, D. (1992) *Blood* **79,** 2322–2329.
6. Kurokawa, T., Iwasa, S., Kakinuma, A., Stassen, J.-M., Lijnen, H. R. & Collen, D. (1991) *Thromb. Haemostasis* **66,** 684–693.
7. Kurokawa, T., Iwasa, S. & Kakinuma, A. (1989) *Bio/Technology* **7,** 1163–1167.
8. Hahn, F. F. (1967) *Antibiotics*, eds. Gottlieb, D. & Shaw, P. D. (Springer, Berlin), Vol. 1, pp. 308–330.
9. Hahn, E. F., Hayes, J. E., Wisseman, C. L., Hopps, H. E. & Smadel, J. F. (1957) *Antibiot. Chemother.* **6,** 531–543.
10. Tijssen, P. (1987) in *Practice and Theory of Enzyme Immunology*, eds. Burdon, R. H. & van Knippenberg, P. H. (Elsevier, New York), pp. 39–41.
11. Traut, R. R. & Monro, R. E. (1964) *J. Mol. Biol.* **10,** 63–72.
12. Shaw, W. V. & Unowsky, J. (1968) *J. Bacteriol.* **95,** 1976–1978.
13. Jardetzky, O. (1963) *J. Biol. Chem.* **238,** 2498–2508.
14. Dunitz, J. D. (1952) *J. Am. Chem. Soc.* **74,** 995–999.
15. Janda, K. D., Benkovic, S. J. & Lerner, R. A. (1989) *Science* **244,** 437–440.
16. Kohler, G. & Milstein, C. (1975) *Nature (London)* **256,** 495–497.
17. Tawfik, D. S., Zemel, R. R., Yellin, R. A., Green, B. S. & Eshhar, Z. (1990) *Biochemistry* **29,** 9916–9921.
18. Herrmann, E. C., Gabliks, J., Engle, C. & Perlman, P. L. (1960) *Proc. Soc. Exp. Biol. Med.* **103,** 625–628.
19. Kleanthous, C. & Shaw, W. V. (1984) *Biochem. J.* **223,** 211–220.
20. Nakagawa, Y., Nitahara, Y. & Miyamura, S. (1979) *Antimicrob. Agents Chemother.* **16,** 719–723.
21. Pollack, S. J., Hsiun, P. & Schultz, P. G. (1989) *J. Am. Chem. Soc.* **111,** 5961–5962.
22. Tramontano, A., Ammann, A. A. & Lerner, R. A. (1988) *J. Am. Chem. Soc.* **110,** 2282–2286.
23. Tramontano, A., Janda, K. D. & Lerner, R. A. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 6736–6740.
24. Fersht, A. (1985) in *Enzyme Structure and Mechanism* (Freeman, New York), pp. 98–118.
25. Kohen, F., Kim, J. B., Lindner, H. R., Eshhar, Z. & Green, B. S. (1980) *FEBS Lett.* **111,** 427–431.
26. Balan, A., Doctor, B. P., Green, B. S., Torten, M. & Ziffer, H. (1988) *J. Chem. Soc. Chem. Commun.*, 106–108.
27. Pollack, S. J. & Schultz, P. G. (1989) *J. Am. Chem. Soc.* **111,** 1929–1931.
28. Tawfik, D. S., Green, B. S., Chap, R., Sela, M. & Eshhar, Z. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 373–377.
29. Priest, F. G. (1977) *Bacteriol. Rev.* **41,** 711–753.
30. Huse, W. D., Sastry, L., Iverson, S. A., Kang, A. S., Alting-Meers, M., Burton, D. R., Benkovic, S. J. & Lerner, R. A. (1989) *Science* **246,** 1275–1281.
31. Skerra, A. & Pluckthun, A. (1988) *Science* **240,** 1038–1041.
32. Better, M., Chang, C. P., Robinson, R. R. & Horwitz, A. H. (1988) *Science* **240,** 1041–1043.
33. Barbas, C. F., Kang, A. S., Lerner, R. A. & Benkovic, S. J. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 7978–7982.