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6. Cell sizes were obtained with the use of a Coulter particle counter and channelizer, which measures the volume (1 fl = 10^{-12} ml) displaced by a particle [see F. Vasi, M. Travisano, R. E. Lenski, *Am. Nat.* **144**, 432 (1994)].
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8. The progenitors were isogenic, except for an arabinose-use marker that enabled us to monitor possible cross-contamination and to score competitors in assays of relative fitness (*5*). Half of the populations were Ara$^-$ and the other half were Ara$^+$. The marker itself had no discernible effect on fitness in the environments used in this study.
9. M. Travisano, F. Vasi, R. E. Lenski, *Evolution*, in press.
10. Fitnesses were measured with fivefold replication for the 12 ancestral genotypes and with 10-fold replication for the 36 derived populations. These assays and all those reported later were run in sets of complete blocks.
11. The variance component due to measurement error is the mean square error. The variance component due to chance indicates variation among populations with the same ancestor that is above and beyond that due to measurement error. The variance component due to history reflects variation among ancestor groups that is above and beyond that due to chance divergence and measurement error. Approximate confidence limits can be placed on these variance components, and they are asymmetric (*34*). We report the square roots of the variance components for chance and history, so that magnitudes and units are directly comparable to the mean change due to adaptation. The t distribution with $n - 1$ df was used to obtain confidence limits for the change in grand mean, where $n = 12$ is the number of independent sets of populations.
12. $F = 24.3$, 11 and 44 df, $P < 0.001$.
13. $t = 5.06$, 11 df, $P < 0.001$.
14. $F = 7.31$, 11 and 24 df, $P < 0.001$.
15. $F = 1.20$, 24 and 314 df, $P = 0.240$.
16. Cell sizes were measured with fivefold replication for both the 12 ancestral genotypes and the 36 derived populations.
17. $r^2 = 0.042$, $n = 12$, $P = 0.525$.
18. $t = 1.71$, 11 df, $P = 0.114$ (*35*).
19. $F = 10.2$, 24 and 139 df, $P < 0.001$; one value was missing.
20. $F = 99.7$, 11 and 44 df, $P < 0.001$.
21. $F = 3.59$, 11 and 24 df, $P = 0.004$.
22. A. F. Bennett, R. E. Lenski, J. E. Mittler, *Evolution* **46**, 16 (1992). Again, half the populations were Ara$^-$ and the other half were Ara$^+$ (*8*).
23. Fitnesses were measured with sevenfold replication for both the 24 ancestral genotypes and the 24 derived populations.
24. $t = 6.10$, 23 df, $P < 0.001$.
25. $F = 1.13$, 23 and 138 df, $P = 0.325$.
26. $F = 2.09$, 23 and 138 df, $P = 0.005$. However, the confidence interval for the initial variance component includes the point estimate of the final variance component. Hence, we cannot confidently claim that the effect of chance-plus-history was amplified during 1000 generations in identical environments, nor can we exclude this possibility.
27. $t = 2.57$, 22 df, $P = 0.017$.
28. Although the confidence intervals for chance-plus-history and for adaptation overlap, neither includes the point estimate of the other. Hence, the difference is significant.
29. Cell sizes were measured with fourfold replication for both the 24 ancestral genotypes and the 24 derived populations.
30. $r^2 = 0.092$, $n = 24$, $P = 0.149$.
31. $F = 19.4$, 23 and 69 df, $P < 0.001$.
32. $F = 32.0$, 23 and 69 df, $P < 0.001$.
33. $t = 2.43$, 23 df, $P = 0.023$ (*35*).
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35. The effect due to adaptation is based on the change in grand mean and thus reflects the directional component of selection on cell size. Stabilizing selection on cell size might be suggested by a reduction in the combined effects of chance and history, but this was not observed.

36. R.E.L. dedicates this paper to the memory of his mother, Jean Lenski. We thank A. Cullum, L. Forney, R. Hudson, S. Kalisz, M. Rose, D. Straney, J. Tiedje, and S. Tonsor for discussions; A. Inouye and B. Korona for assistance in the lab; and two reviewers for comments. Supported by NSF grants DEB-9249916 (to R.E.L.) and IBN-9208662 (to A.F.B. and R.E.L.) and by the NSF Center for Microbial Ecology (BIR-9120006).

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A Proficient Enzyme

Anna Radzicka and Richard Wolfenden

Orotic acid is decarboxylated with a half-time ($t_{1/2}$) of 78 million years in neutral aqueous solution at room temperature, as indicated by reactions in quartz tubes at elevated temperatures. Spontaneous hydrolysis of phosphodiester bonds, such as those present in the backbone of DNA, proceeds even more slowly at high temperatures, but the heat of activation is less positive, so that dimethyl phosphate is hydrolyzed with a $t_{1/2}$ of 130,000 years in neutral solution at room temperature. These values extend the known range of spontaneous rate constants for reactions that are also susceptible to catalysis by enzymes to more than 14 orders of magnitude. Values of the second-order rate constant k_{cat}/K_m for the corresponding enzyme reactions are confined to a range of only 600-fold, in contrast. Orotidine 5′-phosphate decarboxylase, an extremely proficient enzyme, enhances the rate of reaction by a factor of 10^{17} and is estimated to bind the altered substrate in the transition state with a dissociation constant of less than 5×10^{-24} M.

Many enzymes act on substrates at rates that approach the rate of encounter between enzyme and substrate in solution (*1*). Biological reactions vary greatly, however, in their spontaneous rates and in the consequent burden that they place on an efficient catalyst. For example, carbon dioxide is hydrated nonenzymatically within several seconds in neutral aqueous solution at room temperature (*2*); peptide bonds undergo cis/trans isomerization within a few minutes (*3*); chorismic acid is converted to prephenic acid over a period of several hours (*4*); and the hydrolytic deamination of cytidine or adenosine approaches completion only after several centuries (*5*). Observations of reactions in sealed quartz tubes at elevated temperatures, described below, indicate that under physiological conditions in the absence of enzymes, the decarboxylation of orotic acid and the hydrolysis of phosphodiesters proceed on a time scale that dwarfs the life-span of living organisms. Enzymes catalyzing these reactions are correspondingly proficient at lowering the activation barrier for reaction and exhibit a high degree of discrimination between the substrates in the ground state and in the altered form that is present in the transition state, binding the latter species very tightly. Enzymes of this type are expected to be especially sensitive to inhibition by stable analogs of the altered substrate in the transition state.

1,3-Dimethylorotic acid has been shown to undergo decarboxylation slowly in sulfolane at 206°C (*6*). In order to compare this reaction with the enzymatic decarboxylation of orotidine 5′-phosphate (OMP) by

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the pure enzyme from yeast (*7*), we wished to determine the rate of decarboxylation of OMP in neutral aqueous solution in the absence of enzyme. To avoid complications arising from cleavage of the glycosidic bond, 1-methylorotic acid (*8*, *9*) was chosen for this study (Fig. 1A). In dilute aqueous buffers (0.01 to 1.0 M) with pH between 3 and 11, at temperatures between 140° and 200°C, 1-methylorotic acid was converted quantitatively to 1-methyluracil in sealed quartz tubes (*10*), as indicated by high-pressure liquid chromatography analysis, and decarboxylation proceeded to completion with good first-order kinetics. No significant catalysis was observed in the presence of increasing concentrations of potassium acetate, potassium phosphate, or methylamine-HCl buffers (*11*) near their pK_a values at concentrations up to 1 M (at 180°C and at a constant ionic strength of 2.0), nor did the rate of decarboxylation change significantly when the ionic strength was varied between 0.1 and 2.0 by addition of KCl to potassium phosphate buffer (0.05 M, pH 6.8). The rate of decarboxylation decreased sharply with decreasing temperature (Fig. 2), with an enthalpy of activation $\Delta H^{\ddagger} = +44.4$ kcal/mol and an entropy of activation $T\Delta S^{\ddagger} = +5.9$ kcal/mol. Extrapolation of the Arrhenius plot yielded a nonenzymatic rate constant (k_{non}) of 2.8 (± 2) $\times 10^{-16}$ s^{-1} at 25°C (*12*–*14*). Comparison of the present value of k_{non} in neutral aqueous solution at 25°C with the turnover number (39 s^{-1}) reported for yeast OMP decarboxylase at pH 6 and 25°C (*7*) indicates a 1.4×10^{17}–fold rate enhancement (Table 1).

Staphylococcal nuclease catalyzes the hydrolysis of phosphodiester linkages in DNA as well as RNA (*15*). Dimethyl phosphate, a simple phosphodiester, has been reported to decompose slowly in water, with P–O cleavage in neutral solution. From rates observed at lower pH values, at which dimethyl phosphate is more reactive, the rate constant for water attack on the

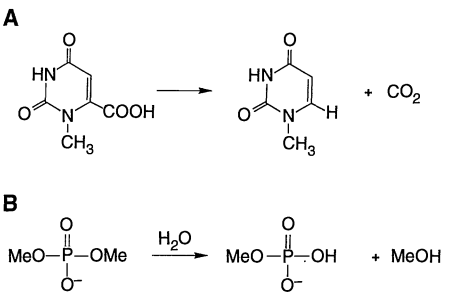

Fig. 1. (**A**) Decarboxylation of 1-methylorotic acid. (**B**) Hydrolysis of dimethyl phosphate.

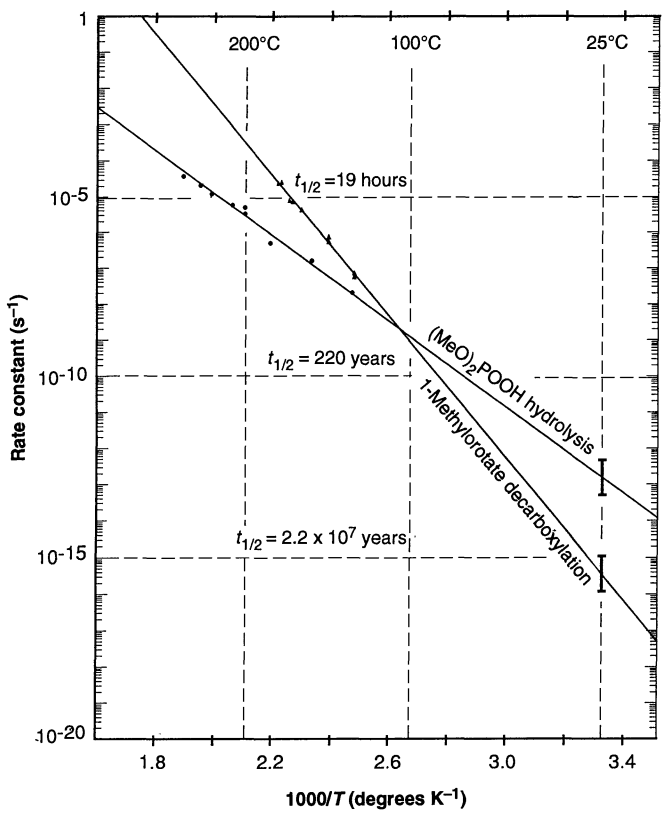

Fig. 2. Effect of temperature on rates of decarboxylation of 1-methylorotic acid (circles) and hydrolysis of dimethyl phosphate (triangles) in potassium phosphate buffer (0.1 M, pH 6.8). Extrapolated values at 25°C are shown with errors estimated from the standard errors of slopes obtained by linear regression of log k (sec^{-1}), plotted as a function of the reciprocal of absolute temperature.

Table 1. Enzymes listed in order of decreasing catalytic proficiency.*

Enzyme	Nonenzymatic $t_{1/2}$*	k_{non}* (s^{-1})	k_{cat}† (s^{-1})	k_{cat}/K_m† (s^{-1} M^{-1})	Rate enhancement (k_{cat}/k_{non})	Catalytic proficiency [(k_{cat}/K_m)/k_{non}] (M^{-1})
OMP decarboxylase	78,000,000 years	2.8×10^{-16}	39	5.6×10^{7}	1.4×10^{17}	2.0×10^{23}
Staphylococcal nuclease	130,000 years	1.7×10^{-13}	95	1.0×10^{7}	5.6×10^{14}	5.9×10^{19}
Adenosine deaminase	120 years	1.8×10^{-10}	370	1.4×10^{7}	2.1×10^{12}	7.8×10^{16}
AMP nucleosidase	69,000 years	1.0×10^{-11}	60	5.0×10^{5}	6.0×10^{12}	5.0×10^{16}
Cytidine deaminase	69 years	3.2×10^{-10}	299	2.9×10^{6}	1.2×10^{12}	9.1×10^{15}
Phosphotriesterase	2.9 years	7.5×10^{-9}	2100	4.0×10^{7}	2.8×10^{11}	5.3×10^{15}
Carboxypeptidase A	7.3 years	3.0×10^{-9}	578	6.6×10^{6}	1.9×10^{11}	2.2×10^{15}
Ketosteroid isomerase	7 weeks	1.7×10^{-7}	66000	3.0×10^{8}	3.9×10^{11}	1.8×10^{15}
Triosephosphate isomerase	1.9 days	4.3×10^{-6}	4300	2.4×10^{8}	1.0×10^{9}	5.6×10^{13}
Chorismate mutase	7.4 hours	2.6×10^{-5}	50	1.1×10^{6}	1.9×10^{6}	4.2×10^{10}
Carbonic anhydrase	5 s	1.3×10^{-1}	1×10^{6}	1.2×10^{8}	7.7×10^{6}	9.2×10^{8}
Cyclophilin, human	23 s	2.8×10^{-2}	13000	1.5×10^{7}	4.6×10^{5}	5.3×10^{8}

*Nonenzymatic reaction rate constants were obtained for OMP decarboxylase and staphylococcal nuclease from the present work, for adenosine and cytidine deaminases from (*5*), for AMP nucleosidase from (*25*), for phosphotriesterase from (*26*), for carboxypeptidase A from (*3*), for ketosteroid isomerase from (*27*), for triosephosphate isomerase from (*28*), for chorismate mutase from (*4*), for carbonic anhydrase from (*2*), and for cyclophilin from (*3*). †Enzyme reaction rate constants were obtained for OMP decarboxylase from (*7*), for staphylococcal nuclease from (*29*), for adenosine deaminase from (*30*), for AMP nucleosidase from (*31*), for phosphotriesterase from (*26*), for carboxypeptidase A from (*32*), for ketosteroid isomerase from (*33*), for triosephosphate isomerase from (*34*), for chorismate mutase from (*4*), for carbonic anhydrase from (*35*), and for cyclophilin from (*36*).

monoanion of dimethyl phosphate (Fig. 1B), the major species in solution at pH 7, was estimated as approximately 0.03 $year^{-1}$ at 100°C, but its dependence on temperature was not investigated (*16*). To determine its rate of hydrolysis at neutral pH and the heat of activation of this reaction, we examined the behavior of dimethyl phosphate in potassium phosphate buffer (0.1 M, pH 6.8) over the temperature range between 150° and 240°C. In sealed quartz tubes, hydrolysis of dimethyl phosphate proceeded directly to inorganic phosphate and methanol, with no accumulation of methyl phosphate, which is in accord with the more rapid hydrolysis of methyl phosphate than of dimethyl phosphate (*16*). Hydrolysis of the diester was monitored by observation of the integrated intensities of proton resonances of dimethyl phosphate in aliquots in which H_2O had been replaced by D_2O. The rate of hydrolysis varied with temperature (Fig. 2), with $\Delta H^{\ddagger} = +26.5$ kcal/mol and $T\Delta S^{\ddagger} = -8.3$ kcal/mol (*17*). Extrapolation of this Arrhenius plot to 25°C yielded $k_{non} = 1.7 (\pm 1) \times 10^{-13}\ s^{-1}$ at 25°C (*13*, *14*). Comparison with the turnover number (95 s^{-1}) reported for staphylococcal nuclease, at pH 7.4 and 23.5°C (*18*), indicates a 5.6×10^{14}–fold rate enhancement under these conditions (Table 1).

An enzyme's proficiency as a catalyst can be appreciated by dividing the second-order rate constant for its action on the substrate (k_{cat}/K_m) by the rate constant of the same reaction, measured in neutral aqueous solution in the absence of enzyme (k_{non}). Catalytic proficiency, defined in this way, measures an enzyme's ability to lower the activation barrier for the reaction of a substrate in solution. Catalytic proficiency, expressed in units of concentration, represents the lower limit of the enzyme's affinity for the altered substrate in the transition state. Thus, enzymes that are proficient by this criterion are expected to be unusually sensitive to a special class of reversible inhibitors, designed to resemble the altered substrate in the transition state (*19*). Table 1 and Fig. 3 compare the present results with rates reported earlier for other nonenzymatic and enzymatic reactions of single substrates that proceed by mechanisms that do not appear to involve double displacement (*20*). Within this group of enzymes, k_{non} is seen to vary by more than 14 orders of magnitude. Catalytic efficiency (k_{cat}/K_m) varies over a range of only 600. Catalytic proficiency [$(k_{cat}/K_m)/k_{non}$], indicated by the length of the vertical bars in Fig. 3, varies over a range of more than 10^{14}, and this variation can be seen to arise almost entirely from variations in k_{non}. For staphylococcal nuclease, the maximal dissociation constant of the altered substrate in the transition state is approximately 1.7×10^{-20} M. The affinity of yeast OMP decarboxylase for the altered substrate in the transition state for decarboxylation is even greater, with a dissociation constant of 5×10^{-24} M or less. OMP decarboxylase is a pure protein catalyst without metals or other cofactors, and other evidence indicates that this remarkable transition state affinity is achieved without formation of covalent bonds between the enzyme and the substrate (*21*–*24*).

In modern organisms, OMP decarboxylase catalyzes the last essential step in the biosynthesis of pyrimidines. This reaction is of such importance for nucleic acid synthesis that the sluggish progress of OMP decarboxylation in the absence of enzyme would seem to have raised a serious barrier to biochemical evolution. If an early organism had developed an ability to enhance the rate of the spontaneous reaction 1000-fold, for example, then the $t_{1/2}$ for production of uridine 5′-phosphate would merely have been reduced to 78,000 years. A partial answer to this paradox may lie in the acute temperature dependence of the rate of the nonenzymatic reaction (Fig. 2). The activation barrier would have been much less severe for a thermophilic organism living at temperatures near 100°C, a temperature at which the $t_{1/2}$ of the nonenzymatic reaction is only 10 years (Fig. 2). If it is assumed that it was possible to generate sufficient binding affinity under these conditions, even a primitive catalyst might have allowed this reaction to proceed at a useful rate.

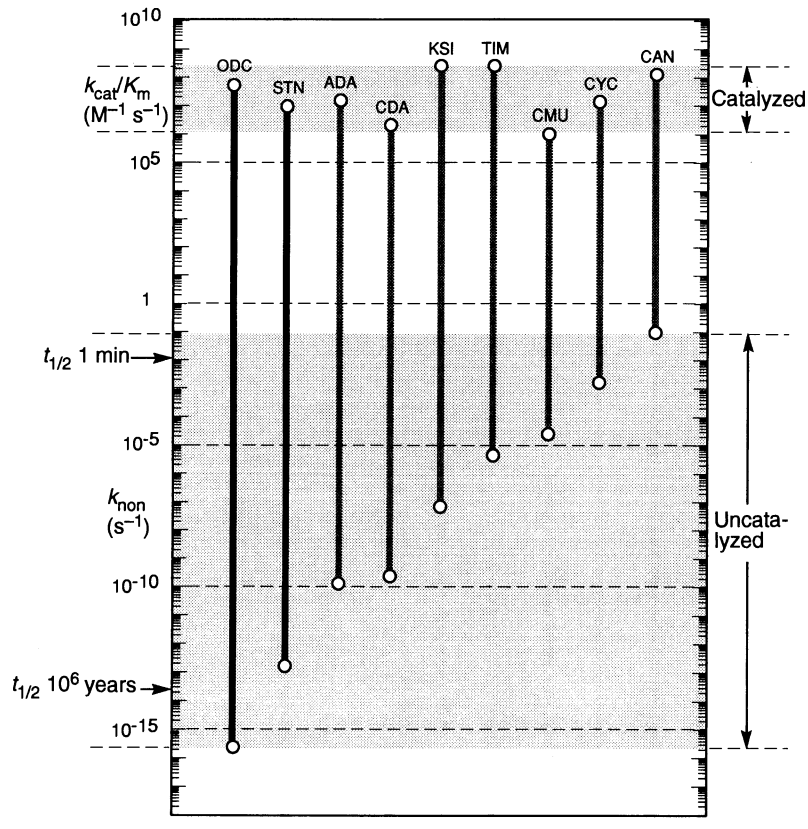

Fig. 3. Rate constants for nonenzymatic (k_{non}) reactions (bottom) in units of s^{-1} and for enzymatic (k_{cat}/K_m) reactions (top) in units of $M^{-1}\ s^{-1}$ in neutral solution at 25°C, from Table 1. Enzyme proficiency [$(k_{cat}/K_m)/k_{non}$] is indicated by the length of the vertical bar for OMP decarboxylase (ODC), staphylococcal nuclease (STN), calf intestinal adenosine deaminase (ADA), bacterial cytidine deaminase (CDA), ketosteroid isomerase (KSI), triosephosphate isomerase (TIM), chorismate mutase (CMU), cyclophilin (CYC), and carbonic anhydrase (CAN). The length of the vertical bars provides a measure of binding affinity in the transition state.

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9. 1-Methylorotic acid exhibits a pK_a value (0.34 ± 0.05) similar to that of orotidine (0.45 ± 0.05), determined spectrophotometrically on Hammett's H_o scale in HCl solutions, which suggests that electron densities at C-6 of the pyrimidine ring are similar for these molecules.

10. At 240°C, the highest temperature investigated, the pressure generated in these tubes amounted to only 33 atm, too low to produce a significant effect on the rates of known reactions in solution [for a survey, see W. J. le Noble, *Prog. Phys. Org. Chem.* **5**, 207 (1967)]. The sealed quartz tubes (1 cm in outside diameter, with a 2-mm wall) used in these experiments proved superior to sealed borosilicate tubes in strength and resistance to solvent attack. In addition, quartz tubes allowed the progress of decarboxylation of 1-methylorotate to be monitored without the opening of the tube, by observation of the ultraviolet absorption spectrum of the contents at intervals by means of a diode array spectrophotometer.
11. The absence of catalysis by methylamine is of special interest, in view of the fact that lysine is the single active site residue whose presence has been identified as essential for the catalytic activity of OMP decarboxylase [J. A. Smiley and M. E. Jones, *Biochemistry* **31**, 12162 (1992)].
12. This rate constant is smaller than a value extrapolated earlier (5×10^{-14} s^{-1}) for 1,3-dimethylorotic acid at 37°C from its behavior in sulfolane at 206° and 226°C (*6*). This difference in rates is probably due at least in part to differences in substrate structure.
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14. Linear Arrhenius plots are almost invariably observed for simple chemical reactions (*13*), but departures from linearity have been reported occasionally for more complex physiological processes in which the mechanism or the rate-determining step changes with temperature [for a review, see F. H. Johnson, H. Eyring, M. J. Polissar, *The Kinetic Basis of Molecular Biology* (Wiley, New York, 1954), pp. 187–285]. In those few cases in which nonlinearity has been observed, the slope at lower temperature is greater than the slope at higher temperature. If the same were true of the simple reactions described here (an unlikely possibility that cannot be tested at present), then the rate of reaction at 25°C would have been overestimated by the present extrapolations.
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17. At temperatures above 100°C, hydrolysis of dimethylphosphate proceeds even more slowly than does decarboxylation of 1-methylorotic acid (Fig. 2). The entropy of activation for decarboxylation is positive (+19.7 cal deg^{-1} mol^{-1}), whereas that of phosphodiester hydrolysis is negative (−27.8 cal deg^{-1} mol^{-1}), so that decarboxylation proceeds much more slowly than does phosphodiester hydrolysis in neutral solution at room temperature. Values of these entropies of activation are in the ranges typically observed for mono- and bimolecular reactions, respectively (*13*).
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19. If there is a difference in mechanism between the enzymatic reaction and the nonenzymatic reaction or if the rate of the enzyme reaction is limited by some event that does not involve bond making or breaking in substrates, such as product release, then these values represent upper limits of the dissociation of the enzyme-substrate complex in the transition state [for a review, see R. Wolfenden, *Annu. Rev. Biophys. Bioeng.* **5**, 271 (1976)].
20. Table 1 is restricted to enzyme reactions that do not appear to proceed by double displacement mechanisms [D. E. Koshland Jr., *Biol. Rev. Camb. Philos. Soc.* **28**, 416 (1953)], because comparisons of enzymatic and nonenzymatic reaction rates do not permit straightforward estimation of transition state affinity for reactions of this type (*19*). Large rate enhancements, although not as large as that for OMP decarboxylase, have also been reported for certain multisubstrate reactions, including those catalyzed by catechol *O*-methyltransferase [I. Mihel, J. O. Knipe, J. K. Coward, R. L. Schowen, *J. Am. Chem. Soc.* **101**, 4349 (1979)] and lactate dehydrogenase [J. W. Burgner II and W. J. Ray, *Biochemistry* **23**, 3636 (1984)].
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23. 5′-Phosphoribosylbarbituric acid, a competitive inhibitor with an inhibition constant value of ~10^{-11} M, is bound noncovalently (*21*) 4 to 5 orders of magnitude more tightly than is the substrate or product, probably because of its resemblance to a nitrogen ylide intermediate in OMP decarboxylation [H. L. Levine, R. S. Brody, F. H. Westheimer, *ibid.* **19**, 4993 (1980)].
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Structure-Based Design of Transcription Factors

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Computer modeling suggested that transcription factors with novel sequence specificities could be designed by combining known DNA binding domains. This structure-based strategy was tested by construction of a fusion protein, ZFHD1, that contained zinc fingers 1 and 2 from Zif268, a short polypeptide linker, and the homeodomain from Oct-1. The fusion protein bound optimally to a sequence containing adjacent homeodomain (TAATTA) and zinc finger (NGGGNG) subsites. When fused to an activation domain, ZFHD1 regulated promoter activity in vivo in a sequence-specific manner. Analysis of known protein-DNA complexes suggests that many other DNA binding proteins could be designed in a similar fashion.

Transcription factors are critical regulators of gene expression. The rational design of transcription factors with novel DNA binding specificities and regulatory activities will provide reagents for both biological research and gene therapy. The recent determination of a series of structures of protein-DNA complexes has facilitated a design strategy that uses computer modeling to predict how DNA binding domains could be combined to generate novel specificities. We explored this strategy by designing and testing a zinc finger–homeodomain fusion protein.

Computer modeling studies were used to visualize how zinc fingers might be fused to the Oct-1 homeodomain. The known crystal structures of the Zif268-DNA (*1*) and Oct-1–DNA (*2*) complexes were aligned by superimposition of the double helices in several different registers. Two arrangements were particularly interesting. In one alignment, the COOH-terminal end of zinc finger 2 was 8.8 Å away from the NH_2-terminal arm of the homeodomain (Fig. 1), which suggested that a short polypeptide linker could connect these domains. In this model, the fusion protein would bind a hybrid DNA site with the sequence 5′-AAATNNTGGGCG-3′. The Oct-1 homeodomain would recognize the AAAT subsite, zinc finger 2 would recognize the TGG subsite, and zinc finger 1 would recognize the GCG subsite. There was no possibility for steric interference between the zinc fingers and the homeodomain in this arrangement. Superimposition of the DNA duplexes in other registers generated a second plausible arrangement for a hybrid protein (*3*); however, this model was not as favorable because there was a risk of steric interference between the zinc fingers and the homeodomain.

The design strategy was tested by construction of a fusion protein, ZFHD1, that contained fingers 1 and 2 of Zif268, a gly-gly-arg-arg linker, and the Oct-1 homeodomain (Fig. 2A). A glutathione-S-transferase (GST) domain was added to facilitate expression and purification, and the DNA binding activity of this fusion protein was

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