mixtures consistent with the scheme: Tempo + $(1\text{-}CO_2)_2 \rightarrow 1^\bullet + CO_2 + 1\text{-}CO_2H$ + Tempo(-H).

Coupled reversed-phase HPLC-MS gave satisfactory resolution of the alkoxyamine components, viz. $1T_{exo}$, 2T, and $1T_{endo}$, in order of elution.[19] The components were identified from (a) HPLC retention times and mass spectra, (b) the "kinetic" relationship between their relative yields, i.e., $(1T_{exo} + 1T_{endo})/2T \alpha$[Tempo], and (c) co-injection of authentic 2T made by treatment of $(2\text{-}CO_2)_2$[15,17] with Tempo. The major unrearranged radical product was presumed to be the exo isomer because trapping at the exo face of $1^\bullet$ would clearly entail less nonbonded interaction (with the cyclopropyl CH_2 group on $1^\bullet$) than trapping at the endo face. This assignment is supported by the reversed-phase HPLC elution order ($1T_{exo}$ before $1T_{endo}$) since the polar N–O group would be more shielded from the solvent[19] in $1T_{endo}$ than in $1T_{exo}$.

Kinetic data, available as Supplementary Material, are consistent with relative rate constants of $k_r^1/k_T = 4.5 \pm 0.5$ M in chlorobenzene and 1.6 ± 0.2 M in 2,2,4-trimethylpentane, both at 37 °C. Radical clock[6,20] and laser flash photolysis measurements[14,21] of k_T indicate that this difference in k_r^1/k_T is due to the solvent dependence of the radical-trapping reaction, i.e., k_T^{alkyl} is about twice as large in alkanes as in aromatic solvents of similar molar mass.

We can assume that $1^\bullet$ will be trapped by Tempo with essentially the same rate constant as a primary alkyl radical,[6,13,14] viz. $k_T = 1.5 \times 10^9$ M^{-1} s^{-1} in 2,2,4-trimethylpentane at 37 °C,[22] and hence the above kinetic data give $k_r^1 = 2.4 \times 10^9$ s^{-1}.[23–25] The 20-fold increase in the rate of rearrangement of $1^\bullet$ relative to the rearrangement of cyclopropylmethyl[5] reflects the large amount of strain in the inner-ring bond[26] which is partly offset by an unfavorable orbital overlap for fission of this bond.[26] With $k_r^1 = 2.4 \times 10^9$ s^{-1} at 37 °C and an assumed preexponential of 10^{13} s^{-1}, we estimate the activation energy $E_A = 5.1$ kcal/mol and $k_r^1 = 1.7 \times 10^3$ s^{-1} at −160 °C, which is fully consistent with EPR data.[10–12]

The product data of Ortiz de Montellano and Stearns[3] suggest that *oxygen rebound* occurs about seven times faster than the rearrangement,[7] $1^\bullet \rightarrow 2^\bullet$, and, if we assume that binding of $1^\bullet$ to the enzyme has little effect on k_r^1,[27,28] we calculate that the *oxygen rebound* rate constant $k_{OH} \approx 7k_r^1 \approx 2 \times 10^{10}$ s^{-1}. Thus *rebound* occurs more rapidly than many typical conformational and configurational changes and also perhaps more rapidly than the gross molecular motion of many enzyme-bound substrates. The numerous reported cases of retention of configuration and stereochemistry and, in particular, the remarkable regioselectivity observed in the hydroxylation of bicyclo[2.1.0]pentane (which undergoes *abstraction and rebound* only at the endo face[3]) are therefore readily explained.

In conclusion, calibration of the radical clock, $1^\bullet \rightarrow 2^\bullet$, suggests that *oxygen rebound* in cytochrome P-450 occurs with an effective rate constant of 2×10^{10} s^{-1} for this substrate/P-450 combination; it would, of course, be interesting to see whether other suitable clock substrates afford similar *rebound* rate constants. Preliminary microsome experiments with 1,1,2,2-tetramethylcyclopropane, following the methodology of Ortiz de Montellano et al.,[3] have yielded mixtures of the corresponding unrearranged and rearranged alcohols. Thus, this substrate may provide a valuable alternative clock for timing the kinetics of *oxygen rebound*. Further work in this area is in progress and will be the subject of a full paper.

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Registry No. 1, 185-94-4; **1**$^\bullet$, 84592-00-7; Tempo, 2564-83-2; P-450, 9035-51-2.

Supplementary Material Available: Table I listing relative hydroxylamine yields and relative rate constants vs [Tempo] in chlorobenzene and in 2,2,4-trimethylpentane (1 page). Ordering information is given on any current masthead page.

(19) 200 × 2 mm ODS column eluted with 77% CH_3CN/H_2O (0.005 M NH_4OAc) at 1.0 mL/min.

(20) Bowry, V. W. Ph.D. Dissertation, Australian National University, 1988.

(21) Bowry, V. W., unpublished laser flash photolysis data.

(22) From $\log k_T^{nonyl} = 10.4 - 1.8/\theta$, where $\theta = 2.3RT$ kcal/mol.[14]

(23) This calculation implicitly assumes that the only reaction between $1^\bullet$ and Tempo is coupling to produce $1T_{exo}$ and $1T_{endo}$. To our knowledge, this is the fastest calibrated alkyl radical rearrangement which involves bond breaking and making. Only the inversions of cyclopropyl[24] and certain 1-substituted cyclopropyl radicals are faster (e.g., for 1-methylcyclopropyl, $k_i = 8.2 \times 10^{10}$ s^{-1} at 37 °C).[25]

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(27) Ring opening of **1** would require only a small amount of solvent/enzyme displacement since (a) the *internal* bond is broken and (b) an early transition state is suggested by the large heat of reaction. Furthermore, there is some evidence (e.g., the lack of substrate specificity in this form of P-450[3] and a substrate binding study for the functionally related LM_2 form[28]) that small hydrocarbon substrates are only loosely bound by hydrophobic forces in relatively capacious binding sites.

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Alkaline Phosphatase Catalyzes the Hydrolysis of Glucose 6-Phosphate via a Dissociative Mechanism

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E. coli alkaline phosphatase catalyzes a multistep reaction in which the hydrolysis of phosphate esters involves a phosphoryl enzyme intermediate. There appear to be two limiting mechanisms by which this intermediate could be formed. These are (1) an associative mechanism in the transition state of which there would be single bonds between the nonbridge oxygens and phosphorus and considerable bond order along the reaction coordinate and (2) a dissociative mechanism in the transition state of which the bond order between the phosphorus and the nonbridge oxygens would be above 1.4, and the bond order along the reaction coordinate would be very low. In the latter case the reaction could proceed via a free metaphosphate intermediate or, more likely, involve an expanded transition state. One way to distinguish between these mechanisms is to determine secondary ^{18}O isotope effects resulting from ^{18}O substitution in the nonbridge oxygens of a phosphate ester. An associative mechanism would show a sizeable normal isotope effect, since the bond order decreases to unity in the transition state, while a dissociative mechanism should show no isotope effect or a slightly inverse one, depending on the degree to which increased P–O bond order for the nonbridge oxygens balances loss of bending O–P–O vibrations in the transition state.[1]

To this end we have measured secondary ^{18}O kinetic isotope effects resulting from ^{18}O substitution in the three nonbridge oxygens on the alkaline phosphatase catalyzed hydrolysis of glucose 6-phosphate at 25 °C. Measurements were made at pH 8, which is on the pH optimum, and at pH 6, which is below the pK of the V/K profile where the chemistry is presumably rate limiting.[2]

^{18}O isotope effects were measured by the remote label method[3] in which 1-[^{13}C]glucose 6-phosphate-[$^{18}O_3$] was mixed with 1-[^{12}C]glucose 6-phosphate to give material with close to natural abundance of ^{13}C at C-1.[4] Since there is presumably no isotope

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Table I. Secondary ^{18}O Isotope Effects on the Alkaline Phosphatase Catalyzed Hydrolysis of Glucose 6-Phosphate[a]

fractnal reactn	pH	isotope effect calculated from: residual glucose 6-phosphate[b]	isotope effect calculated from: glucose product[c]
0.4626	8	0.99893	
		0.99805	
		0.99897	
0.3907		0.99789	
			0.99830
			0.99841
mean	8	0.9984 ± 0.0004	
0.5501	6	1.00317	
		1.00320	
0.5691		1.00410	
			1.00338
mean	6	1.0035 ± 0.0004	

[a] The isotope effects are for three ^{18}O in the nonbridge positions of the phosphate group but have not been corrected for the lack of isotopic purity of the starting materials.[5] [b] Calculated from the expression $\log(1-f)/\log[(1-f)(R_s/R_o)]$, where f is the fraction of reaction and R_s and R_o is the ^{13}C content of the CO_2 from C-1 in the residual glucose 6-phosphate and initial glucose 6-phosphate, respectively. [c] Calculated from the expression $\log(1-f)/\log(1-fR_p/R_o)$, where R_p is the ^{13}C content of the CO_2 from C-1 in the glucose product.

effect on the rate of hydrolysis caused by ^{13}C at C-1, any discrimination between the two species of glucose 6-phosphate results from the ^{18}O substitution. This discrimination is measured by separating residual glucose 6-phosphate and glucose product after approximately 50% hydrolysis and degrading them separately to ribulose 5-phosphate and CO_2 by the action of glucose 6-phosphate and 6-phosphogluconate dehydrogenases (the glucose was phosphorylated to glucose 6-phosphate by hexokinase and MgATP first). The hydrolysis experiments were carried out in 50 mM buffer containing 500 μM each $MgCl_2$ and $ZnCl_2$. The mass ratio in the isolated CO_2 was measured with an isotope ratio mass spectrometer.

The observed isotope effects were 0.9984 ± 0.0004 at pH 8 and 1.0035 ± 0.0004 at pH 6 (see Table I). When these values are corrected for the isotopic purity of the starting materials and the cube root taken,[5] isotope effects of 0.9994 (pH 8) and 1.0012 (pH 6) were obtained for single ^{18}O substitution. Since the dianion of glucose 6-phosphate is the true substrate for alkaline phosphatase, the value at pH 6 must be corrected for the proportion of glucose 6-phosphate which is a dianion. This is done by dividing 1.0012 by the appropriate portion of the ^{18}O equilibrium isotope effect on deprotonation (1.0030).[7] This gives a value for $^{18}(V/K)$ of 0.9982 at pH 6. The value at pH 8 remains 0.9994. Both of these values may contain a contribution from an equilibrium isotope effect on binding to the enzyme; however, the ratio between the two values (0.9988 ± 0.0006) should reflect the effect of making catalysis more rate limiting by changing the pH. The fact that this ratio is inverse strongly supports a dissociative mechanism for phosphoryl transfer catalyzed by alkaline phosphatase.[9]

This conclusion is consistent with the fact that phosphorylated thiols are excellent substrates for alkaline phosphatase.[10] Such phosphorylated thiols are poor substrates for kinases, however, suggesting that there may be different mechanisms for the two classes of enzymes. Experiments are currently under way to differentiate between these possibilities.

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(4) Having the mass ratio close to natural abundance (1.1%) minimizes errors from contaminating CO_2 during the analysis.

(5) The equation used to calculate $^{18}(V/K)$ (the ^{18}O isotope effect for single ^{18}O substitution) at each pH was[6] $1 + W/(^{13}(V/K) - [(1-B)Z/(BX)][W - {}^{13}(V/K)])^{1/3} - 1[1 + 1 - Y)/3]$ where W = observed isotope effect (0.9984 at pH 8 and 1.0035 at pH 6), X = fraction of ^{13}C in the 1-[^{13}C]glucose = 0.99, Y = fraction of $^{18}O_3$ in the [^{13}C,$^{18}O_3$]glucose 6-phosphate = 0.85, Z = fraction of ^{13}C in the 1-[^{12}C]glucose = 0.0001, B = fraction of [^{13}C,$^{18}O_3$]glucose 6-phosphate in the final mixture (natural abundance = 0.0111) = 0.01175, and $^{13}(V/K)$ = the ^{13}C isotope effect at C-1 from analogous alkaline phosphatase hydrolysis experiments with natural abundance glucose 6-phosphate. Because of the broad range of substrate specificity displayed by alkaline phosphatase, this number is assumed to be 1.00.

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A Semisynthetic Catalytic Antibody

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The immune system is unique in its ability to generate highly selective binding sites to a vast array of structurally diverse molecules.[1] It has been demonstrated that the high binding affinity and specificity of antibodies can be exploited in the design of selective catalysts for acyl transfer,[2] pericyclic,[3] photochemical,[4] and redox reactions.[5] In each case, monoclonal antibodies were generated against haptens designed to generate combining sites which acted either to stabilize a transition state, as an entropic trap, or contained a catalytic amino acid side chain. Alternatively, it should be possible to introduce a wide variety of natural or synthetic catalytic groups directly into antibody-combining sites either by generating a cofactor binding site or by selective derivatization of the antibody-combining site.[6] Recently, we reported a general method whereby cleavable affinity labels could be used to site-specifically incorporate a nucleophilic thiol into antibody-combining sites.[7] We now report the first example of a semisynthetic antibody that incorporates a catalytic group in the antibody-combining site. Selective derivatization of a thiol-containing antibody with imidazole affords a selective catalyst for ester hydrolysis. Derivatization of antibodies with other groups such as transition-metal complexes or cofactors should enable us to generate semisynthetic antibodies with a variety of novel functions.

The antibody MOPC 315 binds substituted 2,4-dinitrophenyl (DNP) ligands with association constants ranging from 5×10^4 to 1×10^6 M^{-1}.[8] Lys 52H, which is proximal to the combining site of MOPC 315, has been selectively modified with a unique thiol through the use of cleavable affinity labels (Figure 1).[7,9] Importantly, this thiol acts as a unique handle for the subsequent introduction of catalytic groups into the antibody-combining site.

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