Chemical Equilibrium at an Antibody Binding Site: Catalytic Efficiency Defined by a Haldane Relationship†

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ABSTRACT: This paper describes the first study of a reaction catalyzed in both forward and reverse directions by an antibody. The rates of reversible addition of sulfite to 6-hydroxy-3*H*-xanthen-3-one (**1**) and their pH dependence are influenced by a monoclonal antibody to a fluorescein hapten. The antibody is presumed to recognize the two substrates in a chemically productive prereaction complex with a geometry similar to that between the carboxyl group and the xanthenyl group in fluorescein. Equilibria are determined for the reactions in solution and at the antibody combining site from rate constants for the forward and reverse reactions. The solution equilibrium lies in favor of adduct with $K_A = 1.4 \times 10^7$ M^{-1} at pH 7.5. The corresponding equilibrium at the combining site is much smaller ($K_{Ab} = 8$), and this is attributed to the preferential binding of substrates **1** and sulfite. The magnitude of differential stabilization is determined from the equilibrium constants (2×10^6 M^{-1}). Observed rates at low molar standard state are compared to reveal the acceleration of the process at the antibody combining site versus the bimolecular solution reaction. In the associative direction, the rate factor of 1200 may largely be attributed to the entropy savings for the reaction on the catalyst. This analysis serves to illustrate the potential for catalysis by antibodies due from recognition of structural differences between substrates of different energy on a reaction coordinate and suggests a strategy for inducing antibody catalysts that does not presume knowledge of catalytic mechanisms or transition-state structure.

Complexation of small molecules to protein provides a driving force for many biological events, including signal transduction and enzyme catalysis. An understanding of protein function, and specifically enzyme catalysis, relies on the analysis of binding parameters that correlate with dynamic processes. Recently, antibodies to synthetic haptens have been used to study catalytic activity at their combining sites (Powell & Hansen, 1989; Tramontano & Schloeder, 1989). These experiments are grounded on the theory that enzymes provide a compensating binding energy to reduce the unfavorable activation energy of the reaction. Antibodies produced against analogues of reactive intermediates or transition states have been shown to express an enzyme-like function with varying efficiency of catalysis. Though the activity is presumed to derived from the net stabilization of the transition state relative to substrate, it is difficult to gauge that effect from binding parameters.

Clearly enzymes, and perhaps antibodies, can achieve remarkable rate accelerations by providing alternate mechanisms to a reaction. Catalytic groups at the active site may participate in the reaction, and this masks the contribution of molecular recognition to the process. Attempts to find correlation between hapten/substrate binding free energy and rate accelerations in reactions catalyzed by antibodies (Benkovic et al., 1988) are confounded by the uncertainties of mechanism and assumptions inherent in the equation of kinetic constants with binding constants. Reactions that should have a direct correlation between activation free energies in solution and in the specific binding environment at the active site are not readily analyzed in these terms. For example, two antibodies, produced independently, against the same transition-state analogue showed disparate kinetic activity in the catalysis of the Claisen rearrangement of chorismic acid (Jackson et al., 1988; Hilvert et al., 1988). In general, a quantitative understanding of the role of complexation is difficult to extract from kinetic parameters in enzymatic and antibody-catalyzed reactions. This is due to the problems of accurately measuring binding constants for relevant species and, incidentally, deducing the true affinity between protein and unstable intermediates or transition states.

Fluorescein presents a molecular architecture with discrete fragments oriented in a manner suitable for intramolecular addition or elimination of the carboxylate group from the central carbon of the xanthenone moiety. This reaction occurs readily in acidic solution. The reversible addition of sulfite ion to the central ring of 6-hydroxy-3*H*-xanthen-3-one (**1**) is a rapid and favorable process and constitutes a practical bimolecular counterpart to the fluorescein carboxylate–lactone equilibrium (Figure 1).

The binding of fluorescyl haptens by antibodies has been quite useful for investigation of immunological recognition by various techniques (Herron, 1984; Kranz et al., 1982). It is clear that fluorescein exists in the dianionic, open form in neutral or alkaline solution and at the antibody binding site. A crystal structure of the antibody 4-4-20 complexed to fluorescein has recently been reported (Herron et al., 1989). We have made an investigation of antibodies to the fluorescyl hapten as a model for bisubstrate binding proteins (Janjić et al., 1989). Dye molecules such as **1** are recognized in proximity to a specific carboxylate binding site. It was furthermore shown that the combining site can facilitate the irreversible reduction of a dye molecule by sulfite (Janjić & Tramontano, 1989). We therefore expected that such antibodies would stabilize the dissociated state of substrates undergoing the bimolecular addition–elimination reaction described in Figure 1. We show here than an anti-fluorescyl antibody can catalyze this reaction in both directions, and the position of equilibrium

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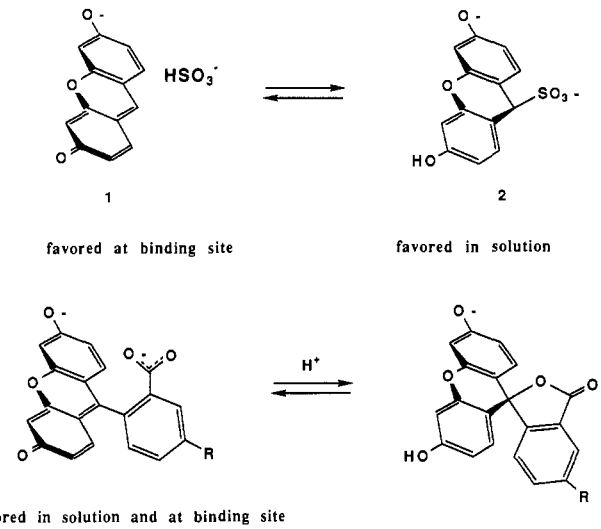

FIGURE 1: Structure of **1** and its sulfite adduct **2**, indicating the analogy of the prereaction complex with the form of fluorescein recognized immunologically.

at the combining site may be determined from the catalytic rates. Differential binding of substrates and products can be quantitated, and the magnitude of binding free energy that may be sequestered to reduce a chemical free energy change is inferred. A comparison of rates for the association of the two substrates at concentrations near K_m suggests the advantage due from intramolecularity in the reaction at the combining site. The model studied here is analogous to a form of one-way catalysis in enzymatic systems where binding is directed to unstable intermediate or products.

Experimental Procedures

Materials. 6-Hydroxyxanthen-3-one (**1**) was prepared as previously described (Janjić et al., 1989). Stock solutions of about 1 mM in dilute aqueous buffer (pH ~8) are stable for several days at room temperature. A concentrated aqueous stock solution of Na_2SO_3 (1 M) containing 0.2 mM EDTA stored in a tightly stoppered amber bottle was used to prepare more dilute stock solutions (1–10 mM).

Complex **2** was prepared by addition of aqueous sodium sulfite to **1** in aqueous medium at neutral or slightly acidic pH. The structure of **2** was determined from the ^1H NMR spectrum in D_2O (excess Na_2SO_3 added to **1**): δ 4.93 (s, 1 H), 6.27 (d, J = 2.1 Hz, 2 H), 6.35 (d of d, J = 2.1, 8.3 Hz, 2 H), 7.08 (d, J = 8.3 Hz, 2 H). The UV–vis spectrum of **2**, with an absorbance maximum at 495 nm ($\epsilon = 3.2 \times 10^3$ au/M at pH 6.5), is similar to that of $NaBH_4$-reduced **1**.

A solution of **2** used in kinetic runs was typically prepared by spectroscopic titration of **1** (25 μM) with sulfite (1 mM) in a cuvette at pH 6.5 (10 mM Bis-Tris). At this pH, the dissociation constant of **2** is small (1.4×10^{-9} M), and equal concentrations of **1** and sulfite (25 μM) form an equilibrium that is greater than 99% in favor of **2**.

Anti-fluorescyl monoclonal antibodies (MAb's) were prepared as described elsewhere (Tramontano & Schloeder, 1989). Those selected for tight binding of fluorescein (including 66D2) also effectively quench the ligand's fluorescence. This property is useful for accurate determination of antibody concentration as well as for evaluation of binding constants of both fluorescent and nonfluorescent ligands (Janjić et al., 1989; Kranz et al., 1982).

Instrumentation. UV–visible absorbance measurements were done on a Perkin-Elmer Model Lambda 3B or HP Model 8452A diode array spectrophotometer equipped with a Lambda RM6 water-circulating thermostat unit. Fluorescence readings were taken on a Perkin-Elmer Model LS-5 spectrofluorometer. NMR spectra were obtained on a Bruker 300-MHz spectrometer. All pH measurements were taken on a Fisher Model 825 MP pH meter in conjunction with a Ag/AgCl glass electrode.

Kinetic Measurements. Initial rates of formation or decomposition of adduct **2** can be conveniently measured spectrophotometrically at the absorbance maximum of **1** (λ = 488 nm). The molar absorbance varying between $\epsilon_{488} = 9.0 \times 10^4$ at pH >8.5 and 0.9×10^4 at pH <4 was used for calculation of kinetic constants. The apparent pK_a of **1** was determined by spectrophotometric titration at λ = 488 nm. Catalytic rates were obtained by correcting the total observed rate for the uncatalyzed rate. Under the experimental conditions, the uncatalyzed rate accounted for less than 10% of the total rate. All kinetic measurements were done at 25.0 ± 0.1 °C.

Results

The reaction between sulfite ion and xanthenone **1** gives an adduct assigned as **2** on the basis of its ^1H NMR and ultraviolet absorbance spectra. The reaction is conveniently monitored spectrophotometrically at the absorbance maximum of **1**. The formation of **2** was found to be first order in both sulfite and **1**, with a second-order rate constant of $k_1 = 1.33 \times 10^3$ M^{-1} s^{-1} at pH 7.5 and 25.0 °C (5 mM Tris, 80 μM EDTA). Xanthenone **1** is regenerated when a stock solution of adduct **2** (pH 6.5) is diluted into aqueous buffer. The reaction rate determined by initial rates appeared to be first order in adduct **2**, with k_{-1} of 9.4×10^{-5} s^{-1} under the conditions noted above.

The pH dependences of the logarithm of rate constants are linear for both k_1 (slope = −0.4, r = 0.997) and k_{-1} (slope = 1.3, r = 0.999) in a range between pH 7 and 9. The rapid decomposition of sulfite in base precludes accurate determinations at higher pH and vanishing concentrations of **1** at low pH limit the acidic range. The equilibrium constant, calculated as $K_A = k_1/k_{-1}$, is in favor of adduct **2** throughout the range studied and increases with decreasing pH. The pK_A of xanthenone **1**, determined by spectrophotometric titration, is near 6.3.

Previously we reported anti-fluorescyl antibody binding affinities for xanthene dyes determined by quenching of dye fluorescence upon binding. Displacement of the dye by small anionic ligands allows determination of affinity for the carboxylate site indirectly. Scatchard plots for binding of **1** in the absence or presence of a small anion allow estimates of both dissociation constants (Janjić et al., 1989).

Monoclonal antibodies against fluorescein isothiocyanate were obtained as described elsewhere (Tramontano & Schloeder, 1989). Ten out of 14 of these antibodies accelerated conjugate addition of sulfite to **1**. The activity of one of these (66D2) is described as follows. Saturation kinetics were observed with both sulfite and **1**, as evidenced from double-reciprocal plots (Figure 2a). The family of plots at various concentrations has a common intercept above the x-axis, suggesting that the two binding sites are weakly cooperative (α = 0.15) (Segel, 1975). The effect of antibody 66D2 on the rate of dissociation of **2** was investigated similarly, observing formation of **1** from the preformed adduct. The net antibody-catalyzed reaction shows saturation kinetics up to a concentration of about 3 μM in **2**. With increasing substrate concentrations the rate falls off rapidly (data not shown), presumably because of inhibition by products or uncomplexed **1** present in stock solutions of adduct **2**. Nevertheless, a useful double-reciprocal plot can be obtained (Figure 2b). Rate

Table I: Kinetic Parameters and Equilibrium Constants for Antibody-Catalyzed and Uncatalyzed Forward and Reverse Addition of Sulfite to Xanthenone (**1**)[a]

	k_1 (s^{-1})	k_{-1} (s^{-1})	$K_{A(Ab)}$[c]	K_{m1} (μM)	K_{mS} (μM)	K_{m2} (μM)
catalyzed (66D2)	0.88[b]	0.11[b]	8	0.33	10	0.77
uncatalyzed	1.33×10^3 M^{-1}	9.4×10^{-5}	1.4×10^7 M^{-1}			

[a] For conditions see legend to Figure 2. [b] Same as the saturation rate constant k_{cat}. [c] Equilibrium constant on mAb 66D2 is K_{Ab} in the text.

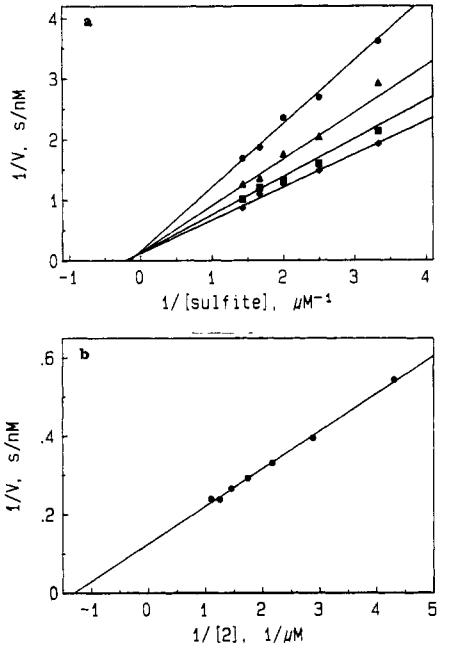

FIGURE 2: (a) Lineweaver–Burk plot for conjugate addition of sulfite to **1** catalyzed by antibody 66D2 at pH 7.5 and 25 °C in 5 mM Tris. The experiment was performed by varying the total concentration of sulfite ($[SO_3^{2-}] + [HSO_3^-]$) at 0.04 (●). 0.06 (▲), 0.08 (■), and 0.11 (◆) μM **1**. The concentration of immunoglobulin 66D2 in the kinetic assays was 22 nM. Linear regression values for the y-intercept and the slope of the double-reciprocal plots were used in a secondary plot to obtain the values of k_{cat}, K_m's, and α. The lines drawn have slope and intercept values that were calculated from the corresponding catalytic constants determined in secondary plots. (b) Lineweaver–Burk plot for the decomposition of adduct **2** catalyzed by 72 nM 66D2 under conditions described in (a).

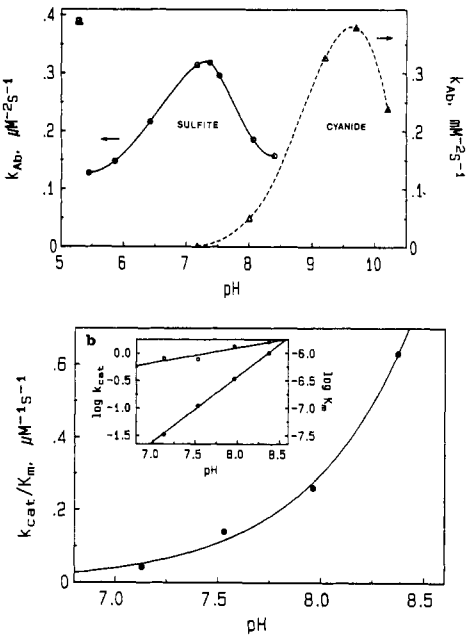

FIGURE 3: (a) pH dependence of k_{Ab} for 66D2-catalyzed addition of sulfite (solid curve, left y-axis) or cyanide (dashed curve, right y-axis) to **1**. The reaction was first order in 66D2; the observed rate could be described as $V_{obs} = \{k_{uncat} + k_{Ab}[Ab]\}[\mathbf{1}][\text{nucleophile}]$, where the concentration terms refer to the sum of all ionization states. At each pH, the catalytic constant, k_{Ab}, was calculated from the slope of the V_{obsd} vs [Ab] plots. Kinetics were done in 2 mM Bis-Tris (●), Tris (○, △), and borate (▲) buffers at 25.0 °C. (b) pH dependence of k_{cat}/K_m for 66D2-catalyzed dissociation of **2** in 5 mM Tris buffers containing 80 μm EDTA at 25.0 °C. The inset shows the relative change in k_{cat} (●) vs K_m (○) over the pH range.

constants and kinetic parameters under the conditions of these plots are summarized in Table I.

Both the forward and reverse reactions catalyzed by 66D2 are completely inhibited in the presence of fluorescein. An inhibition constant, K_i, of 10^{-10} M, obtained from a Henderson plot for 66D2-catalyzed formation of **2**, is in fair agreement with the dissociation constant K_d of 1.5×10^{-11} M, determined by fluorescence quenching (Janjić et al., 1989). The antibody-catalyzed addition reaction is also inhibited by a variety of small anions, as is the redox reaction catalyzed by this antibody (Janjić & Tramontano, 1989). Chloride concentration maintained at 5 mM throughout these experiments has a negligible inhibitory effect (K_i = 20 mM).

The position of equilibrium for the formation of sulfite adduct **2** at the combining site of 66D2 and in solution is calculated from the respective forward and reverse rate constants determined under the same conditions (Table I). These quantities are related through a thermodynamic cycle:

$$\begin{array}{ccc} \text{Ab} + \mathbf{1} + \text{S} & \overset{K_A}{\rightleftharpoons} & \text{Ab} + \mathbf{2} \\ K_1K_S \downarrow\uparrow & & \uparrow\downarrow K_2 \\ \text{Ab}\,[\mathbf{1} + \text{S}] & \overset{K_{Ab}}{\rightleftharpoons} & \text{Ab}\,[\mathbf{2}] \end{array}$$

K_1K_s is the product of association constants for binding of substrates **1** and sulfite, and K_2 is the association constant for binding of adduct **2** by the antibody. This constraint, defined also by the Haldane relationship, allows that $K_A/K_{Ab} = K_1K_S/K_2$, the difference in stabilization of reactants and products by complexation to the antibody, which in this system at pH 7.5 is 1.7×10^6 M^{-1}. The ratio can also be approximated from the respective K_m's and the interaction term α at pH 7.5 as $K_{m2}/(\alpha K_{m1}K_{mS}) = 1.6 \times 10^6$ M^{-1}, in good agreement with the value from the thermodynamic cycle.

The pH dependences for the catalyzed reaction rates were examined within the limits defined. The forward reaction exhibited a bell-shaped profile of k_{Ab} vs pH. The maximum rate was observed near pH 7.2 (Figure 3a). The reverse reaction showed an increase in rate at high pH with maximal rates outside the region where measurements are practical (Figure 3b). The binding differential is not significantly affected by pH in this range ($K_A/K_{Ab} = 9.4 \times 10^5$ M^{-1} at pH 8.4).

The effect of antibody on other nucleophilic reactions with xanthenone **1** was also examined. The reaction of **1** with aqueous cyanide was found to form an adduct, as judged by spectrophotometric changes similar to those caused by sulfite. Its rate is much slower than the corresponding sulfite reaction rate at the same pH. Antibody 66D2 catalyzed this reaction efficiently above pH 8.0. The catalyzed rate had a pH dependence similar to the 66D2-catalyzed sulfite addition, showing a bell-shaped profile (Figure 3a) with a maximum

rate near pH 9.7 (k_{Ab} = 1.76 × 10^{-2} M^{-1} s^{-1}). The shift in the maximum rate to a higher pH is in proportion to the pK_a difference between hydrogen cyanide (pK_a = 9.3) and bisulfite (pK_a = 6.9). Since the term k_{Ab} is independent of total **1**, sulfite, or cyanide concentration, the acidic leg of the bell-shaped pH dependence may reflect the ionization of **1**, HSO_3^-, or HCN, where the antibody binds the more ionized form as substrate. Whereas the pH dependence of the sulfite reaction may be due to ionization of HSO_3^- or **1**, the cyanide reaction clearly demonstrates the requirement for a negatively charged nuleophile. The decrease in activity at higher pH is presumably due to ionization of a group on the antibody.

The pH dependences of the solution reaction rates indicate the different catalytic requirements of the forward and reverse processes. In particular, the reverse process has a near first order (1.3) dependence on hydroxide, suggesting specific base catalysis. The antibody, with binding interaction directed to the fluorescyl dianion, may also employ general base catalysis in the reverse process. Proton transfer from a phenolic group of **2** to a basic group in the combining site would allow delocalization in an ion pair in the complex (containing **1** as product in this case). Such an ion pair (involving arginine and histidine) has been suggested to characterize the interaction of a high-affinity anti-fluorescyl antibody with its ligand (Bedzyk et al., 1990). The probable substrate in the antibody-catalyzed fragmentation is therefore the phenolate **2** (Figure 1). The greater dependence of k_{cat} than K_m on pH is in agreement with this interpretation (Figure 3b).

The implication of electrostatic interactions at the xanthenone site as well as at the sulfite site serves to emphasize the important role of such forces in both recognition and catalysis at enzyme active sites. While these observations help explain how the protein can favor the dissociation reaction through a specific pathway, the thermodynamic analysis and subsequent discussion eschew these considerations by regarding all bound species at equilibrium with substrates in solution.

Discussion

The complementarity of antibodies for antigens can be compared to the complementarity of enzymes for transition states (Pauling, 1948). Recent successes in demonstrating catalytic activity by antibodies validate the structure–function correlate as a basis for enzymatic catalysis (Kraut, 1988). A quantitative understanding of such effects on proteins might be expected to reveal the magnitude of catalytic power that is attributable to differential stabilization of species on a reaction coordinate. A related question that also seems approachable by study of catalysis by antibodies is whether entropic factors on proteins that restrict or channel molecular motion toward a reaction pathway are sufficient to account for the large rate accelerations of enzymes.

We have investigated a chemical equilibrium at the combining site of an anti-fluorescyl antibody through a thermodynamic cycle. The reaction is catalyzed by mAb 66D2 in both the forward and reverse directions. The binding differential of substrates and products may be determined from the equilibrium constants or from apparent binding constants and kinetic parameters. In solution the reaction of **1** and sulfite lies in favor of adduct **2** with K_A = 1.4 × 10^7 M^{-1}. At the antibody combining site this equilibrium is much less favorable, K_{Ab} = 8, at the same pH. If free energy changes are compared, without regard to activation energies and therefore considerations of mechanism, an estimate is obtained for the antibody's ability to discriminate between chemically interconvertible species. Substrates and products are stabilized by different extent. From the thermodynamic cycle that quantity $K_1K_S/K_2 = K_A/K_{Ab}$ is about 1.7 × 10^6 M^{-1}, equal to a free energy change of −8.5 kcal/mol in favor of dissociation. This value is in good agreement with that defined by the ratio of K_m constants.

Relation of Binding Differential to Catalysis. Regarded as a model for transition-state theory applied to enzymes (Lienhard, 1972; Schowen, 1978), the equilibrium between the sulfite–xanthenone complex and adduct **2** at the anti-fluorescyl antibody binding site illustrates the influence of protein–ligand interactions on reactivity. These antibodies recognize the open form of an analogous air of species related by the fluorescyl lactone–carboxylate interconversion. Relative to the solution reaction, the more favorable equilibrium in the dissociative direction is attributable to the better complementarity of antibody with the xanthenone–sulfite complex rather than with adduct **2**. The antibody provides binding energy to drive the dissociative reaction, and the magnitude of this contribution (1.7 × 10^6 M^{-1}) is a significant fraction of the intrinsic affinity of the antibody, which may be taken as its affinity for fluorescein (K_a = 6.7 × 10^{10} M^{-1}). In terms of molecular recognition, this differentiation may be due to differences in shape (ring puckering), ionization states, and charge localization between the complex of **1** and sulfite and adduct **2**.

Several examples of catalysis by monoclonal antibodies have been documented since reports published in 1986 (Tramontano et al., 1986; Pollack et al., 1986). Rate accelerations or efficiencies, determined with respect to the reaction occurring in the absence of the catalyst, can range over 6 orders of magnitude or more. While these examples clearly establish that antibodies can be quite versatile as catalysts, it becomes of interest to extract a quantitative understanding of the role of binding energy in catalysis. One approach takes the ratio of the Michaelis constant and hapten inhibition constant, K_m/K_i, as an index of differential binding of substrate and transition state (Benkovic et al., 1988). Since antibodies presumably bind optimally to their antigen among all possible ligands, hapten affinity or inhibition constants may overestimate actual transition-state affinity. This is recognized as a source of error when the observed rate differential k_{cat}/k_{uncat} is less than that predicted by K_m/K_i but does not explain the incongruence when $k_{cat}/k_{uncat} > K_m/K_i$. The assumption that the reactions occurring in solution and at the active site share the same mechanism presents a more serious difficulty. Certain reactions, such as a Claisen rearrangement, might be expected to derive catalytic advantage exclusively from nonbonded interactions between the combining site and the specific molecular shape of the transition state. However, it is difficult to explain rate accelerations as large as 10^4 from the apparent differential binding of 58 (Jackson et al., 1988). The analysis presented here, based on equilibrium values, is not dependent on assumptions of transition-state affinity and structure. The results imply that rate accelerations of more than 10^6 could result from interactions directed to features of an intermediate or transition state that distinguish it from substrate. In a related study an antibody was shown to discriminate between the reduced and oxidized form of a flavin by a factor of 10^5 (Shokat et al., 1988). However, flavin-binding antibodies do not recognize all substrates and products of a redox process at the combining site. The observed differential binding may therefore be due in part to interactions other than those resulting from chemical and structural changes in the reaction.

Entropy Trap Advantage. Because the associative reaction involves the combination of two molecules into one, an unfavorable entropy change may accompany this process. The

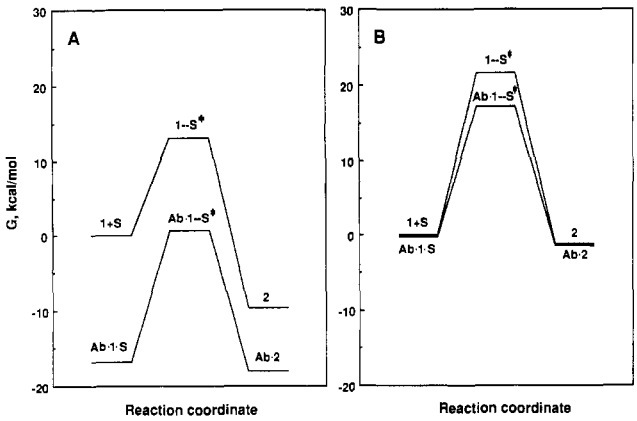

FIGURE 4: Free energy diagram for the reversible addition of sulfite to **1** in solution (upper profile) and on MAb 66D2 (lower profile) at a standard state of 1 M (panel A) and 5.7×10^{-7} M (panel B). Free energies were calculated from the rate and equilibrium constants given in Table I. The association steps are not explicitly shown.

reaction at the combining site should proceed without this unfavorable component. A comparison of rates on the antibody to those in solution, at 1 M standard state, does not reveal any advantage from intramolecularity (effective molarity = 6.8×10^{-4} M). However, this comparison is informative only if ΔH is similar for the two processes. Compensating enthalpy and entropy changes can occur in either the binding or chemical steps. Furthermore, comparison of bimolecular reaction rates in solution with those on an enzyme will depend on the choice of standard state (Schowen, 1978).

The antibody's ability as an entropy trap is more evident at low molar standard states where the affinity for substrates is weakened. At a concentration A where the positions of equilibrium in solution and at the combining site are equal ($A = K_{Ab}/K_A = 5.7 \times 10^{-7}$ M), the binding of substrates is negligible (K_m values for xanthenone dye **1** and its sulfite adduct are 3.3×10^{-7} M and 7.7×10^{-7} M, respectively). If the associative reaction may formally be divided into entropic and enthalpic steps (Jencks, 1975), the rate constants in the presence and absence of antibody will reveal a catalytic efficiency that approximates the entropy trap advantage for the antibody process (Figure 4). This rate factor $k_1^{cat}/(k_1 A)$ is found to be about 1200.[1] The argument assumes that binding interactions directed specifically to **1** and sulfite should capture any entropy advantage that may be due solely to the juxtapositioning of substrates in the productive complex and that little additional entropy is lost on going to the transition state.

Effective molarities as great as 10^8 M are attributed to the entropic advantage in intramolecular reactions (Page & Jencks, 1971). Enzymatic rate accelerations, expressed as effective molarity, support the premise that an entropic difference between free and bound substrates can provide a major driving force for a reaction. Antibodies have shown only modest effects as yet. Our results suggest that, without additional binding forces applied to strain or destabilize substrates toward the transition state, high effective molarities should not be expected. Alternate explanations for the small effect seen here may come from large solvation changes in the uncatalyzed reactions that do not participate in the reaction at the combining site or from the small entropy requirements of this particular reaction.

[1] This quantity is equivalent to k_{-1}^{cat}/k_{-1}, the concentration-independent factor for the dissociative rtes. This will be true when a standard state is chosen such that $\Delta\Delta G = \Delta G_A - \Delta G_{Ab} = 0$. Then the forward and reverse reactions must be accelerated by the same degree.

Conclusion

The reversibility of the reaction studied allows the determination of equilibrium constants on and off the antibody in a straightforward manner. Analysis based on the Haldane relationship provides the most direct measure yet of the differential binding energy available to antibodies for chemical catalysis. The quantity K_A/K_{Ab} may be compared with similar factors found for other enzymatic reactions. For example, the equilibrium between ADP + P_i and ATP on myosin versus in solution reveals a binding differential of nearly 10^5 (Bagshaw & Trentham, 1973; Lawson & Veech, 1979). In purine hydration by adenosine deaminase, an equilibrium in favor of the 1,6-dihydrate on the enzyme reveals a remarkable binding differential of at least 10^8 that the enzyme can use to stabilize its transition state (Jones et al., 1989). The extent to which the factor of 10^6 M^{-1} is representative of an antibody's catalytic potential or can be expressed in high effective molarity values may not be obvious from available information.

The comparison to equilibrium constants at enzyme active sites prompts further theoretical speculation. Whereas the solution equilibrium value is concentration dependent, the antibody succeeds to a large degree in balancing the energy of the two ground states for the reaction to an "internal" equilibrium constant near unity. Such a constraint is presumed to govern the functional evolution of enzymes (Albery & Knowles, 1976). While this observation should not be accorded any direct significance, the point may be made that the fluorescyl hapten elicits a monoclonal antibody that selectively stabilizes the high-energy complex of xanthenone–sulfite (at 1 M standard state). In this respect we may conclude that the use of a "reactive intermediate analogue" can suffice to produce an efficient abzyme even though knowledge of a mechanism is limited.

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Monofunctional Chorismate Mutase from *Bacillus subtilis*: Kinetic and ^{13}C NMR Studies on the Interactions of the Enzyme with Its Ligands†

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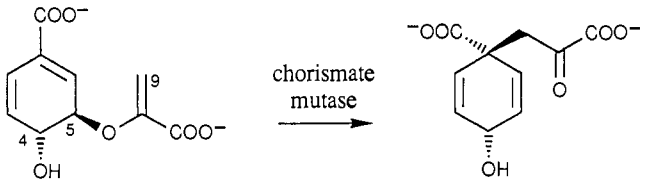
ABSTRACT: The interaction of the monofunctional chorismate mutase from *Bacillus subtilis* with chorismate and prephenate has been studied kinetically and by NMR spectroscopy with ^{13}C specifically labeled substrates. Prephenate dominates the population of enzyme-bound species, and the "off" rate constant ($\sim$60 s^{-1}) obtained from line-broadening experiments is close to the value of k_{cat} for chorismate (50 s^{-1}) determined kinetically. The calculated "on" rate constant for prephenate (8×10^5 M^{-1} s^{-1}) is similar to the value of k_{cat}/K_m for chorismate (5×10^5 M^{-1} s^{-1}). The kinetic parameters of the *Bacillus* mutase are remarkably insensitive to pH over a wide range and display no solvent isotope effect. These results suggest that the enzyme-catalyzed reaction may be encounter controlled (slowed from the diffusion limit by some feature of the enzyme's active site) and that k_{cat} for chorismate is determined by the product off rate. There is now no evidence to suggest that the skeletal rearrangement on the enzyme surface occurs by a pathway other than a pericyclic process.

The intramolecular rearrangement of chorismate (**1**) to prephenate (**2**) is catalyzed by chorismate mutase, the enzyme that lies at the branch point in the shikimate pathway that leads to the biosynthesis of the three aromatic amino acids, phenylalanine, tyrosine, and tryptophan. The reaction is formally a Claisen rearrangement and constitutes the only example in primary metabolism of what appears to be a pericyclic process. The uncatalyzed rearrangement occurs readily in aqueous solution and seems to be a concerted, asynchronous reaction (Addadi et al., 1983). In contrast, little is known about the mechanism of the enzyme-catalyzed transformation, or about the origins of the 10^6-fold rate acceleration over the uncatalyzed process (Andrews et al., 1973; Görisch, 1978).



chorismate — prephenate

1 **2**

Most mechanistic investigations of chorismate mutase have focused on the bifunctional "T" protein from *Escherichia coli*, in which the chorismate mutase activity is linked to that of prephenate dehydrogenase (Koch et al., 1971). This bifunctional enzyme is a homodimer of subunit M_r = 42 000, in which the mutase activity apparently derives from the N-terminal third of the protein (Hudson & Davidson, 1984; Maruya et al., 1987). The stereochemical course of the enzyme-catalyzed reaction has been determined and involves a transition state of chair-like geometry, as has also been found for the uncatalyzed transformation (Sogo et al., 1984; Copley et al., 1985). In contrast to the uncatalyzed reaction, the enzymic rearrangement is insensitive to tritium substitution either at C-5 or at C-9 of chorismate (**1**), suggesting that some transition state *before* that involving the chemical transformation limits the reaction rate when the substrate concentration is low (i.e., under V_{max}/K_m conditions; Addadi et al., 1983). These results do not illuminate the nature of the chemical rearrangement at the enzyme's active site, nor do they identify the rate-limiting transition state. The observation of a solvent deuterium kinetic isotope effect on V_{max} and of a small inverse secondary tritium isotope effect at C-4 of chorismate has been used to argue for a rate-limiting heterolysis on the enzyme surface (Guilford et al., 1987). This stepwise pathway was formulated as involving the attack of an enzymic nucleophile at C-5 of the substrate chorismate, thus generating a covalent enzyme-bound intermediate that subsequently collapses to prephenate in an S_N2'-like process. No observations bear directly on the nature of the pathway of the enzymic rearrangement, however, and this question must be regarded as unresolved. Much of the uncertainty derives from our ignorance both of the identity of the rate-limiting transition state(s) under saturating and subsaturating conditions and of the nature of the substrates when enzyme bound. The relatively large size of the bifunctional T protein, as well as the presence on it of multiple binding sites for substrates,

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