# Enzymatic Hydration of an Olefin: The Burden Borne by Fumarase

Stephen L. Bearne* and Richard Wolfenden*

*Department of Biochemistry and Biophysics*
*University of North Carolina*
*Chapel Hill, North Carolina 27599-7260*

*Received June 21, 1995*

The rate enhancement produced by any catalyst depends on its ability to discriminate between the substrate in the ground state and the altered substrate in the transition state, binding the latter species with greater affinity and reducing the activation barrier of the reaction.[1,2] An upper limit can be estimated for the dissociation constant of an enzyme's complex with the altered substrate in the transition state ($K_{tx}$) by dividing the rate constant for the reaction in the absence of enzyme ($k_{non}$) by the catalytic efficiency of the enzyme ($k_{cat}/K_m$).[2] We have used this approach to estimate the magnitude of the transition state stabilization achieved by fumarase and have observed that binding of the altered substrate in the transition state by the enzyme is associated with a substantial gain of entropy.

Fumarase catalyzes the reversible hydration of fumarate to (*S*)-malate (Scheme 1) with high efficiency under physiological conditions, acting on its substrate with an apparent second-order rate constant ($k_{cat}/K_m = 2.4 \times 10^8\ M^{-1}\ s^{-1}$)[3-5] that approaches the rate of encounter between enzyme and substrate in solution.[6] The corresponding nonenzymatic reaction, involving hydration of a carbon−carbon double bond, proceeds so slowly that, even in the presence of hydronium ion[7] or hydroxide ion[8] catalysis, it has been observed only in superheated aqueous solutions.[9,10]

To determine the rate of fumarate hydration in the absence of enzyme, we have measured the nonenzymatic rate of fumarate hydration in neutral solution at elevated temperatures,[11-13] obtaining by extrapolation a rate constant of $2.5 \times 10^{-13}\ s^{-1}$ for the nonenzymatic reaction at 37 °C (Figure 1). Comparison with the turnover number ($k_{cat} = 880\ s^{-1}$) reported for fumarase[3] indicates a rate enhancement of ($3.5 \times 10^{15}$)-fold at pH 6.8 and 37 °C. Comparison with the reported second-order rate constant indicates that the upper limit of the dissociation constant of the enzyme−substrate complex in the transition state ($K_{tx}$) is approximately $1.0 \times 10^{-21}$ M.[14]

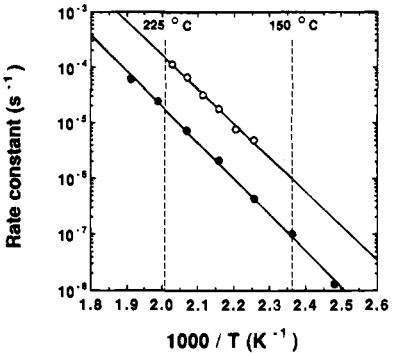
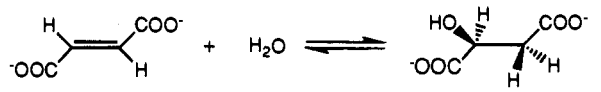



**Figure 1.** Effect of temperature on the rates of hydration of fumaric acid at pH 4.47 (○) and pH 6.82 (●). Curves shown are linear regression lines of $k$ ($s^{-1}$) plotted as a logarithmic function of the reciprocal of absolute temperature. The enthalpy and entropy of activation at pH 6.82 are 28.9 (±0.6) kcal $mol^{-1}$ and −23.0 (±0.7) cal $mol^{-1}\ K^{-1}$, respectively. At pH 4.47, the enthalpy and entropy of activation are 28 (±1) kcal $mol^{-1}$ and −21 (±1) cal $mol^{-1}\ K^{-1}$, respectively.

**Scheme 1**




Accordingly, fumarase stabilizes the altered substrate in the transition state ($\Delta G_{tx}$) by at least 30 kcal/mol (Figure 2). By the principle of microscopic reversibility, the transition state for the reverse reaction, involving carbon−hydrogen bond scission, is also stabilized by 30 kcal/mol in free energy.[21] This value is substantially greater than that observed for triosephos-

* Corresponding authors.

(1) Polanyi, M. *Z. Elektrochem.* **1921**, *27*, 143−150.

(2) For a review, see: Wolfenden, R. In *Transition States in Biological Processes*; Gandour, R. D., Schowen, R. L., Eds.; Plenum Press: New York, 1978; pp 555−578.

(3) Brant, D. A.; Barnett, L. B.; Alberty, R. A. *J. Am. Chem. Soc.* **1963**, *85*, 2204−2209. (Values quoted have been adjusted to pH 6.8 and also to reflect the homotetrameric structure of the enzyme.[4])

(4) Teipel, J. W.; Hill, R. L. *J. Biol. Chem.* **1968**, *243*, 5679−5683.

(5) Alberty, R. A.; Peirce, W. H. *J. Am. Chem. Soc.* **1957**, *79*, 1526−1530.

(6) Alberty, R. A. In *The Enzymes*, *5*, 2nd ed.; Boyer, P. D., Lardy, H., Myrbäck, K., Eds.; Academic Press: New York, 1961; pp 531−544.

(7) Rozelle, L. T.; Alberty, R. A. *J. Phys. Chem.* **1957**, *61*, 1637−1640.

(8) Erickson, L. E.; Alberty, R. A. *J. Phys. Chem.* **1959**, *63*, 705−709.

(9) (a) Bender, M. L.; Connors, K. A. *J. Am. Chem. Soc.* **1961**, *83*, 4099−4100. (b) Bender, M. L.; Connors, K. A. *J. Am. Chem. Soc.* **1962**, *84*, 1980−1986.

(10) Weiss, J. M.; Downs, C. R. *J. Am. Chem. Soc.* **1922**, *44*, 1118−1125.

(11) Fumaric acid solutions (0.5 M) were buffered at pH 4.47 and 6.82 by fumarate and 0.1 M potassium phosphate, respectively. Ionic strength was maintained at 1.7 with KCl. Reactions were conducted in sealed quartz tubes at temperatures ranging between 130 and 250 °C in a Thermolyne 47900 furnace. The hydration of fumaric acid was monitored by observation of the integrated signal intensities of proton resonances of the malic and fumaric acids. At pH 4.47, the reaction proceeded cleanly, and first-order rate constants for the conversion of fumarate to malate were obtained by following the reaction to equilibrium.[12] This was not possible at pH 6.82 due to the rapid formation of numerous side products as evidenced in the proton NMR spectrum. However, no significant production of side products occurred during the first 10% of the hydration reaction, and first-order rate constants were obtained from measurements of initial rates of reaction.[12]

(12) Jencks, W. P. *Catalysis in Chemistry and Enzymology*; Dover Publications, Inc.: New York, 1987; pp 555−590.

(13) The rate constants for fumarate hydration at 170 °C in the absence and presence of 0.1 M phosphate buffer, pH 6.82, are 3.9 (±0.1) × $10^{-7}$ $s^{-1}$ and 4.3 (±0.1) × $10^{-7}$ $s^{-1}$, respectively, indicating that the phosphate buffer has little effect on the spontaneous rate of hydration. Because the reaction rate depends on the pH,[9] the temperature dependence of the first-order rate constants was also determined at pH 4.47. The values of the free energy changes required to reach the transition state at pH 4.47 and 6.82 are 35 (±6) kcal/mol and 36 (±1) kcal/mol, respectively, at 37 °C. It is unlikely that any decrease in pH due to changes in the ionization of the phosphate buffer as the temperature is increased would significantly alter the observed rates of hydration at pH 6.82.

(14) If there is a difference in mechanism between the nonenzymatic and enzymatic reactions, or if the rate of the enzyme reaction is limited by some event that does not involve bond making or breaking in substrates, then the value of $K_{tx}$ represents an upper limit for the dissociation of the enzyme−substrate complex in the transition state.[2] The rate-limiting step of the fumarase-catalyzed reaction, believed to proceed by a carbanionic mechanism,[15,16] may involve product release[16,17] or proton exchange accompanied by a slow conformational isomerization.[18,19] Because the C−H bond of malate is difficult to break, as underscored by the present findings, it seems reasonable to suppose that the C−H-cleaving step may be only a little faster than the rate-limiting conformational change.[20]

(15) Porter, D. J. T.; Bright, H. J. *J. Biol. Chem.* **1980**, *255*, 4772−4780.

(16) Blanchard, J. S.; Cleland, W. W. *Biochemistry* **1980**, *19*, 4506−4513.

(17) Sweet, W. L.; Blanchard, J. S. *Arch. Biochem. Biophys.* **1990**, *277*, 196−202.

(18) Rose, I. A.; Warms, J. V. B.; Kuo, D. J. *Biochemistry* **1992**, *31*, 9993−9999.

(19) Rose, I. A.; Warms, J. V. B.; Yuan, R. G. *Biochemistry* **1993**, *32*, 8504−8511.

(20) Albery, W. J.; Knowles, J. R. *Biochemistry* **1976**, *15*, 5631−5640.

(21) The equilibrium constant for the reversible formation of malate from fumarate is 3.3 at pH 6.8 and 37 °C,[22] and therefore $k_{non}$ for malate dehydration is $7.6 \times 10^{-14}\ s^{-1}$. The corresponding value of $k_{cat}/K_m$ in this direction is $7.1 \times 10^7\ M^{-1}\ s^{-1}$.[3]

(22) Bock, R. M.; Alberty, R. A. *J. Am. Chem. Soc.* **1953**, *75*, 1921−1925.

0002-7863/95/1517-9588$09.00/0 

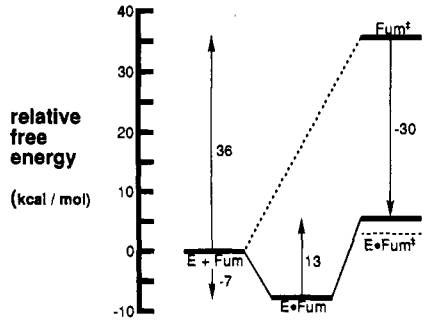



**Figure 2.** Free energy profile (pH 6.8, 37 °C) for the hydration of fumarate (Fum) in the absence of enzyme (dashed line) and in the presence of fumarase (solid line, from ref 3). The enzyme−substrate complex in the transition state (E·Fum‡) is shown at an arbitrary free energy close to the free energy of the transition state of the rate-limiting step of the enzyme-catalyzed reaction.[14]

phate isomerase ($\Delta G_{tx}$ = −19 kcal/mol)[23,24] and ketosteroid isomerase ($\Delta G_{tx}$ = −21 kcal/mol),[25,26] which also catalyze C−H bond cleavage. The only enzyme for which a comparable degree of transition state stabilization has been observed is OMP decarboxylase ($\Delta G_{tx}$ = −32 kcal/mol), which catalyzes the cleavage of a carbon−carbon bond.[27]

An apparent enthalpy ($\Delta H_{tx}$ = −24 kcal/mol) and entropy ($\Delta S_{tx}$ = 19 cal mol$^{-1}$ K$^{-1}$) for formation of the enzyme−substrate complex in the transition state[28] are obtained by comparing the variation of $k_{non}$ with temperature observed in the present experiments with the variation of $k_{cat}/K_m$ with temperature reported by Brant *et al.*[3,28] Of particular interest is the finding that binding of the altered substrate in the transition state is accompanied by an overall *gain* of entropy of 19 cal mol$^{-1}$ K$^{-1}$. The temperature dependence of the kinetic parameters of both the enzymatic and nonenzymatic reactions has also been reported for two other enzymes, ribonuclease A[30,31] and carbonic anhydrase,[32,33] allowing similar calculations for these enzymes as well. The results (Table 1) indicate that ribonuclease A and carbonic anhydrase stabilize their altered substrates in the transition state by 16 kcal/mol and 13 kcal/mol, respectively, considerably less than the value (30 kcal/mol) that we observe for fumarase. In contrast to the behavior of fumarase, the apparent change in entropy that accompanies transition state binding is negative for both ribonuclease A ($\Delta S_{tx}$ = −14 cal mol$^{-1}$ K$^{-1}$) and carbonic anhydrase ($\Delta S_{tx}$ = −15 cal mol$^{-1}$ K$^{-1}$).

The observation that transition state binding by fumarase is accompanied by a gain in entropy may seem surprising in view of the loss of transitional and rotational freedom that is expected when two molecules combine.[34,35] In aqueous solution, however, compensating entropic effects may arise from changing solvation. Electrostatic interactions, like hydrophobic interactions, probably involve a release of ordered water molecules from the region between the interacting groups, resulting in a positive contribution to the observed $\Delta S$;[36] complete protonation of the dianion of fumarate, for example, is accompanied by a gain of entropy of 37 cal mol$^{-1}$ K$^{-1}$.[37] The binding of fumarate by fumarase, in the ground state, was shown in earlier work to be accompanied by a small increase in entropy ($\Delta S$ = +4 cal mol$^{-1}$ K$^{-1}$).[3,38] Electrostatic stabilization of the greater negative charge, developed by fumarate in a carbanionic intermediate[15,16] approaching the transition state, could explain the much greater gain in entropy that is observed for binding the altered substrate in the transition state relative to the ground state. Entropic effects from changes in solvation could also arise, presumably, from changes in the conformation of fumarase.[19,35]

To stabilize the altered substrate in the transition state most effectively, an enzyme might be expected to maximize enzyme−ligand interactions, including hydrogen-bonding, electrostatic, and hydrophobic effects, and these interactions tend to be accompanied by a gain of entropy. It will be of interest to determine whether large positive entropy changes in the transition state, such as that observed for fumarase, tend to be characteristic of enzymes that are unusually proficient. In the course of maximizing these contacts, one might expect that the active site might tend to surround the ligand as completely as possible.[41] The nature of the enzyme−ligand interactions will be clearer when structural information concerning this remarkable catalyst becomes available.[42]

**Table 1.** Apparent Thermodynamic Parameters for Enzymes Binding Their Altered Substrate in the Transition State[a]

| enzyme | $\Delta G_{tx}$ (kcal/mol) | $\Delta H_{tx}$ (kcal/mol) | $\Delta S_{tx}$ (cal mol$^{-1}$ K$^{-1}$) |
|---|---|---|---|
| fumarase[b] (fumarate hydration) | −30 | −24 | **19** |
| ribonuclease A[c] (cCMP hydrolysis) | −16 | −21 | −14 |
| carbonic anhydrase[d] ($CO_2$ hydration) | −13 | −18 | −15 |

[a] The apparent enthalpy and entropy of association are calculated from the temperature dependence of the dissociation constant $K_{tx}$.[28] The $K_{tx}$ values were calculated from the temperature dependence of $k_{cat}/K_m$ and $k_{non}$ for the different enzymatic and nonenzymatic reactions, respectively: [b]*this* study; [c]*data* from refs 30 and 31; [d]*data* from refs 32 and 33.

**Acknowledgment.** S.L.B. is grateful to the Natural Sciences and Engineering Research Council of Canada for support through a postdoctoral fellowship. This research was supported by NIH Grant GM-18325.

JA952024P

(23) Hall, A.; Knowles, J. R. *Biochemistry* **1975**, *14*, 4348−4352.
(24) Putman, S. J. *Biochem. J.* **1972**, *129*, 301−310.
(25) Hawkinson, D. C.; Eames, T. C. M.; Pollack, R. M. *Biochemistry* **1991**, *30*, 10849−10858.
(26) Pollack, R. M.; Zeng, B.; Mack, J. P. G.; Eldin, S. *J. Am. Chem. Soc.* **1989**, *111*, 6419−6423.
(27) Radzicka, A.; Wolfenden, R. *Science* **1995**, *267*, 90−93.
(28) The steady state kinetic parameters for fumarase were determined by Alberty and co-workers,[3] over the range between 5 and 37 °C. Combining those values of $k_{cat}/K_m$ with the values of the nonenzymatic rate constants for fumarate hydration (this study), $K_{tx}$ values may be calculated and their temperature dependence analyzed in the form of a van't Hoff plot.[29] Because the rates of C−H bond cleavage and the rate-limiting conformational change are expected to be similar,[14] the observed thermodynamic parameters are likely to resemble those that would be observed for proton abstraction, if those could be observed directly.
(29) Eisenberg, D.; Crothers, D. *Physical Chemistry with Applications to the Life Sciences*; Benjamin/Cummings Publishing Co., Inc.: Menlo Park, CA, 1979; pp 158−160.
(30) Eftink, M. R.; Biltonen, R. L. *Biochemistry* **1983**, *22*, 5134−5140.
(31) Eftink, M. R.; Biltonen, R. L. *Biochemistry* **1983**, *22*, 5140−5150.
(32) (a) Edsall, J. T.; Wyman, J. *Biophysical Chemistry 1*; Academic Press, Inc.: New York, 1958; p 583−590. (b) Roughton, J. F. W. *J. Am. Chem. Soc.* **1941**, *63*, 2930−2934.
(33) Ghannam, A. F.; Tsen, W.; Rowlett, R. S. *J. Biol. Chem.* **1986**, *261*, 1164−1169.
(34) Page, M. I.; Jencks, W. P. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1678−1683.
(35) Jencks, W. P. *Adv. Enzymol.* **1975**, *43*, 219−410.
(36) Kauzmann, W. *Adv. Protein Chem.* **1959**, *14*, 1−63.
(37) Christensen, J. J.; Izatt, R. M.; Hansen, L. D. *J. Am. Chem. Soc.* **1967**, *89*, 213−222.
(38) Small positive or negative changes of entropy have also been observed to accompany substrate binding by other enzymes.[39,40]
(39) Laidler, K. J. *The Chemical Kinetics of Enzyme Action*; Oxford University Press: London, 1958; pp 194−205.
(40) Laidler, K. J.; Peterman, B. F. *Methods Enzymol.* **1979**, *63*, 234−257.
(41) Wolfenden, R. *Mol. Cell. Biochem.* **1974**, *3*, 207−211.
(42) Weaver, T.; Levitt, D.; Banaszak, L.; Donnelly, M.; Wilkens-Stevens, P. *FASEB J.* **1995**, *9*, A1466.