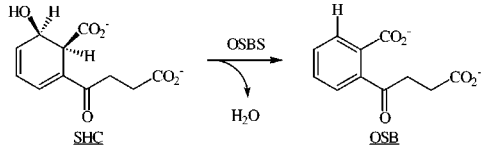
The Lesser "Burden Borne" by *o*-Succinylbenzoate Synthase: An "Easy" Reaction Involving a Carboxylate Carbon Acid

Erika A. Taylor, David R. J. Palmer,§ and John A. Gerlt*

Departments of Biochemistry and Chemistry
University of Illinois, Urbana, Illinois 61801

Received April 5, 2001

Enzymes that initiate reactions by abstraction of the α-proton of a carboxylate anion are ubiquitous in biochemistry. Such reactions have attracted considerable recent attention because the thermodynamics for formation of the enolic/enolate anion intermediate on the reaction coordinates are highly unfavorable due to the high pK_a of the substrate proton (>29) and the low pK_a of the conjugate acid of the active site general basic catalyst (<7). Our studies of the enolase superfamily suggest that one strategy for stabilization of the intermediates is coordination of the substrate carboxylate group to a divalent metal ion so that electrostatic stabilization occurs as the proton is abstracted and negative charge develops on the carboxylate/enediolate oxygens.[1] *o*-Succinylbenzoate synthase (OSBS), a member of the enolase superfamily, catalyzes the dehydration of 2-succinyl-2,4-cyclohexadiene-1-carboxylate (SHCHC) to yield *o*-succinylbenzoate (OSB), an intermediate in the biosynthesis of menaquinone cofactors that are required for anaerobic growth of eubacteria and at least some archae.[2] As described in this Communication, the "burden borne"[3] by OSBS is considerably less than that borne by fumarase[3] as well as other members of the enolase superfamily[4] that catalyze reactions initiated by abstraction of the α-proton of a carboxylate anion.



The efficiencies of enzyme-catalyzed reactions can be quantitated by comparing either k_{cat} or k_{cat}/K_m with k_{non}, the "spontaneous rate." The former ratio (k_{cat}/k_{non}; unitless) is designated the "rate acceleration;" the latter ratio [$(k_{cat}/K_m)/k_{non}$; units of M^{-1}] is designated "proficiency"[5] or "efficiency"[6] and quantitates the total energetic cost associated with catalysis. Wolfenden and his colleagues have quantitated values of k_{non} for a variety of nonenzymatic reactions[3−5] by measuring the temperature dependence of the rate constant ($\Delta H^{\ddagger}$) and extrapolating to ambient temperature. Pertinent to the OSBS-catalyzed reaction, they measured k_{non} for two reactions initiated by abstraction of the α-proton from carboxylate carbon acid substrates: the dehydration reaction catalyzed by fumarase[5] and the racemization reaction catalyzed by mandelate racemase (MR),[6] the latter a member of the enolase superfamily.[1] The values for k_{non}, 1.1×10^{-14} sec^{-1} and 3×10^{-13} sec^{-1}, respectively, are similar, reflecting the high pK_a's of the α-protons[7] in reactions whose reactants and products are nearly isoenergetic.[8] These values may be used to calculate the values of the rate accelerations and proficiencies of the fumarase- and MR-catalyzed reactions; these are displayed in Table 1. For both enzymes, the values of the rate accelerations and proficiencies are similar.

Table 1. Kinetic Constants

	OSBS[a]	fumarase[b]	MR[c]
k_{cat} (s^{-1})	29 ± 0.8	1240	500
k_{cat}/K_m (M^{-1} s^{-1})	$(1.8 \pm 0.2) \times 10^{6}$	2.3×10^{8}	1.3×10^{6}
k_{non} (s^{-1})	$(1.6 \pm 1.0) \times 10^{-10}$	1.1×10^{-14}	3×10^{-13}
k_{cat}/k_{non}	$(1.8 \pm 1.1) \times 10^{11}$	1.1×10^{17}	1.7×10^{15}
$[(k_{cat}/K_m)/k_{non}]$ (M^{-1})	$(1.1 \pm 0.7) \times 10^{16}$	2.1×10^{22}	4.3×10^{18}

[a] This work; pH 8.0 and 25 °C. [b] Calculated from refs 3 (k_{non}) and 15 (k_{cat} and k_{cat}/K_m); pH 6.82 and 25 °C. The values for k_{cat} and k_{cat}/K_m are the averages of the values at 21 °C and 29 °C. [c] Reference 4; pH 7.5 and 25 °C.

Although the literature suggested that SHCHC, the substrate for the OSBS-catalyzed reaction, would be too unstable to isolate and characterize,[9] we have found that chorismate can be converted to SHCHC in 60% yield using the coupled actions of purified isochorismate mutase and SHCHC synthase; purification by anion-exchange chromatography affords the dianion of SHCHC that is stable at neutral pH and ambient temperature.[10] At alkaline pH and ambient temperature, SHCHC is converted to *o*-succinylbenzoate (OSB); at acidic pH, SHCHC is converted to succinylbenzene (SB).[9] At moderately elevated temperatures (80−120 °C) at pH 8.0, the pH optimum for the reaction catalyzed by OSBS from *Escherichia coli*, SHCHC is converted to a 1:(6.3 ± 0.2) mixture of OSB and SB with half-times ranging from 1 h to 2 days.[11] The decomposition of SHCHC is first-order and not significantly catalyzed by the phosphate buffer. The first-order rate constant (k_{non}) for formation of OSB at 25 °C, $(1.6 \pm 1.0) \times 10^{-10}$ sec^{-1}, was obtained by extrapolation of a plot[12] of ln(k/T) versus $1/T$ that describes the temperature dependence of the rate constant for formation of OSB (Figure 1); the slope, $-\Delta H^{\ddagger}/R$, and intercept, $23.76 + \Delta S^{\ddagger}/R$, yield values of $+28.6 \pm 0.1$ kcal/mol for $\Delta H^{\ddagger}$ and -7.4 ± 0.2 cal/mol deg for $\Delta S^{\ddagger}$. The decomposition of [1-^{2}H]-SHCHC is also first-order [$k_{non} = (5.9 \pm 5.0) \times 10^{-11}$] and yields a 1:(18 ± 0.7) mixture of OSB and SB.

The value of the primary deuterium kinetic isotope effect, 2.7 ± 0.13, is consistent with a mechanism involving an early transition state for the initial abstraction of the α-hydrogen.[13] An

§ Current address: Department of Chemistry, University of Saskatchewan, 110 Science, Place, Saskatoon, SK, Canada S7N 5C9.

(1) Babbitt, P. C.; Hasson, M.; Wedekind, J. E.; Palmer, D. R. J.; Rayment, I.; Ringe, D.; Kenyon, G. L.; Gerlt, J. A. *Biochemistry* **1996**, *35*, 16489−16501.
(2) Meganathan, R. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*; Neidhart, F. C., Curtis, R., Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M., Umbarger, H. E., Eds.; ASM Press: Washington, DC, 1996; pp 642−656.
(3) Bearne, S. L.; Wolfenden, R. *J. Am. Chem. Soc.* **1995**, *117*, 9588−9589.
(4) Bearne, S. L.; Wolfenden, R. *Biochemistry* **1997**, *36*, 1646−1656.
(5) Radzicka, A.; Wolfenden, R. *Science* **1995**, *267*, 90−93.
(6) Bruice, T. C.; Benkovic, S. J. *Biochemistry* **2000**, *39*, 6267−6274.
(7) Gerlt, J. A.; Gassman, P. G. *J. Am. Chem. Soc.* **1993**, *115*, 11552−11569.
(8) The equilibrium constant for the fumarase-catalyzed reaction is 3.3: Bock, R. M.; Alberty, R. A. *J. Am. Chem. Soc.* **1953**, *75*, 1921−1925. The equilibrium constant for the MR-catalyzed reaction is 1.0.
(9) Popp, J. L.; Berliner, C.; Bentley, R. *Anal. Biochem.* **1989**, *178*, 306−310.
(10) Palmer, D. R. J.; Garrett, J. B.; Sharma, V.; Meganathan, R.; Babbitt, P. C.; Gerlt, J. A. *Biochemistry* **1999**, *38*, 4252−4258.
(11) A stock solution (7.5 mL) of 2 mM phosphate buffer and 0.4 mM SHCHC was aliquoted into 15 0.5 mL conical reaction vials; the vials were incubated, with stirring, in an oil bath at 80, 90, 100, 110, and 120 °C. Reactions were performed in triplicate. Vials were removed at appropriate times and cooled to 0 °C to terminate the reaction. The contents were diluted to 800 μL with 50 mM Tris-HCl, pH 7.5, and the UV spectrum from 220 to 340 nm was recorded. The slope of a plot of log OD_{293} vs time is the first-order rate constant for the disappearance of SHCHC. The distribution of SB vs OSB was determined by integration of the NMR spectrum of the completed reaction and used to obtain the rate constant for the conversion of SHCHC to OSB.; the distribution of products was observed to be independent of temperature within the estimated error. The distribution of SB vs OSB is pH independent from 7 to 9, suggesting that the dehydration reaction measured at pH 8 is water-catalyzed rather than specific base-catalyzed.
(12) Cleland, W. W.; Northrup, D. B. *Methods Enzymol.* **1999**, *308*, 3−27.
(13) Hammond, G. S. *J. Am. Chem. Soc.* **1955**, *77*, 334.

10.1021/ja010882h CCC: $20.00
Published on Web 05/26/2001

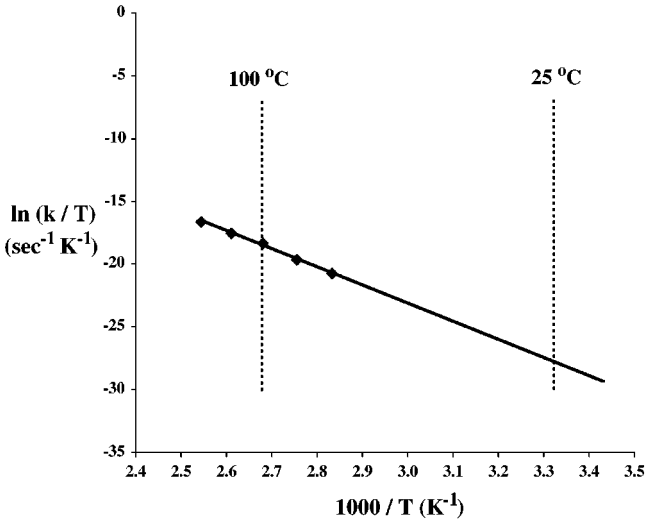


Figure 1. Temperature dependence of the dehydration of SHCHC in 2 mM potassium phosphate buffer, pH 8.0.

alternate mechanism involving initial departure of hydroxide ion to generate an allylic carbenium ion is expected to be accompanied by rapid loss of the very acidic α-hydrogen. We also measured the kinetic constants for the OSBS from *E. coli* at pH 8 and 25 °C using both protiated and deuterated samples of SHCHC. The values of k_{cat} and k_{cat}/K_m are subject to large primary deuterium isotope effects, 6.1 ± 0.4 and 4.4 ± 1.3, respectively, that reflect a mechanism dominated by rate-determining abstraction of the α-hydrogen. Although the latter values are larger than those for the nonenzymatic reaction and may reflect a somewhat different transition-state geometry due to stabilization of the enolate anion intermediate, we conclude that a comparison of the rates of the nonenzymatic and OSBS-catalyzed dehydration of SHCHC is relevant.

The values of k_{cat} as well as of k_{cat}/K_m are similar for the OSBS-, fumarase-, and MR-catalyzed reactions (Table 1), with the values of k_{cat}/K_m approaching the diffusion-controlled limit.[14] In contrast, the values for the rate acceleration and proficiency for the OSBS-catalyzed reaction are significantly less than those for the other reactions initiated by abstraction of the α-proton of a carboxylate anion (Table 1).

A comparison of the free energy changes associated with the OSBS- and fumarase-catalyzed[3,15] reactions is presented in Figure 2; the changes for the MR-catalyzed reaction are similar to that for the fumarase-catalyzed reaction. This analysis reveals that the unusual values for both the rate acceleration and proficiency of

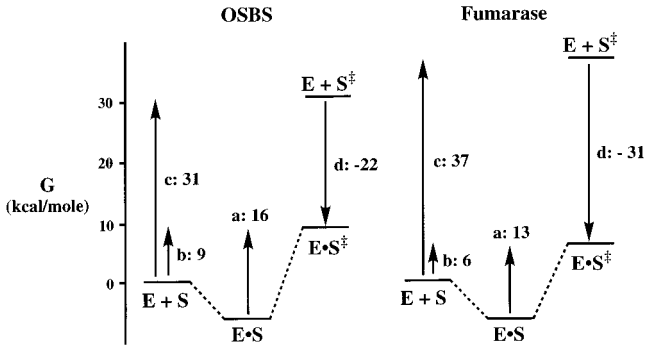


Figure 2. Free energy changes associated with the reactions catalyzed by OSBS (pH 8 and 25 °C) and fumarase (pH 6.82 and 25 °C) assuming 1M standard states.[3] The numbers refer to the free energy changes associated with k_{cat} (arrows marked "a"), k_{cat}/K_m ("b"), k_{non} ("c") and $(k_{cat}/K_m)/k_{non}$ ("d"). The values of the free energies associated with k_{cat} and k_{non} were calculated from $\Delta G^{\ddagger} = -RT \ln(kh/k_bT)$, where k is k_{cat} or k_{non}, k_b is Boltzmann's constant and h is Planck's constant.

the OSBS-catalyzed reaction result from the fact that the spontaneous dehydration of SHCHC is more facile that the spontaneous dehydration of malate or racemization of mandelate. While the reactions catalyzed by fumarase and MR have equilibrium constants that do not differ significantly from unity,[8] that for the OSBS-catalyzed reaction necessarily lies far in the direction of dehydration as a result of OSB being aromatic. Although the value of the pK_a of the α-proton of SHCHC is unknown, the exergonicity of dehydration will allow the transition state for the reaction to be early on the reaction coordinate,[13] in agreement with the value of the substrate deuterium kinetic isotope effect, thereby providing a plausible explanation for the "unusual" reactivity of SHCHC as compared to the other carbon acids.

Both OSBS and enolase are ubiquitous in microorganisms. We have noted that the sequences of OSBS's are highly diverged, with the exception of the active-site residues that are involved in catalysis by either binding the essential Mg(II) or acting as general acid/base catalysts in mediating abstraction of the α-proton and departure of the β-OH group; in contrast, the sequences of enolases are highly conserved.[10,16] The lower energetic cost of the OSBS-catalyzed reaction compared to that of the enolase-catalyzed reaction may allow the observed greater divergence of sequence for OSBS's and, perhaps, a less precise active-site geometry without compromising the values of either k_{cat} or k_{cat}/K_m.[17]

JA010882H

(14) Knowles, J. R.; Albery, W. J. *Biochemistry* **1976**, *15*, 5631−5640.
(15) Brant, D. A.; Barnett, L. B.; Alberty, R. A. *J. Am. Chem. Soc.* **1963**, *85*, 2204−2209.
(16) Thompson, T. B.; Garrett, J. B.; Taylor, E. A.; Meganathan, R.; Gerlt, J. A.; Rayment, I. *Biochemistry* **2000**, *39*, 10662−10676..
(17) Supported by NIH GM-52594. We thank Professors Patricia C. Babbitt, University of California, San Francisco, and Ivan C. Rayment, University of Wisconsin, for helpful discussions.