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Deuterium Isotope Effects in the Unusual Addition of Toluene to Fumarate Catalyzed by Benzylsuccinate Synthase[†]

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Abstract

The first step in the anaerobic metabolism of toluene is a highly unusual reaction: the addition of toluene across the double bond of fumarate to produce (*R*)-benzylsuccinate that is catalyzed by benzylsuccinate synthase. Benzylsuccinate synthase is a member of the glycyl radical-containing family of enzymes, and the reaction is initiated by abstraction of a hydrogen atom from the methyl group of toluene. To gain insight into the free energy profile of this reaction, we have measured the kinetic isotope effects on $V_{\max}$ and $V_{\max}/K_m$ when deuterated toluene is the substrate. At 30 °C the isotope effects are 1.7 ± 0.2 and 2.9 ± 0.1 on $V_{\max}$ and $V_{\max}/K_m$, respectively, at 4 °C they increase slightly to 2.2 ± 0.2 and 3.1 ± 0.1 respectively. We compare these results with the theoretical isotope effects on $V_{\max}$ and $V_{\max}/K_m$ that are predicted from the free energy profile for the uncatalyzed reaction, which has previously been computed using density functional theory. The comparison allows us to draw some conclusions on how the enzyme may catalyze this unusual reaction.

Keywords

toluene metabolism; free radicals; enzyme kinetics; *Tauera. aromatica*; glycyl radical enzyme

Toluene and related aromatic compounds represent an important class of pollutants that are long-lived in the environment and have relatively high water solubility. This is a particular problem in environments where oxygen is scarce, such as ground water supplies. It was initially thought that the only routes to degrade such compounds were through oxidative pathways that require molecular oxygen to functionalize otherwise inert aromatic hydrocarbons. However, the recent discovery in bacteria of anaerobic pathways for the degradation of aromatic compounds has aroused much interest, both for the potential they offer for bio-remediation in situations where oxygen is scarce, and because of the novel chemistry involved (1,2).

Various denitrifying and sulfate-reducing bacteria such as *Thauera aromatica* and *Desulfobacula toluolica* are able to live on toluene as their sole source of carbon and energy under anaerobic conditions (3,4). The first step in this pathway is the addition of toluene across the double bond of fumarate to produce (*R*)-benzylsuccinate, as shown in scheme 1, in which hydrogen in the methyl group of toluene is retained in the product (5). This remarkable chemical transformation is catalyzed by benzylsuccinate synthase¹ (BSS).

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¹The abbreviations used are: BSS, benzylsuccinate synthase; $D(V/K)$, the deuterium kinetic isotope effect on $V_{\max}/K_m$; D_V , the deuterium kinetic isotope effect on $V_{\max}$.

BSS as a member of the growing class of glycy radical-containing enzymes that includes pyruvate formate-lyase (PFL) and anaerobic ribonucleotide reductase (6-11). EPR studies show that the resting enzyme harbors an organic radical similar to that observed in these enzymes (12-14). Like other glycy radical enzymes, BSS is extremely oxygen sensitive, and exposure to air results in oxidative cleavage of the α subunit at the site of the presumed glycy radical (6).

We recently investigated the stereochemistry of hydrogen transfer using deuterated toluene (15). We showed that when fumarate was the co-substrate, deuterium was transferred from toluene to the C-3 pro-(*S*) position of benzylsuccinate, implying that the addition of toluene to the double bond of fumarate was *syn*. However, when maleate (which is also recognized by the enzyme (16)) was the co-substrate, the addition of toluene occurs in an *anti* fashion. These observations provide evidence for the formation of the C-3 radical of benzylsuccinate as an intermediate, in which rotation about the C-2, C-3 bond can occur to relieve the sterically unfavorable *cis* conformation of the carboxylate groups when maleate is the co-substrate.

Based on the observations above, the mechanism shown in Figure 1 is proposed. This involves the initial transfer of the glycy radical to an active site cysteine residue, which then abstracts hydrogen from toluene to generate a benzylic radical. The benzylic radical subsequently undergoes addition to fumarate to generate the C-3 radical of (R)-benzylsuccinate. The abstracted hydrogen is transferred to the product to generate benzylsuccinate and the cysteinyl radical, and lastly the radical is transferred back to glycine.

The rapid loss of activity encountered during attempts to purify the enzyme and its extreme oxygen sensitivity (6) has severely hindered efforts to study the enzyme. As a result, virtually nothing is known experimentally regarding the kinetics or energetics of this highly unusual enzyme reaction. However, a density functional theoretical study has evaluated the BSS reaction and computed the relative stabilities of the radical intermediates and activation energies of each step in the putative mechanism (17). The rate-determining step in the reaction is predicted to be addition of the benzyl radical to fumarate, which is effectively irreversible ($\Delta G = -10.1$ kcal/mol). To test this prediction and gain insight into the nature of the rate-determining step in BSS we have measured the deuterium kinetic isotope effects on $V_{\max}$ and $V_{\max}/K_m$ for the addition of deuterated toluene to fumarate.

MATERIALS AND METHODS

Growth of Thauera Aromatica

T. Aromatica was grown at 30 °C under a N₂ atmosphere in the minimal medium described by Coschigano (7), containing 0.5 mM toluene as the carbon source and 5 mM nitrate as an oxidizing agent. The toluene and nitrate concentrations were maintained at their approximate starting concentrations by additional feedings during growth. Cells were grown to an OD₆₀₀ of 0.7 – 0.9 and harvested under anaerobic conditions by centrifugation at 6,000 g for 30 min in sealed nitrogen-flushed centrifuge tubes. The cell pellet was suspended in degassed buffer containing 10 % glycerol, 0.5 mM of sodium dithionite and the cells pelleted again at 6,000 g for 30 min. The cell pellet was frozen with liquid nitrogen and stored at –80 °C.

Preparation of cell extracts

All experiments were performed in a glove box (Coy Laboratory Products) in a 3% H₂/ 97% N₂ atmosphere, at 4 °C in the cold room, with oxygen concentration not higher than 5 ppm. Typically, one volume of cell pellet was resuspended in 2.5 volumes of a buffer comprising 40 mM triethanolamine/HCl, 10 mM fumaric acid and 10% glycerol at pH 8.0. The resuspended cells were sonicated at 0 °C with four bursts (13 W $\times$ 30 s), with a 1 min rest between each

burst to prevent the solution over heating. The lysate was centrifuged at 14,000 g at 4 °C for 10 min to remove the cell debris and the supernatant containing the soluble proteins was used immediately.

Enzymatic synthesis of the benzylsuccinic acid

Typically 400 – 600 μ L of the cell extract was added to 500 μ L aqueous toluene solution (5.75 mM) and the reaction mixture was incubated at 4 °C or 30 °C. At various times 200 μ L of the reaction solution was removed and the reaction quenched by addition of 50 μ L 4 M HCl. The protein residue was removed by centrifugation at 14,000 g at ambient temperature for 15 min, and the supernatant was further analyzed by reverse phase HPLC.

HPLC analysis of benzylsuccinic acid

Chromatography was performed at room temperature with detection at 254 nm using a Vydac reverse-phase C-18 column (5 μ M, 4.6 $\times$ 250). The column was equilibrated in 0.3% formic acid and compounds were eluted with an ascending gradient of CH₃CN: 0 – 35 % over 6 mL; 35 – 47 % over 12 mL; and 47 – 100 % over 5 mL at a flow rate of 1 mL/min. Using this gradient, benzylsuccinic acid eluted at 11.8 min. The identity of the benzylsuccinic acid was confirmed by mass spectrometry as described below. The area of the benzylsuccinic acid peak was determined after subtraction of the base line from the $t = 0$ chromatograph and the amount of benzylsuccinic acid formed determined by reference to standard curves constructed with authentic commercially available benzylsuccinic acid.

Electrospray ionization mass spectroscopy

The deuterium content of benzylsuccinic acid was analyzed by ESI-MS spectroscopy under the negative ion mode. Samples collected after HPLC were directly analyzed by time-of-flight electrospray mass spectrometry (LCT Micromass) under the negative ion mode. The samples were introduced into the spectrometer through an in-line HPLC pump, in a carrier solvent of 90% methanol/10% water at 0.1 mL/min. The desolvation temperature was 150 °C. The spectral data were analyzed using the MassLynx 4.0 software suite. Mass calculated for C₁₁H₁₂O₄ (M⁻): 208.07, found: 207.04; mass calculated for C₁₁H₄D₈O₄ (M⁻): 216.12, found: 215.10.

Analysis of deuterated toluene mixtures

To accurately determine the ratio of C₇H₈ to C₇D₈ in experiments to measure $V_{\max}/K_m$ isotope effects, aqueous toluene solutions were analyzed by GC/MS spectroscopy using a Finnigan Trace MS gas chromatograph/mass spectrometer.

RESULTS AND DISCUSSION

Isotope effects are a powerful tool to study the chemical mechanisms and energetics of enzyme reactions. For BSS a theoretical study has calculated the free energy profile for the enzyme-catalyzed reaction, providing predictions about the rate-determining step that may be experimentally tested by measuring isotope effects. We therefore decided to measure the deuterium kinetic isotope effect for the BSS-catalyzed reaction when deuterium was present in the methyl group of toluene. In these experiments we have employed cell-free extracts of *T. aromatica*, because, although the purification of the enzyme has been reported (6), large losses of activity occurred during purification and the purified enzyme was highly unstable. We were not been able to obtain active preparations of the enzyme using the reported purification procedure.

Kinetic analyses are generally performed using highly purified enzymes to guard against the risk of interference due to contaminating enzymes or inhibitors. However, since isotope effects

involve purely a comparison between the rates of reaction of labeled and unlabeled substrates they can be reliably measured in crude enzyme preparations provided no other isotopically-sensitive reactions are available to either the substrate or products of the reaction, which could lead to the results being skewed. In the present case these criteria are met, because BSS is the only enzyme for which toluene is a substrate under anaerobic conditions. The product of the reaction, benzylsuccinate, is the substrate for benzylsuccinyl-CoA transferase, which catalyzes the transfer of coenzyme A from succinyl-CoA; in the absence of this reaction there are no other fates for benzylsuccinate. As discussed below, we found no evidence for benzylsuccinyl-CoA transferase activity. This is not surprising because any endogenous succinyl-CoA in the cell-free extract is likely to be rapidly hydrolysed under the slightly basic buffer conditions employed in the reaction.

Enzyme assay

We developed an HPLC-based assay for BSS activity, in place of the radioactivity-based assay used previously (6,13). This allowed us both to reliably quantify the amount of benzylsuccinate produced, as shown in Figure 2, and subsequently analyze the deuterium content of the benzylsuccinate peak by mass spectrometry. Furthermore, it allowed us to assess whether any additional products were being formed during incubation of the substrates with the cell-free extract. We were unable to detect the formation of any other metabolites – no other peaks appeared or disappeared – during the incubation period.

In the assay, the substrate concentrations were typically 2 mM for toluene and 10 mM for fumarate; reducing the concentration of either substrate by half did not change the rate of reaction, so these concentrations may be assumed to be saturating. (The K_m for toluene is not known accurately, but is below 100 μ M; L. L. unpublished data.) Under the conditions of the assay, the formation of benzylsuccinate was linear with time for up to 20 min, as shown in Figure 3. The activity of the enzyme was measured at both 4 °C and 30 °C. The specific activity of the cell extract at 4 °C was 2.4 nmol/min/mg of protein, whereas at 30 °C the specific activity rose to 7.4 nmol/min/mg. This level of activity is similar to that reported in permeabilized cells of *Azoracoccus sp* (18), and some 4 orders of magnitude higher than that reported for purified enzyme from *T. aromatica* (6). As judged by SDS-PAGE, the BSS α -subunit, a conspicuous band of M_r 98,000, comprised about 3.5 % of the total proteins present in the cell free extract, which allows us to estimate k_{cat} for BSS as approximately 0.11 s⁻¹ at 4 °C and 0.35 s⁻¹ at 30 °C.

Deuterium kinetic isotope effects

The isotope effects on V_{max}/K_m were measured in competition experiments in which an equimolar mixture of toluene and d₈-toluene was incubated with enzyme-containing cell-free extract for various times. The reaction was quenched, and the amount of benzylsuccinic acid in the sample was determined by HPLC; the deuterium content of the benzylsuccinic acid was subsequently determined by mass spectrometry (Figure 4). In these experiments apparent kinetic isotope effects were measured at relatively low extents of reaction of between 3 and 15 %. Under these conditions the isotopic composition of the starting toluene varies approximately linearly with the extent of reaction. The kinetic isotope effect on $^D(V/K)$ was therefore calculated by linear extrapolation of the apparent kinetic isotope effect to zero extent of reaction (Figure 4). $^D(V/K) = 2.9 \pm 0.1$ at 30 °C, a value which rises slightly to 3.1 ± 0.1 at 4 °C

The isotope effects on V_{max} , were determined by direct comparison of the initial rates at which toluene and d₈-toluene were converted to benzylsuccinate (Figure 3). At 30 °C $^DV = 1.7 \pm 0.2$, whereas at 4 °C the isotope effect rises to 2.2 ± 0.2 . These isotope effects are significantly lower than the corresponding $^D(V/K)$ isotope effects, suggesting that although hydrogen abstraction from toluene is a kinetically significant step it is not fully rate-determining. There

are two isotopically sensitive steps in the BSS reaction – abstraction of hydrogen from toluene and return of hydrogen to benzylsuccinate – however, it is more likely that the isotope effect is manifested on the former step because the latter step occurs after the addition of the benzylic radical to fumarate, which is expected to be essentially irreversible.

Comparison of isotope effects predicted by the calculated free energy profile with experimentally determined isotope effects

The free energy profile for the BSS-catalyzed reaction has been modeled computationally using density functional theory calculations at the B3LYP level of theory (17). The model included the transfer of hydrogen between the active site cysteine residue and the glycyl radical, but otherwise no information about the protein was assumed because the structure of the enzyme is not known. The calculated free energy profile is shown in Figure 5 and may be considered as an approximation to that for the uncatalyzed reaction, assuming that all the reacting species have been brought into a reasonable alignment for the reaction to proceed.

We wished to use our experimentally-determined isotope effects on $V_{\max}$ and $V_{\max}/K_m$ to compare how the true free energy profile for BSS may differ from the calculated one, and thereby gain insights into how the enzyme effect catalysis in the reaction. This may be achieved as follows: First, the $\Delta G^\ddagger$ calculated for each transition state of the reaction mechanism can be used to calculate the corresponding elementary rate constants the mechanism. Second, the expressions for k_{cat} and k_{cat}/K_m can be derived in terms of the elementary rate constants for the mechanism, and from these the corresponding expressions for the isotope effects on $V_{\max}$ and $V_{\max}/K_m$ can also be derived. Third, although we do not know the exact value of the intrinsic isotope effect on C-H cleavage is for the reaction, by making a reasonable guess we can evaluate whether the calculated free energy profile predicts isotope effects on $V_{\max}$ and $V_{\max}/K_m$ that are in qualitative agreement with those measured experimentally. In particular, we can determine whether the energy profile predicts the isotope effects to be: **a)** fully suppressed, i.e. $^D V = ^D(V/K) = 1$; **b)** fully expressed, i.e. $^D V = ^D(V/K) = ^D V_{\text{int}}$; **c)** $^D V$ suppressed relative to $^D(V/K)$ i.e. $^D V < ^D(V/K)$; or **d)** in the most unusual case, $^D(V/K)$ suppressed relative to $^D V$ i.e. $^D V > ^D(V/K)$.

Expressions for k_{cat} and k_{cat}/K_m

Expressions for k_{cat} and k_{cat}/K_m for the mechanism shown in Figure 1 can be derived using the method of net rate constants (19), among other methods. To simplify matters, we assume, based on the computed free energy profile, that formation of the carbon-carbon bond between benzyl radical and fumarate (k_4) is essentially irreversible, in which case subsequent steps in the mechanism can be neglected from the analysis. This is a reasonable assumption because a strong carbon-carbon sigma bond is formed at the expense of a weaker carbon-carbon pi bond. We also assume that neither substrate binding nor product release is rate-limiting, which is also reasonable in view of the isotope effects we have measured. The expressions for k_{cat} and k_{cat}/K_m are given by equations 1 and 2 respectively, where the elementary rate constants refer to the steps shown in Figure 1; k_{-1} in Equation 2 is the off rate for substrate binding.

$$k_{\text{cat}} = \frac{k_2 k_3 k_4}{k_2 k_3 + k_2 k_{-3} + k_2 k_4 + k_{-2} k_{-3} + k_{-2} k_4 + k_3 k_4} \quad \text{Equation 1}$$

$$\frac{k_{\text{cat}}}{K_m} = \frac{k_1 k_2 k_3 k_4}{k_{-1} k_{-2} k_{-3} + k_{-1} k_{-2} k_4 + k_{-1} k_3 k_4 + k_2 k_3 k_4} \quad \text{Equation 2}$$

Expressions for $^D V$ and $^D(V/K)$

The expressions for the observed isotope effects on k_{cat} and k_{cat}/K_m can be derived by assuming that only k_3 and k_{-3} are altered in the reaction with deuterated toluene. They are obtained by dividing equations 1 and 2 by the equivalent expressions in which k_3 and k_{-3} are replaced with $^D k_3$ and $^D k_{-3}$ respectively to give equations 3 and 4.

$$V_{\text{obs}} = \frac{k_3}{k_3} \cdot \frac{k_2 k_3 + k_2 k_{-3} + k_2 k_4 + k_{-2} k_{-3} + k_{-2} k_4 + ^D k_3 k_4}{k_2 k_3 + k_2 k_{-3} + k_2 k_4 + k_{-2} k_{-3} + k_{-2} k_4 + k_3 k_4} \quad \text{Equation 3}$$

$$\left(\frac{V}{K}\right)_{\text{obs}} = \frac{k_3}{k_3} \cdot \frac{k_{-1} k_{-2} k_{-3} + k_{-1} k_{-2} k_4 + k_{-1} k_3 k_4 + k_2 k_3 k_4}{k_{-1} k_{-2} k_{-3} + k_{-1} k_{-2} k_4 + k_{-1} k_3 k_4 + k_2 k_3 k_4} \quad \text{Equation 4}$$

These equations may be rewritten by substituting $^D k_3$ by $k_3/^D V_{\text{int}}$ and $^D k_{-3}$ by $k_{-3}/^D V_{\text{int}}$, where $^D V_{\text{int}}$ is the intrinsic isotope effect on hydrogen transfer, to give equations 5 and 6; this assumes that any equilibrium isotope effects are small enough to be ignored. These equations describe how the observed isotope effects on V_{max} and V_{max}/K_m change as a function of the elementary rate constants for the various steps in the mechanism.

$$V_{\text{obs}} = \frac{V_{\text{int}} k_4 (k_2 k_{-2}) + k_3 (k_2 + k_{-2}) + k_{-3} (k_2 - k_4)}{k_4 (k_2 + k_{-2}) + k_3 (k_2 + k_{-2}) + k_{-3} (k_2 + k_4)} \quad \text{Equation 5}$$

$$^D \left(\frac{V}{K}\right)_{\text{obs}} = \frac{^D V_{\text{int}} k_{-1} k_{-2} k_4 + k_{-1} k_{-2} k_{-3} + k_{-1} k_3 k_4 + k_2 k_3 k_4}{k_{-1} k_{-2} k_4 + k_{-1} k_{-2} k_{-3} + k_{-1} k_3 k_4 + k_2 k_3 k_4} \quad \text{Equation 6}$$

Comparison of theory and experiment

The elementary rate constants for each step in the BSS reaction were calculated from the computationally determined free energies at 4 °C and at 30 °C using the standard equation derived from basic transition state theory (Equation 7). We made the usual assumption that the transmission coefficient is unity; i.e. no attempt was made to account for the fact that the Arrhenius pre-exponential factors may be different for the individual transition states in the mechanism.

$$k_i = \left(\frac{kT}{h}\right) \exp\left(\frac{-\Delta G_i^\ddagger}{RT}\right) \quad \text{Equation 7}$$

The rate constants are tabulated in Table 1. From these, k_{cat} for the overall reaction was calculated using equation 1 to be 8.4 s^{-1} at 4 °C and 96 s^{-1} at 30 °C. This is well within the range of turnover numbers expected for a “typical” enzyme, although significantly higher than the values of about 0.1 s^{-1} at 4 °C and 0.35 s^{-1} at 30 °C for k_{cat} that we calculate from our experiments. However, given that the computationally-determined free energy profile includes no explicit knowledge of the protein environment and does not account for substrate binding/product release or protein conformational changes, the numbers agree reasonably well.

To compare our experimentally-determined isotope effects with those predicted by the computationally-determined free energy profile, we used equations 5 and 6 with the data in Table 1 to calculate the extent to which intrinsic isotope effect, $^D V_{\text{int}}$, is masked by other steps in under V_{max} and V_{max}/K_m conditions. Although equation 6 requires us to assume a value for k_{-1} , the substrate off-rate, if the term $k_2 k_3 k_4$ in equation 6 is small enough to be neglected then k_{-1} will cancel from equation 6. In practice, if k_{-1} is greater than $\sim 50 \text{ s}^{-1}$ this criterion is met and the value of k_{-1} does not influence $^D(V/K)_{\text{obs}}$. (If k_{-1} were to be very slow, corresponding

to a “sticky” substrate, this would lead to the unusual case in which $^D(V/K)_{\text{obs}}$ is actually *smaller* than $^DV_{\text{obs}}$, which is the opposite to our experimental finding.)

Our calculations show that at 30 °C $^DV_{\text{obs}} = 0.03 \text{ } ^DV_{\text{int}} + 0.97$ and, assuming k_{-1} is not limiting, $^D(V/K)_{\text{obs}} = 0.03 \text{ } ^DV_{\text{int}} + 0.97$, whereas at 4 °C $^DV_{\text{obs}} = 0.02 \text{ } ^DV_{\text{int}} + 0.98$ and $^D(V/K)_{\text{obs}} = 0.02 \text{ } ^DV_{\text{int}} + 0.98$. Thus the calculated free energy profile predicts that $^DV_{\text{obs}}$ and $^D(V/K)_{\text{obs}}$ would not only be almost fully suppressed at both 30 °C and 4 °C, but they would have the *same* value. In contrast, we find that $^D(V/K)_{\text{obs}}$ both is significantly larger than $^DV_{\text{obs}}$ and, although the value of 3 for $^D(V/K)_{\text{obs}}$ is unlikely to represent the full intrinsic isotope effect, it is probably significantly more than 2 – 3 % of $^DV_{\text{int}}$. For example, if the free energy profile was correct, then a value of 3 for $^D(V/K)_{\text{obs}}$ would imply that $^DV_{\text{int}}$ would be ~ 65 at 30 °C and ~ 100 at 4 °C!

Such large values are clearly much higher than those predicted for deuterium isotope effects by the semi-classical theory, and would suggest that the calculated free energy profile is incompatible with the experimental data. However, we should note that deuterium isotope effects in the range of 30 - 50 have been measured in several enzymes that catalyze hydrogen atom abstractions from carbon, for example: lipxygenase, galactose oxidase and B₁₂-dependent isomerases (20-25), in reactions that proceed with a high degree of quantum tunneling. Given that the BSS-catalyzed reaction involves radical chemistry similar to the enzymes mentioned above, the possibility that the intrinsic deuterium isotope effects are indeed extremely large cannot be entirely excluded. However, even allowing for a very large intrinsic isotope effect, the computed free energy profile is unable to account for the experimental observation that $^D(V/K)_{\text{obs}}$ is significantly larger than $^DV_{\text{obs}}$.

To be consistent with our data, the free energy profile must qualitatively reproduce the observations that $^D(V/K)_{\text{obs}}$ is larger than $^DV_{\text{obs}}$ and that both isotope effects are to a significant degree suppressed from the intrinsic value. These requirements are not sufficient to uniquely specify the free energy profile, but they do place a number of constraints on the relative heights of the energy barriers.

To account for our data the calculated free energy profile needs to be altered as follows. First, for $^D(V/K)_{\text{obs}}$ to be larger than $^DV_{\text{obs}}$ a kinetically significant step in the reaction must occur *after* the isotopically sensitive step. Therefore k_4 , or more probably a later step such as product release, must be either of a similar magnitude or slower than either k_3 or k_2 . Secondly, k_3 , which is the isotopically sensitive step, must be either of a similar magnitude to, or slower than, k_2 , otherwise this step is not kinetically significant. Lastly, k_2 must be of a similar magnitude to k_{-2} because if $k_2 \gg k_{-2}$ (i.e. by more than a factor of 10) then $^D(V/K)_{\text{obs}}$ will tend become *smaller* than $^DV_{\text{obs}}$, corresponding to a “sticky” substrate. A free energy profile that qualitatively reproduces our experimental observations is illustrated in Figure 5.

In physical terms, the main difference between the experimentally deduced free energy profile (Figure 5) and the calculated one (Figure 6) is that starting glycyl radical and the intermediate cysteinyl and benzylic radicals appear to be much closer in energy on the enzyme than on paper. The relative heights of the energy barriers between the intermediates are the same in the two free energy profiles. It may be that the calculations reproduce the energies of the radicals poorly, but we think this is unlikely as in general DFT produces reliable results for the ground state energies of small molecules. More interestingly, this comparison suggests that the enzyme plays a role in stabilizing the cysteinyl and benzylic radicals relative to the glycyl radical, or, equally, it could destabilize the glycyl radical. This may be an important and general mechanism for controlling the reactivity of radicals in this class of enzymes. This would represent another example of a generally observed principle of enzymic catalysis: that enzymes act to shift the internal equilibrium constants for the conversion of bound reaction intermediates

towards unity. Indeed, enzymes that utilize free radicals in catalysis appear to be particularly adept at stabilizing free radical species in their active sites (9).

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Scheme 1.
Reaction catalyzed by benzylsuccinate synthase

Figure 1.
Proposed mechanism for benzylsuccinate synthase.

Figure 2. HPLC assay of BSS activity in cell-free extracts of *T. aromatica*. *Top* Typical HPLC chromatograph with position of the benzy succinic acid peak indicated. *Bottom* Enlargement of the region between 10 and 13 min illustrating the time-dependent increase in benzy succinic acid.

Figure 3.

Determination of the isotope effect on $V_{\max}$ for BSS. Initial velocity plots for BSS, reacting with saturating concentrations of toluene. The symbols represent the reactions of unlabelled toluene (triangles) or d_8 -toluene (squares) at 4 °C (closed symbols) or 30 °C (open symbols).

Figure 4.

Determination of V/K isotope effect for BSS *Top* Representative electrospray mass spectra (negative mode) showing the ratio of benzylsuccinate ($M-1 = 207$) to d_8 -benzylsuccinate ($M-1 = 215$) produced by incubation of BSS with a 1:1 mixture of toluene and d_8 -toluene at 30 °C. *Bottom* Determination of the isotope effect on $V_{\max}/K_m$ by extrapolation of the apparent isotope effect to zero extent of reaction. Data shown for 4 °C (□) or at 30 °C (○).

Figure 5.

Free energy profile for the BSS reaction calculated by density functional theory. The data used to construct the figure are taken from reference (17). The heights of the energy barriers (kcal/mol) are indicated by the side of each barrier; the bold numbers refer to the enzyme species shown in Figure 1; the steps subject to a deuterium isotope effect are indicated by dashed lines.

Figure 6.

Qualitative free energy profile for the initial steps in the BSS-catalyzed reaction that is consistent with the experimentally observed isotope effects on $V_{\max}$ and $V_{\max}/K_m$. The numbers refer to the enzyme species shown in Figure 1; the step subject to a deuterium isotope effect is indicated by dashed lines.

Table 1

Calculated values for elementary rate constants in the BSS-catalyzed reaction at 4 °C and 30 °C.

elementary rate constant ^a	$\Delta G^\ddagger$ ^b (kcal/mol)	value at 4 °C (10 ³ s ⁻¹)	value at 30 °C (10 ³ s ⁻¹)
k_2	10.7	20.9	121
k_{-2}	9.5	185	888
k_3	8.5	1,140	4,670
k_{-3}	7.3	10,100	34,300
k_4	6.4	51,500	152,000

^a rate constants refer to the steps shown in Figure 1

^b data taken from reference (17)