Catalytic Mechanism of Benzylsuccinate Synthase, a Theoretical Study

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Density functional theory calculations using the hybrid functional B3LYP have been performed to study the catalytic mechanism of benzylsuccinate synthase. This enzyme catalyzes the novel addition of the methyl carbon of toluene to fumarate, forming benzylsuccinate and thereby initiating the anaerobic metabolism of toluene in denitrifying bacteria. Benzylsuccinate synthase was suggested to contain a stable glycyl radical, based on sequence similarity to the two known glycyl radical containing enzymes pyruvate-formate lyase and class III anaerobic ribonucleotide reductase. This suggestion was recently confirmed by electron paramagnetic resonance experiments. The calculations demonstrate that an overall homolytic radical mechanism is thermodynamically very plausible. The radical is transferred from the stable glycyl radical to toluene via a cysteinyl radical in two hydrogen atom transfer steps. The rate-limiting step is shown to be the addition of benzyl radical to fumarate, forming a benzylsuccinyl radical intermediate. A full potential energy surface for the benzylsuccinate synthase reactions is presented.

I. Introduction

Benzylsuccinate synthase (BSS) belongs to the growing family of enzymes that utilize a glycyl radical in their catalytic process.[1−4] The first glycyl radical enzyme discovered and characterized was pyruvate formate lyase (PFL),[5] which catalyzes the reversible conversion of pyruvate and coenzyme A into formate and acetyl-coenzyme A. This was followed by the identification and characterization of the glycyl radical in class III anaerobic ribonucleotide reductase (ARNR),[6] which catalyzes the reduction of ribonucleotides to deoxyribonucleotides under strict anaerobic conditions.

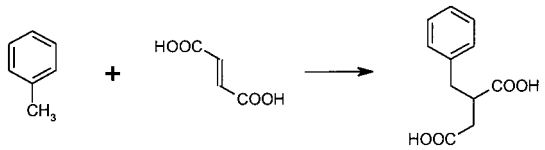
Benzylsuccinate synthase initiates the anaerobic toluene degradation in denitrifying bacteria by catalyzing the addition of toluene to fumarate, forming benzylsuccinate (Scheme 1).

BSS is composed of three nonidentical subunits ($\alpha_2\beta_2\gamma_2$) with molecular masses of 98, 8.5, and 6.6 kDa, for the α, β, and γ subunits, respectively.[7] The enzyme was first suggested to contain a glycyl radical based on strong homology between the α-subunit and PFL and ARNR. Very recently, the existence of the glycyl radical in BSS was confirmed by Krieger et al. using electron paramagnetic resonance spectroscopy.[8]

The three glycyl radical containing enzymes have in common that they are extremely oxygen sensitive. Exposure to oxygen is known to irreversibly inactivate the enzymes by cleavage of the polypeptide chain at the glycyl radical site, a process that has been studied in detail, both experimentally[9] and theoretically.[10] Also in common for the enzymes is that they are catalytically inactive when synthesized and are activated each by its own special activating enzyme.

The glycyl radical in these enzymes is remarkably stable and robust. This stability is usually explained using the so-called capto-dative effect,[11,12] which occurs when the radical center is located between an electron donor (the amino group) and an electron acceptor (the amide carbonyl). The combined effect of these two groups results in an enhanced radical resonance stability.

SCHEME 1: Reaction Catalyzed by Benzylsuccinate Synthase



The suggested mechanistic role of the glycyl radical in all three enzymes is to function as radical storage site. The glycyl radical is believed to abstract a hydrogen atom from a cysteine residue in the proximity, creating a transient thiyl radical, which then starts the substrate reactions. In the case of ARNR, the transient thiyl radical abstracts the 3′ hydrogen of the ribose, while in PFL the radical attacks the keto group of the pyruvate, forming a tetrahedral oxy radical intermediate.

In earlier work,[13] we showed that the peptide bonds from neighboring amino acids weaken the capto-dative effect, making the glycyl radical in proteins less stable. This way, the C−H bond strength of protein-bound glycine is perfectly tuned to function as a radical storage site. It is only a few kcal/mol more stable than the cysteinyl radical, making the latter readily accessible when needed upon substrate binding.

On the basis of what is known about BSS spectroscopically and biochemically,[14] the following mechanism has been proposed.[7,8] First, the stable glycyl radical (presumably at position 828 in *Azoarcus* sp. Strain T numbering) abstracts a hydrogen atom from a neighboring cysteine residue (presumably Cys492), creating the transient thiyl radical. The thiyl radical in turn abstracts a hydrogen atom from the toluene methyl, creating a benzyl radical intermediate. This benzyl radical then attacks the double bond of the fumarate, yielding a benzylsuccinyl radical intermediate, which is subsequently quenched by hydrogen atom transfer from the cysteine, giving the benzylsuccinate product.

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The cysteinyl radical then either abstracts a hydrogen atom from the glycine residue, regenerating the stable glycyl radical, or starts a new catalytic cycle by abstracting a hydrogen atom from a new toluene substrate.

In the present study, we use quantum chemical calculations to probe the energetics of this reaction mechanism. The method employed is the hybrid Hartree−Fock/density functional theory method B3LYP.[15] The same approach has previously been used to study the catalytic mechanisms of the other members of the glycyl radical enzyme family, PFL[16] and ARNR,[17] as well as numerous other enzyme mechanisms.[18]

II. Methods

All geometries and energies presented in the present study are computed using the B3LYP[15] density functional theory method as implemented in the Gaussian98 program package.[19] Geometry optimizations were performed using the double-ζ plus polarization basis set 6-31G(d,p), followed by single point energy calculation using the larger basis set 6-311+G(2d,2p). Spin densities reported are calculated using Mulliken population analysis. Hessians were calculated at the B3LYP/6-31G(d,p) level of theory. Hessians provide a control that the stationary points localized are correct, with no imaginary frequencies for minima and one imaginary frequency for transition states, and also allow evaluation of the zero-point vibrational effects on energy. Electrostatic solvent effects were modeled using the conductor-like solvation model COSMO[20] at the B3LYP/6-31G-(d,p) level. In this model, a cavity around the system is surrounded by polarizable dielectric continuum. The dielectric constant for the protein is chosen to the standard $\epsilon = 4$. The choice of the dielectric constant is somewhat arbitrary. However, the effects are seen to be rather small (less than 4 kcal/mol), and the energies should therefore not be very sensitive to the choice of ϵ. All energies discussed below include zero-point energy (ZPE) and solvation effects.

III. Results and Discussion

a. Chemical Models. In line with previous studies,[16−18] we use methylthiol (CH_3SH) to model the cysteine residue. This small model was found to be adequate to model the S−H bond strength correctly.

To model the C_α–H bond of glycine correctly, it was previously found to be necessary to include the peptide bonds at both sides of the C_α, as mentioned above.[13,16] The minimal model of protein-bound glycine is CHO−NH−CH_2–CO−NH_2 and is used in the present study.

Another point here is that we have chosen to work with charge-neutral models. This means that fumarate is modeled using fumaric acid, and accordingly, benzylsuccinate is modeled using benzylsuccinic acid. This can be justified by noting that proteins in general have low dielectric constants ($\epsilon \approx 4$), which means that charge separation, if present, should be very small, unless the system is specifically set up to create and enhance charge separation, as in, for example, photosystem II. Also, these kinds of charged carboxylate groups are often found to hydrogen bind to positively charged groups, such as arginine and lysine. Using gas-phase quantum chemical methods to model protein reactions involving this kind of charged groups, we have found that this approach yields accurate reaction energies and barriers, as for instance in the study of the related enzyme of PFL.[16]

b. Step 1. Creation of Thiyl Radical. The first step of the catalytic cycle proposed for BSS is the creation of a transient thiyl radical by hydrogen atom transfer to the glycyl radical. This step is a common initial step in the reaction mechanisms of glycyl radical enzymes. We calculate the barrier for this step to be 10.7 kcal/mol.[21] The reaction is slightly endothermic (3.4 kcal/mol); i.e., the glycyl radical is more stable than the cysteinyl radical, as previously found.[16] The optimized transition state structure (TS1) is given in Figure 1. The spin at the transition state is distributed mainly on the sulfur and the glycyl C_α centers (0.42 and 0.43, respectively). The critical C−H and S−H bond lengths are 1.42 and 1.55 Å, respectively.

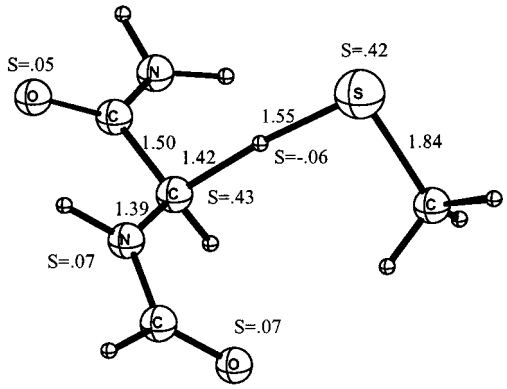


Figure 1. Optimized transition state structure (TS1) for the hydrogen atom transfer from cysteine to glycyl radical (step 1). Bond lengths are given in angströms.

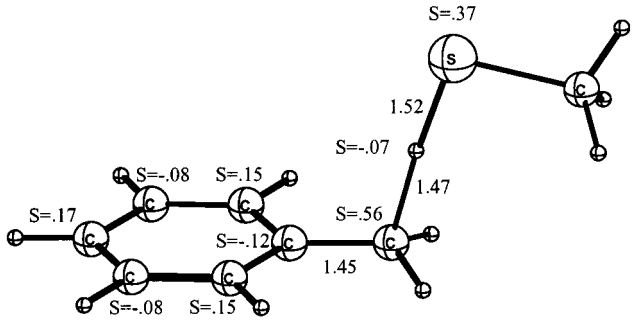


Figure 2. Optimized transition state structure (TS2) for the hydrogen atom transfer from toluene to cysteinyl radical (step 2).

c. Step 2. Formation of Benzyl Radical. Once the thiyl radical is formed, it is proposed to abstract a hydrogen atom from the methyl group of the toluene molecule. This step is expected to be feasible, since the resulting benzyl radical is stabilized by resonances similar to those in phenoxyl radical, lowering the toluene C−H bond dissociation energy. Indeed, the reaction of transferring a hydrogen atom from toluene to cysteinyl is calculated to be endothermic by 3.1 kcal/mol with a barrier of 9.5 kcal/mol. Added to the endothermicity of the previous step (3.4 kcal/mol), these energies become 6.5 and 12.9 kcal/mol, for the endothermicity and barrier, respectively. The optimized structure of the transition state (TS2) is displayed in Figure 2. The structure has one imaginary frequency of 1194 cm^{-1}, corresponding to the transfer of the hydrogen atom between the carbon and the sulfur. At TS2, the critical C−H bond is 1.47 Å and the S−H bond is 1.52 Å, both similar to the distances involved in the hydrogen atom transfer between glycine and cysteine (step 1). The spin is shared between the two species, the sulfur center of the cysteine having 0.37 of the spin and the methyl carbon of the toluene 0.56. The rest is of the spin is delocalized to the benzene ring in the familiar odd-alternant pattern.

d. Step 3. Addition of Benzyl Radical to Fumarate. In Steps 1 and 2, the radical is transferred from glycine to the toluene substrate, via the cysteine residue. The fact that these two steps are calculated to be endothermic is consistent with the finding

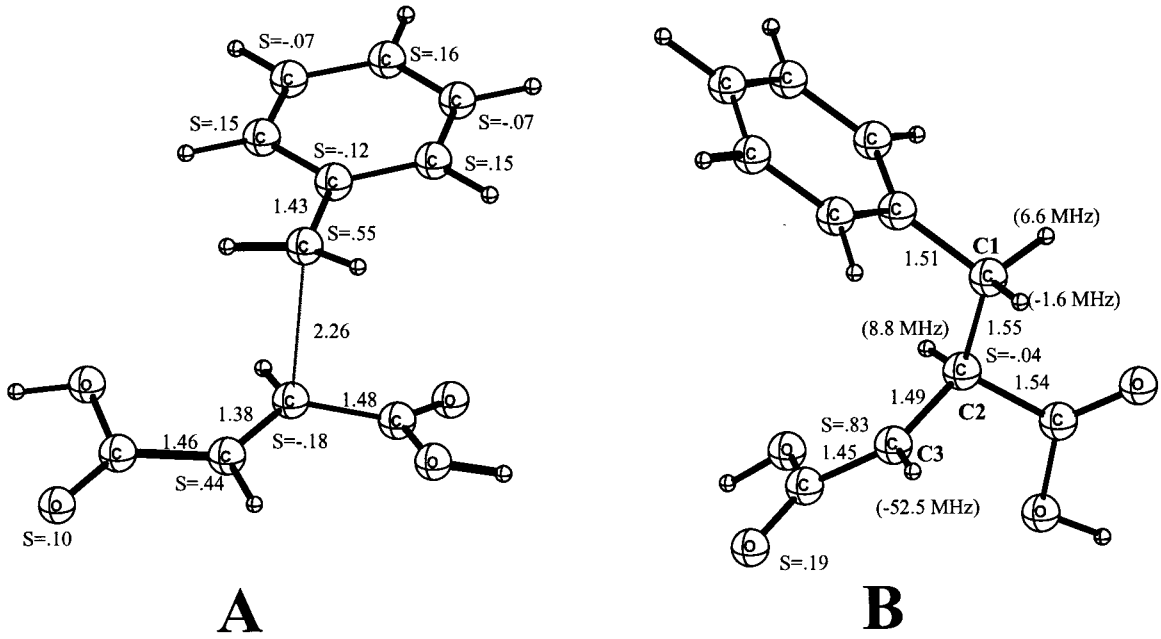


Figure 3. Optimized structures for (A) the transition state for addition of benzyl radical to fumarate (TS3), and (B) the resulting benzylsuccinyl radical (step 3). In parentheses, isotropic ^{1}H hyperfine coupling constants are given.

that incubation of BSS with toluene as sole substrate does not change the EPR spectrum of the glycyl radical.[8] That is, without fumarate present, the equilibrium remains at the glycyl radical.

The next step in the proposed mechanism is the benzyl radical attack on the double bond of the fumarate, forming benzylsuccinyl radical intermediate. We calculate the barrier for this reaction to be 8.5 kcal/mol, which, added to the total endothermicity of the previous steps (6.5 kcal/mol), yields a feasible total barrier of 15.0 kcal/mol. The reaction step is exothermic by 10.1 kcal/mol, bringing the energy down to 3.6 kcal/mol lower than the starting point of the reactions. Optimized structures of the transition state (TS3) and the benzylsuccinate radical intermediate are shown in Figure 3. TS3 has one imaginary frequency of 386 cm^{-1}, corresponding to the formation of the C−C bond. At TS3 the C−C bond to be formed has a length of 2.26 Å, and a considerable amount of unpaired spin has transferred to the fumarate.

In the resulting benzylsuccinyl radical, the spin is quite localized to the C3 carbon center (0.83); see Figure 3B for numbering. There is, however, an important resonance with the adjacent carbonyl group of the carboxylic moiety, which causes delocalization of the spin to the carbonyl oxygen (0.19). This resonance contributes to the stability of the benzylsuccinyl radical and hence the overall exothermicity of the reaction step.

In Figure 3B we also provide further characterization of the benzylsuccinyl radical intermediate by means of its isotropic proton hyperfine coupling constants, as calculated at the B3LYP/6-311+G(2d,2p) level. The largest coupling is found for the H3 proton with a magnitude of −52.5 MHz, reflecting the high spin concentration at that carbon. The H2 coupling is 8.8 MHz, while the methylene protons at C1 have couplings of 6.6 and −1.6 MHz. These methylene couplings are, however, strongly dependent on the rotational angle about the C1−C2 bond.

It is interesting to compare these values to the related steady-state radical intermediate observed in the lysine 2,3-aminomutase reaction.[22] This is a lysine-based α-carbon radical with adjacent carboxylate group, similar hence to the benzylsuccinate radical. The H_α (H3 in our numbering) coupling there was measured to be 60.8 MHz and the H_β (H2 in our numbering) to be 14.9 MHz. These couplings compare well with our calculated couplings of −52.5 and 8.8 MHz, respectively. Using the McConnell relation, the spin density at the C_α center was estimated to be ca. 0.8, in perfect agreement with our calculated 0.83.

e. Step 4. Quenching of Benzylsuccinate Radical. The benzylsuccinyl radical is calculated to be 5.8 kcal/mol less stable than the cysteinyl radical, i.e., to transfer a hydrogen atom from cysteine to benzylsuccinyl radical is exothermic by 5.8 kcal/mol. We calculate the barrier for this reaction to be the quite low, 7.0 kcal/mol. The optimized structure of the transition state (TS4) is shown in Figure 4A. The transition state has one imaginary frequency of −1076 cm^{-1}. The unpaired spin is again shared mainly between the sulfur center (0.36) and the carbon center (0.57). Also here, the oxygen of the adjacent carboxyl has some spin (0.10). The S−H and C−H distances are 1.49 and 1.52 Å, respectively, very similar to those in the two other transition states involving hydrogen atom transfer between a carbon center and a sulfur center (TS1and TS2).

f. Step 5. Regeneration of Glycyl Radical. At this point, the benzylsuccinate product is formed and the radical is at the cysteine residue. The cysteinyl radical can either initiate another catalytic turnover by abstracting a hydrogen atom from a new toluene molecule (step 2) or regenerate the glycyl radical to complete the catalytic cycle. The latter case is just the reverse of step 1 (TS1R). The reaction has, now in the backward direction, a barrier of 7.3 kcal/mol and an exothermicity of 3.4 kcal/mol.

An alternative to steps 4 and 5 could be a hydrogen atom transfer from glycine directly to the benzylsuccinyl radical, without the intermediacy of the cysteinyl radical, forming the benzylsuccinate product and regenerating the stable glycyl radical. This reaction has a calculated barrier and exothermicity of 17.5 and 9.2 kcal/mol, respectively. The barrier is much higher than the barrier for the hydrogen atom transfer from the cysteine residue (7.0 kcal/mol), clearly favoring the latter case. However, since the active site structure is unknown, the substrates could be bound to the enzyme in such a way that it is impossible to proceed through that pathway. The calculations here merely establish that with a barrier of 17.5 kcal/mol, a direct hydrogen atom transfer from the glycine residue to the

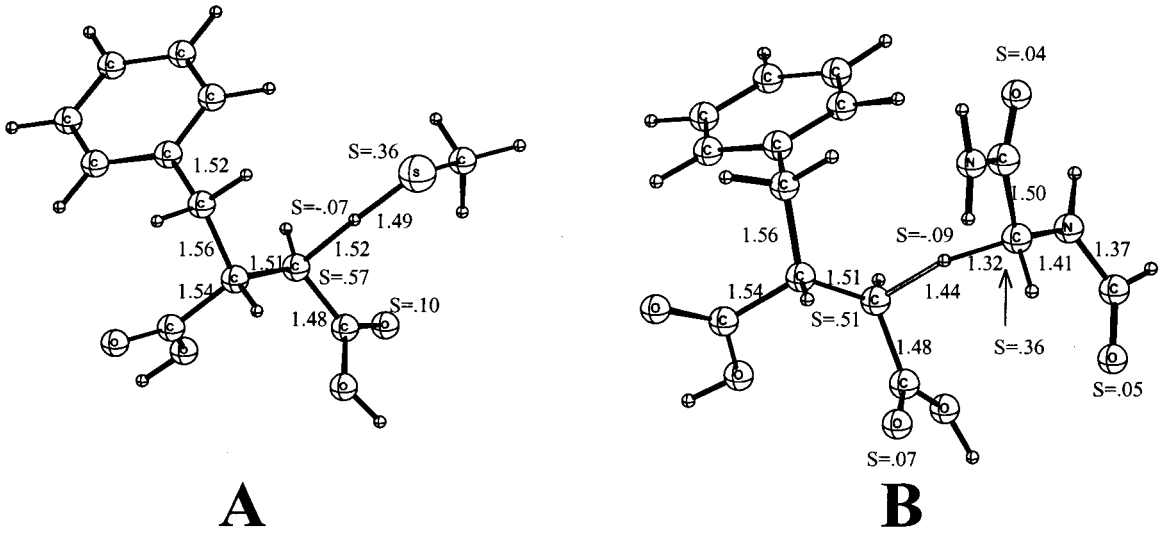
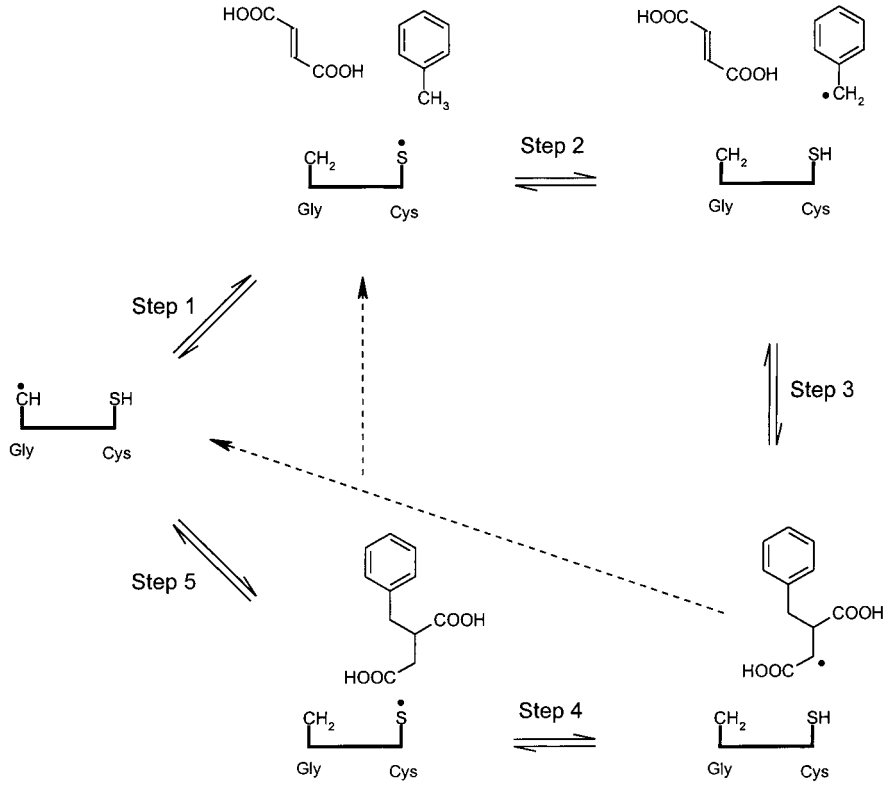


Figure 4. Optimized transition state structure for the hydrogen atom transfer from (A) cysteine (TS4) and (B) glycine (TS5) to benzylsuccinyl radical.

SCHEME 2: Summary of the BSS Reaction Mechanism Studied in the Present Paper[a]



[a] Dashed arrows indicate possible short cuts as discussed in the text.

benzylsuccinyl radical could be feasible, should that be dictated by the structural arrangement at the active site. The optimized transition state structure (TS5) is shown in Figure 4B. The critical C−H distances are 1.44 and 1.32 Å, and the spin is shared mainly between the two carbon centers. The structure has one imaginary frequency of 1627 cm^{-1}.

IV. Conclusions

We have in the present study examined the energetics of the catalytic mechanism of benzylsuccinate synthase by means of density functional theory. The steps considered are depicted in Scheme 2, and the calculated energies and barriers are summarized in Table 1 and displayed in Figure 5.

The calculations provide strong support for the experimentally proposed mechanism, in that all the steps have favorable reaction energies and activation barriers. The rate-limiting step is found to be the addition of benzyl radical to fumarate (step 3), with a total barrier of 15.0 kcal/mol.

Prior to that, to create the benzyl radical involved in the addition, we have shown that the two hydrogen atom transfer steps, form cysteine to glycyl and from toluene to cysteinyl, occur with relatively low barriers (10.7 and 12.9 kcal/mol, respectively). We have also demonstrated that quenching of the benzylsuccinate radical intermediate can be performed by hydrogen atom transfer from either cysteine or glycine. The former case is clearly favored over the latter thermodynamically,

TABLE 1: Summary of the Energies and Barriers (kcal/mol) Calculated for the Various Individual Reaction Steps of the BSS Mechanism

reaction	barrier	energy
Gly radical + Cys → Gly + Cys radical	10.7	3.4
Cys radical + toluene → benzyl radical + Cys	9.5	3.1
benzyl radical + fumarate → benzylsuccinyl radical	8.5	−10.1
benzylsuccinyl radical + Cys → benzylsuccinate + Cys radical	7.0	−5.8
Cys radical + Gly → Cys + Gly radical	7.3	−3.4
Benzylsuccinyl radical + Gly → benzylsuccinate + Gly radical	17.5	−9.2
Overall reaction: toluene + fumarate → benzylsuccinate	15.0[a]	−12.8

[a] Rate-limiting barrier.

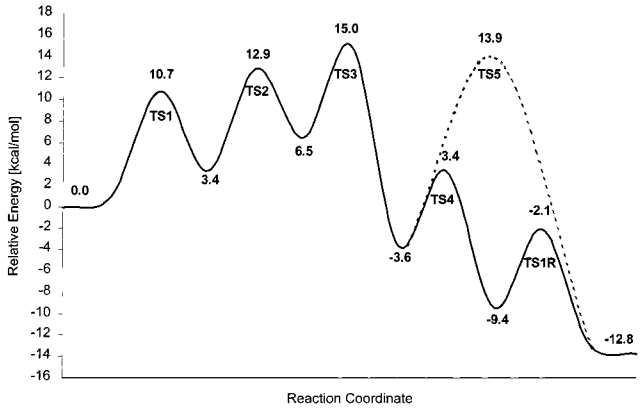


Figure 5. Potential energy surface for the reactions of benzylsuccinate synthase.

but since it is not known how the groups are arranged at the active site, both possibilities are viable. The overall reaction is exothermic by 12.8 kcal/mol, constituting a considerable driving force.

The relatively low barriers calculated in the present work for the C−C bond formation chemistry is a remarkable fact and testament to the ingenuity of nature in using radicals to carry out this kind of complicated chemistry that usually requires involvement of transition metal ions.

It is important here to remember that due to the lack of detailed structural information for BSS, we have in the calculations assumed that the reacting groups can move freely relative to each other. In the enzyme, the reacting groups might be strained one way or another, resulting in somewhat different reaction barriers and energies. This would not have any significant effect on the conclusions drawn the present work.

Another effect that we have neglected and that is likely to raise the barriers is entropy. This is particularly of concern for step 3, where two particles merge to one. We have reasoned in the following way. It can be argued that the entropy penalty is paid from the affinity when the two substrates bind to the enzyme. After that point, the enzyme−substrate complex can be considered as one particle. Also, accurate determination of the entropy effects is difficult using the quantum chemical methods employed here. We believe, thus, that calculating the entropies of these reactions, in particular without any structural information about the enzyme, will only serve to introduce more uncertainty to the results.

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