# A Birch-like Mechanism in Enzymatic Benzoyl-CoA Reduction: A Kinetic Study of Substrate Analogues Combined with an ab Initio Model†

Henrik Möbitz and Matthias Boll*

*Institut für Biologie II, Mikrobiologie, Universität Freiburg, Schänzlestrasse 1, D-79104 Freiburg, Germany*

*Received July 2, 2001; Revised Manuscript Received November 30, 2001*

ABSTRACT: Benzoyl-CoA reductase from the anaerobic bacterium *Thauera aromatica* catalyzes the ATP-driven two-electron reduction of the aromatic moiety of benzoyl-CoA. A Birch mechanism involving alternate one-electron and one-proton transfer steps to the aromatic ring was previously proposed for benzoyl-CoA reductase. Due to the high redox barrier, the first electron transfer step yielding a radical anion is considered the rate-limiting step in this reaction. Focusing on the mechanism of substrate reduction, this work combines the kinetic analysis of a number of substrate analogues with a model based on the ab initio calculated electron density of the radical anion of benzoyl-CoA, a transition state model of the proposed Birch mechanism. Both $K_m$ and $k_{cat}$ of ortho-substituted benzoyl-CoA increased in parallel with the substituent's acceptor strength (F > Cl = H > OH > $NH_2$). Among the isomers of monofluorobenzoyl-CoA, reduction rates decreased in the following order: ortho > meta > para; the $K_m$ values increased in the following order: meta > ortho > para. Five-ring heteroaromatic acid thiol esters were reduced in the following order: thiophene > furan > pyrrole; the 2-isomers are reduced much faster than the 3-isomers. Most of these results could be rationalized by the model. A Hammett plot indicated that the reaction mechanism is only slightly polar, suggesting the involvement of a partial protonation of the carbonyl oxygen of benzoyl-CoA and/or a simultaneous transfer of the first electron and proton. Surprisingly, benzoyl-CoA reductase exhibited a hydrogen kinetic isotope effect on $k_{cat}$ for pyridine-2-carbonyl-CoA (2.1) but only a negligible one for benzoyl-CoA (1.2), indicating that pyridine-2-carbonyl-CoA reduction proceeds according to a varied mechanism.

In organic chemistry, the so-called Birch reduction is a widely used synthetic tool for achieving selective 1,4-dihydro-addition in aromatic compounds (*1*). Due to the extremely negative single-electron redox potential, especially for aromatic systems that do not bear an acceptor substitutent, this reaction makes use of solvated electrons, the most potent reductant known (obtained by dissolving alkali metals in liquid ammonia). Taking into account these harsh and completely unphysiological reaction conditions, it was surprising to discover an enzyme catalyzing a similar reaction: benzoyl-CoA reductase catalyzes the two-electron reduction of benzoyl-CoA to cyclohexa-1,5-diene-1-carbonyl-CoA. Remarkably, the product of benzoyl-CoA reductase is a conjugated diene and not the expected 1,4-diene from a classical Birch reduction for which the protonation steps are kinetically controlled. The transfer of two electrons from ferredoxin to the substrate is coupled to the hydrolysis of two molecules of ATP to ADP and $P_i$ (*2*−*4*) (Figure 1). Benzoyl-CoA reductase catalyzes a reaction analogous to that of nitrogenase, which similarly couples ATP hydrolysis to electron transfer to overcome kinetic limitations. In addition, there are intriguing problems involved in the mechanism. Benzoyl-CoA reductase has to provide a very low-potential electron donor and at the same time shield this donor from an environment where protons abound. In analogy to the Birch reduction, a mechanism has been postulated that proceeds via alternate steps of one-electron and proton transfer and involves radical intermediates (Figure 2). In this reaction sequence, the first electron transfer to the aromatic substrate yielding a radical anion is considered rate-limiting. In a

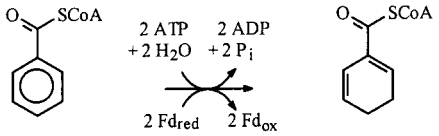


FIGURE 1: Reaction catalyzed by benzoyl-CoA reductase from *T. aromatica*. Benzoyl-CoA is reduced in a two-electron reduction, the electrons being supplied by ferredoxin, with two ATP molecules being hydrolyzed for each cyclohexa-1,5-diene-1-carbonyl-CoA molecule formed.

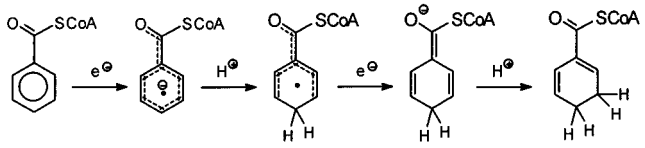


FIGURE 2: Postulated Birch mechanism for the reduction of benzoyl-CoA by benzoyl-CoA reductase. Transfer of the first electron is the rate-governing step with an in vitro redox potential of approximately −1.9 V, yielding the radical anion. Subsequent steps of proton and electron transfers give the product cyclohexa-1,5-diene-1-carbonyl-CoA.

† This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

* To whom correspondence should be addressed: Institut für Biologie II, Mikrobiologie, Universität Freiburg, Schänzlestr. 1, D-79104 Freiburg, Germany. Telephone: 44 7612032685. Fax: 44 7612032626. E-mail: boll@uni-freiburg.de.

10.1021/bi0113770 CCC: $22.00 
Published on Web 01/18/2002

previous study, the redox potential for this first step was −1.9 V as determined for the model compound *S*-ethylthiobenzoate (*4*). The thiol ester functionality has been suggested to play an essential role in this step by stabilizing a ketyl radical anion intermediate (*5*).

Benzoyl-CoA reductase serves as a central enzyme in the anoxic metabolism of aromatic compounds in bacteria; most of the known low-molecular mass aromatic compounds are converted to benzoyl-CoA (*6*). So far, the biochemistry of the 170 kDa protein benzoyl-CoA reductase has exclusively been studied in the denitrifying bacterium *Thauera aromatica*. In analogy to the Fe protein of nitrogenase, two subunits (29 and 49 kDa) form a module with two ATP-binding sites and an interfacially ligated [4Fe-4S] cluster. Amino acid sequence comparisons revealed that the molecular architecture of this module is most similar to the monodimeric activating enzyme (activase) of 2-hydroxyglutaryl-CoA dehydratase (*7*). Notably, a mechanism involving a ketyl radical has also been proposed for this enzyme system (*5*). The remaining subunits of benzoyl-CoA reductase (44 and 48 kDa) carry two further [4Fe-4S] clusters and are involved in binding benzoyl-CoA. The midpoint potentials of the three cysteine-ligated [4Fe-4S] clusters are in the range from −500 to −600 mV (*8*), still leaving a large gap to that of a thiol ester of benzoic acid (*4*). An EPR study of benzoyl-CoA reductase has identified an unsual $S = {}^{7}/_{2}$ high-spin state of a $[4Fe\text{-}4S]^{+}$ cluster. Its formation is strictly dependent on ATP hydrolysis and concomitant conformational changes (*8*). This cluster is likely to play a key role in the transformation of chemical into redox potential. It is important to notice that the ATP-dependent processes account for more than half of the catalytic cycle as determined in single-turnover studies (*9*). Even in the case of a complete conversion of the energy to be gained from hydrolysis of two molecules ATP, the maximal lowering of the redox potential of an electron would be approximately −1.0 V, still leaving a large gap to the potential required for the formation of the radical anion. Therefore, further activation of benzoyl-CoA by suitable solvation in the active site is required to enable electron transfer to this substrate. A "partial protonation" of benzoyl-CoA at its carbonyl moiety has been proposed to shift the required redox potential of the aromatic substrate to a more positive value (*5*). For a carbonyl compound that cannot be protonated at physiological pH, high-level ab initio calculations showed that partial protonation can lower the transition state barrier to almost the same extent as covalent protonation (*10*).

So far, no experimental evidence has been given for a Birch mechanism in enzymatic aromatic ring reduction. Our work presents a first approach, combining the kinetic data obtained for several substrate analogues of benzoyl-CoA reductase with the predictions derived from an ab initio calculation for the rate-limiting transition state. The model for the mechanism draws on the electron density of the radical anion as an approximation for the transition state and considers the effect of an acceptor substituent on $k_{cat}$ and $K_m$ in terms of its position at the aromatic moiety. The results that we obtained support what we term a "Birch-like" mechanism in benzoyl-CoA reductase catalysis, in which the transfer of the first electron is assisted by a partial protonation at the carbonyl atom and/or a simultaneous covalent protonation.

## EXPERIMENTAL PROCEDURES

*Growth of Bacterial Cells*. *T. aromatica* (DSM 6984) was grown anoxically at 28 °C in a mineral salt medium. 4-Hydroxybenzoate and nitrate in a ratio of 1:3.5 served as sole sources of energy and cell carbon. Continuous feeding of the substrates, cell harvesting, storage, and preparation of cell extracts were carried out as described previously (*11*).

*Protein Purification and Sample Storage.* Partial purification of benzoyl-CoA reductase from *T. aromatica* (wet cell mass of 200 g) was performed under strictly anaerobic conditions in a glovebox under an $N_2/H_2$ (95:5 by volume) atmosphere as described previously (*2*). The enzyme used througout this study was more than 90% pure. Protein samples were immediately frozen in liquid nitrogen and stored anaerobically at −196 °C for several months.

*Synthesis and Purification of Coenzyme A Thiol Esters*. Benzoyl-CoA was synthesized from CoA and benzoic acid anhydride (*12*). All other thiol esters were synthesized starting from the corresponding acids according to the general method of Gross and Zenk (*13*). The crude product of thiol ester synthesis was further purified on a preparative HPLC column (250 mm × 20 mm, Lichrosphere C-18, Merck), using similar conditions as in analytical HPLC (see below).

*Miscellaneous Syntheses.* 2-Cyanobenzoic acid was not available commercially and was therefore synthesized as reported previously (*14*). Its NMR spectrum was in accordance with that of the expected product. *N*-Methylpyridine-3-carboxylic acid was synthesized by adding 30 mmol of iodomethane in 5 mL of ethanol to 50 mL of a 0.4 M methylpyridine-3-carboxylate in 80% ethanol. After being stirred for 1 h at room temperature, the mixture was lyophilized. This crude product (HPLC, >95% conversion) was used for thiol ester synthesis.

*HPLC Analysis of CoA Thiol Esters and Products.* For the analysis of thiol ester purity, as well as for product analysis, samples were applied to an analytical RP-C18 column (120 mm × 4 mm, Grom-Sil 120 ODS-4 HE, 5 μM; using a Waters HPLC system), preequilibrated with 2% acetonitrile in 50 mM potassium phosphate (pH 6.8) at a flow rate of 1 mL/min. A gradient from 2 to 15% acetonitrile in the same buffer over the course of 25 min was applied. Elution was monitored with a photodiode array detector. To characterize each thiol ester, the retention times and spectra of the pure substances were recorded.

*Spectrophotometric Assay of Benzoyl-CoA Reductase (Oxidation of Reduced Methyl Viologen)*. The MgATP- and benzoyl-CoA-dependent oxidation of methyl viologen was measured in a spectrophotometric assay at 675 nm ($\epsilon_{MV[675]} = 4.9\ mM^{-1}\ cm^{-1}$) as described previously (*2*), applying 7.5 μmol of enzyme activity per minute per assay.

*Stoichiometry of Substrate-Dependent Methyl Viologen Oxidation*. The stoichiometry was measured spectrophotometrically at 730 nm [$\epsilon_{MV[730]} = 2.4\ mM^{-1}\ cm^{-1}$ (*15*)] as the change in absorption per amount of substrate applied. An ATP-regenerating system consisting of pyruvate kinase (1 unit) and phosphoenolpyruvate (10 mM) was added to the standard assay. For each substrate that was tested, at least four measurements of varying concentrations were performed. Only substrates that could be converted in <1 h were tested.

*Kinetic Analysis*. The reaction was studied under pseudo-first-order conditions with all substrates except the thiol ester present in saturating amounts (from here on, only thiol esters will be termed substrates). The kinetic parameters were determind by fitting the data to the Michaelis−Menten equation, which was followed well at low rates and low substrate concentrations (up to $2-5K_m$). To obtain one $K_m$ value, at least four different measurements at a minimum of four different substrate concentrations were performed; two independent measurements were performed (the standard deviations of both the apparent $K_m$ and $k_{cat}$ were in the range of 10%). $k_{cat}$ was determined at saturating amounts of substrate as $V_{max}$/[E]. As different aliquots from the same batch of enzyme exhibited small variations in specific activity, the $k_{cat}$ of benzoyl-CoA reductase/substrate is reported relative to the $k_{cat}$ of benzoyl-CoA reductase/benzoyl-CoA measured on the same occasion (typically 0.85 $s^{-1}$). The thiol ester concentration was determined before and after the measurements. In the case of substrates that are susceptible to hydrolysis, aliquots for three to four measurements were frozen in liquid nitrogen and kept at −20 °C until they were used. As pyridine-4-carbonyl-CoA exhibited a pH-dependent, nonenzymatic reduction, the pH was chosen to minimize it (pH 7.7).

*Kinetic Isotope Effect*. To test the possible involvement of a proton in the rate-determining step of the reaction, a determination of the rates in $H_2O$ versus $D_2O$ of benzoyl-CoA and pyridine-2-carbonyl-CoA reduction at the respective maxima was performed (under saturating substrate concentrations). The maxima were determined in the pH range of 6.5−8.0 and the pD range of 7.2−8.6 and found to be similar for both substances (pH 7.0 and pD 7.6) with the typical shift of 0.6 unit on changing to $D_2O$. For buffers in $D_2O$, the pD was adjusted with KOH in $D_2O$, where the reading of the glass pH electrode, $pH_{nom}$, was corrected by 0.40 unit, so that $pD = pH_{nom} + 0.40$ (*16*). Each rate was measured four times (two sets of measurements).

*Further Determinations.* Protein concentrations were determined according to the Bradford method, using BSA as a standard (*17*). Concentrations of thiol esters were assayed with Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)] on the hydrolyzed versus unhydrolyzed probe. Hydrolysis was quantitatively affected after 5 min at 25 °C by mixing the probe (approximately 1 mM) with 0.25 equiv (volume) of 4 M KOH.

*Computational Methods.* Optimum geometries and their energies were calculated with the B3LYP/6-31+G(d,p) hybrid Hartree−Fock/density functional method (*18*), using the GAUSSIAN94 program package (*19*). Preliminary studies on the B3LYP/3-21 level gave similar geometries but a negative electron affinity. The large CoA thiol ester moiety of benzoyl-CoA was modeled by a methylthiol ester group. No symmetry constraints were imposed during the calculations. Vibrational analyses were performed with the same method to confirm that the optimum structures were stationary points. In addition, different input structures with varying dihedral angles between carbonyl group and the benzene plane (0°, 30°, 60°, and 90°) led to the same structure. Electron densities and electrostatic potentials were calculated with the CUBE function of GAUSSIAN94 and visualized using the MOLEKEL program (*20*).

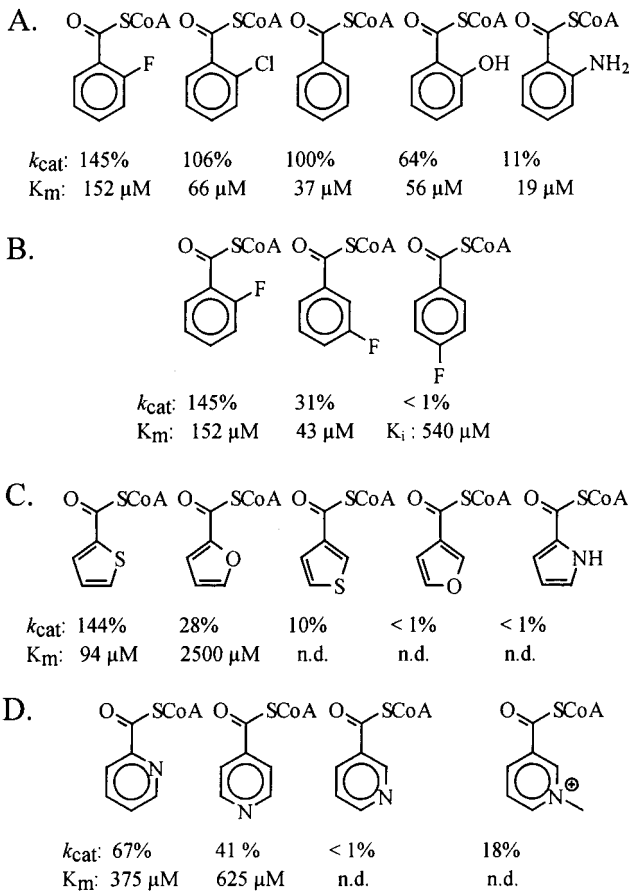


FIGURE 3: $k_{cat}$ of enzymatic reduction (relative to benzoyl-CoA) and apparent $K_m$ values for ortho-substituted benzoyl-CoA (A), for the position isomers of monofluorinated benzoyl-CoA (B), for various heteroaromatic five-ring CoA thiol esters (C), and for the position isomers of pyridine carbonyl-CoA (D).

## RESULTS

*Kinetic Properties of Ortho-Substituted Benzoyl-CoA.* To study the polar influence of substituents, 2-cyano-, 2-fluoro-, 2-chloro-, 2-hydroxy-, and 2-aminobenzoyl-CoA were synthesized and their kinetic properties were determined (Figure 3A). Both $k_{cat}$ and $K_m$ increased in parallel with the acceptor strength of the substituent. The acceptor substituent fluorine enhanced the reaction rate to 145%, whereas the rate with 2-chlorobenzoyl-CoA (106%) was similar to that of non-substituted benzoyl-CoA (100% refers to the rate of benzoyl-CoA reduction). In contrast, the donor groups hydroxy and amino slowed the rate to 64 and 11% of its original value, respectively. In contrast, variations in $K_m$ seem to be dominated by electronic effects. Introduction of, for instance, fluorine in the ortho position resulted in a 4-fold increase in the apparent $K_m$ (152 $\mu$M), despite the almost identical van der Waals radii of fluorine and hydrogen. Remarkably, the donor substituent $NH_2$ lowered the $K_m$ (19 $\mu$M) below that of benzoyl-CoA in spite of its bulkier size. For 2-cyanobenzoyl-CoA, a determination of the kinetic properties was not feasible due to a fast, nonenzymatic reduction of the substrate by methyl viologen. This reaction was first-order, obscuring the enzymatic reaction. The apparent $K_m$ was extrapolated to be above 2 mM. For the slowly reduced substrate 2-aminobenzoyl-CoA (11%), the apparent $K_m$ was confirmed by determination of its $K_I$ in a pseudoinhibition study. The

typical pattern of competitive inhibition was observed in the double-reciprocal plot (increasing slopes with increasing concentrations of 2-aminobenzoyl-CoA, converging on the ordinate; not shown).

*Kinetic Properties of the Isomers of Fluorobenzoyl-CoA.* To test the susceptibility of the different aromatic positions to electronic changes, the kinetics of the isomers of fluorobenzoyl-CoA were analyzed (Figure 3B). Reduction rates decreased in the following order: ortho > meta > para; the $K_m$ values decreased in the following order: meta > ortho > para. Unexpectedly, 4-fluorobenzoyl-CoA did not react at all. To probe whether the low reactivity of 4-fluorobenzoyl-CoA might be due to unfavorable binding properties, an inhibition study was performed. A competitive inhibtion was found for 4-fluorobenzoyl-CoA (apparent $K_I = 540$ $\mu$M), establishing that 4-fluorobenzoyl-CoA does indeed bind to benzoyl-CoA reductase, although with a lower affinity.

In previous studies, it has been shown that both 3-hydroxy- and 3-methylbenzoyl-CoA were converted by benzoyl-CoA reductase at 12% of the rate obtained for benzoyl-CoA (*2*). Together with the rate of 3-fluorobenzoyl-CoA reduction determined in this work (31%), the meta position shows less variation in $k_{cat}$ than the ortho position. Furthermore, the $K_m$ value for 3-fluorobenzoyl-CoA (43 $\mu$M) is hardly changed with respect to benzoyl-CoA (37 $\mu$M). This is consistent with the smaller susceptibility of the meta position for electronic variations derived in the model below. The trend of $K_m$ values of the three fluoro isomers is in accordance with the radical anion transition state model (see below and Discussion).

*Kinetic Properties of Five-Ring Heteroaromatic CoA Thiol Esters.* The kinetic properties of both isomers of thiophene and furan carbonyl-CoA, as well as pyrrole-2-carbonyl-CoA, were analyzed (Figure 3C). Surprisingly, thiophene-2-carbonyl-CoA (together with 2-fluorobenzoyl-CoA) proved to be the most reactive of all the substrates that were tested (144% relative rate), whereas the 3-isomer was converted at a rate that was only 10% of that of benzoyl-CoA. For the furan compounds, the 2-isomer (28%) was significantly more reactive than the 3-isomer. Both furan-3-carbonyl-CoA and pyrrole-2-carbonyl-CoA were not converted. All results were in accordance with the ease of reduction of five-ring heteroaromatic compounds found in Birch reduction (*21*). The significantly greater reactivity of the 2-substituted isomers compared to the 3-substituted isomers can be rationalized by the radical anion transition state model below. The variation of the $K_m$ for the almost isosteric 2-isomers of thiophene (94 $\mu$M) and furan (2500 $\mu$M) is yet another example of the strong influence of electronic changes on binding.

*Kinetic Properties of the Isomers of Pyridine Carbonyl-CoA.* As an additional approach to studying both polar and mesomeric effects, the kinetic properties of the isomers of pyridine carbonyl-CoA were analyzed (Figure 3D). The reduction rates decreased in the following order: ortho (67%) > para (41%) ≫ meta (<1%). All $K_m$ values were increased by more than 1 order of magnitude with respect to that of benzoyl-CoA, which may partly be due to an unfavorable interaction of the nitrogen lone electron pair. As presented in detail below, the reduced species might rather be the N-protonated form. This helps explain the strong variation in rates, which are in parallel with the redox potentials reported for an analogous group of compounds, the pyridinium cations (*22*). Of these, only the meta derivative does not form a stable one-electron adduct (like NADH) and is less easily reduced (*23*). From the pH dependence of the pseudo-first-order rates of the nonenzymatic reduction of pyridine carbonyl-CoA isomers by methyl viologen (data not shown), the same order of the redox potential was inferred: ortho > para ≫ meta. Yet, if the rate-limiting protonation is circumvented by N-methylation (equivalent to 100% protonation), even the meta derivative is reducible (18%) and its rate is increased at least 20-fold with respect to that of the nonmethylated form.

Table 1: Stoichiometry of Substrate Reduction to Methyl Viologen Oxidation[a]

| substrate | methyl viologen oxidized/ substrate applied |
|---|---|
| benzoyl-CoA | 1.9 ± 0.2 |
| furan-2-carbonyl-CoA | 1.8 ± 0.1 |
| 2-fluorobenzoyl-CoA | 1.9 ± 0.2 |
| 3-fluorobenzoyl-CoA | 2.0 ± 0.2 |
| pyridine-2-carbonyl-CoA | 2.2 ± 0.3 |

[a] In a photometric assay, the change in the extinction of methyl viologen was related to the amount of substrate added.

*Stoichiometry and Products of the Enzymatic Reduction of Alternative Substrates.* The reaction rates were calculated assuming a ratio of 2 methyl viologen oxidized per substrate added. So far, this has only been shown for benzoyl-CoA (*3*). To establish whether this holds true generally, the stoichiometry was determined for a number of substrates. The results (Table 1) were found to be in accordance with the assumed stoichiometry. Two-electron reduction seems to be a general feature of *T. aromatica* benzoyl-CoA reductase.

In an attempt to identify the nature of the products formed from some alternative substrates, HPLC and NMR techniques were employed. Unfortunately, it turned out that the dienes formed from these substrate analogues were extremely unstable, preventing their clear identification. In case of 2-fluorobenzoyl-CoA, a preliminary $^{19}F$ and $^{1}H$ NMR analysis of the single product that formed indicated the presence of a reduced fluorinated diene, possibly 2-fluorocyclohexa-1,5-diene-1-carbonyl-CoA (unpublished data). This result could not be fully confirmed due to a rapid degradation at 4 °C. 3-Fluorobenzoyl-CoA gave a single, even more unstable, product. In case of 2-amino- or 2-hydroxybenzoyl-CoA, the expected amino- and hydroxydiene products are converted further nonenzymatically into the corresponding cyclohexenone compounds (with the enamine intermediate being rapidly hydrolyzed) (*24*). In the case of the six- and five-ring heteroaromatic substrates, complex product patterns were observed by HPLC. This class of compounds is known to form fragmentation products during Birch reduction (*21*).

*Kinetic Isotope Effect.* To probe whether proton transfer is a rate-liming step in the mechanism of benzoyl-CoA reductase, the hydrogen kinetic isotope effects (KIEs),[1] $k_{cat,H}/k_{cat,D}$, for benzoyl-CoA and pyridine-2-carbonyl-CoA were determined. For benzoyl-CoA, the KIE was determined to be 1.2 ± 0.1, a value that can be ascribed to a pure solvent isotope effect. In contrast to this, pyridine-2-carbonyl-CoA

[1] Abbreviations: KIE, kinetic isotope effect; LFER, linear free energy relation; SOMO, singly occupied molecular orbital.

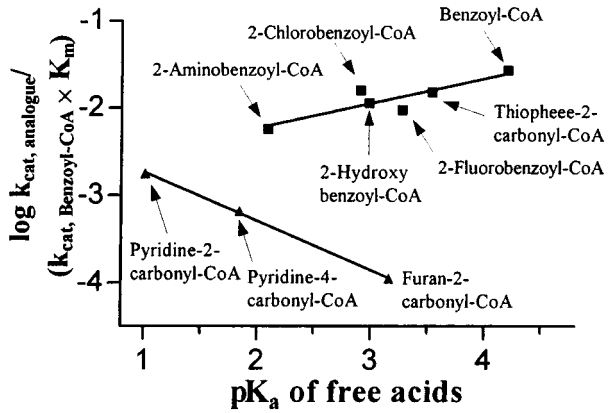


FIGURE 4: Hammett plot ($K_m$ in micromolar) for heteroaromatic (▲) and ortho-substituted (■) substrate analogues. With the exception of thiophene-2-carbonyl-CoA, the two classes of compounds form two distinct correlations. For that of the aromatic compounds, the slope was 0.28 ($r^2 = 0.78$), whereas for the heteroaromatics, it was −0.56 ($r^2 = 1.00$).

displays a total KIE of 2.1 ± 0.1. Although small, this effect is in the range of primary KIEs.

*A Hammett Plot for the Reduction of Different Substrates by Benzoyl-CoA Reductase.* In a Hammett plot, two correlations between log $k_{benzoyl-CoA-analogue}/k_{benzoyl-CoA}$ [where the rate constant $k$ is extrapolated to $k_{cat}/K_m$ ($K_m$ in micromolar)] and log $K_a$ values of the free acids could be obtained (Figure 4). As, in organic chemistry, substituent constants are only defined for the meta and para positions, we refer to original p$K_a$ values (*25*). The prerequisite of negligible entropic contributions is ascribed to the small extent of steric effects on the $K_m$ and the small steric requirements of both electron and proton transfers. Apparently, the compounds group into two separate correlations: one with a slope $\rho$ of 0.28 ($r^2 = 0.78$) and the other with a $\rho$ of −0.56 ($r^2 = 1.00$). Interestingly, the latter group consists of heteroaromatics only.

*Computational Studies on Benzoyl-CoA and Its Radical Anion.* As a basis for a rational understanding of the kinetic data in terms of a Birch mechanism, the optimum geometries and electron distributions for the benzoyl-CoA model benzoic acid methylthiol ester and its radical anion were calculated ab initio (Figure 5). The B3LYP/6-31+G(d,p) electron affinity of benzoic acid methylthiol ester was calculated to be 12 kcal/mol (no zero-point correction), which is in the range of the experimental value of benzaldehyde (9 kcal/mol) (*26*). Figure 5A shows the optimum geometry and the electron density of the benzoic acid methylthiol ester radical anion. The geometries of benzoic acid methylthiol ester (not shown) and benzoic acid methylthiol ester radical anion are both essentially planar, a finding that is consistent with earlier ab initio calculations of analogous acceptor-substituted benzene radical anions (*27*). The difference electron density between the neutral and radical anion state demonstrates how the density of the additional electron is distributed (Figure 5B). Notably, the additional electron is located preferentially at the carbonyl C, at the alternating ortho and para positions, and on the S and O atoms. The difference electron densities can be rationalized by a Jahn−Teller distortion along the pseudomirror plane perpendicular to the ring yielding a *p*-quinonoid system and by the inductive effects of the thiol ester group. Both effects result in a shortening of the bonds between the ortho and meta positions and the bond between the carbonyl group and the ring (whereas the other ring bonds and the carbonyl bond are stretched).

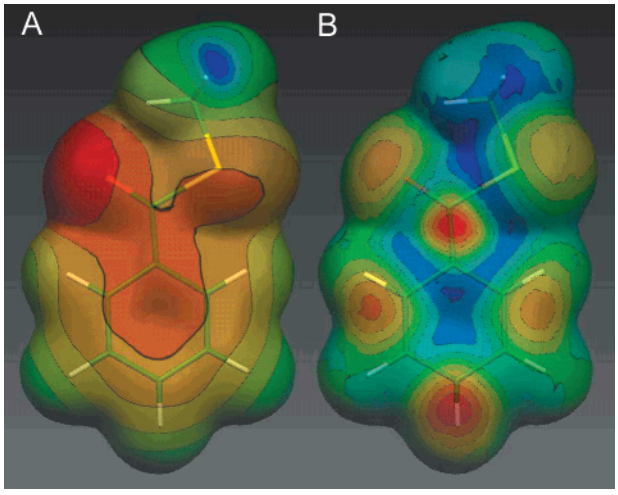


FIGURE 5: (A) B3LYP/6-31+G(d,p) optimum geometry and electron density of benzoic acid methylthiol ester radical anion. The electron density is illustrated by the electrostatic potential mapped onto the 0.002 electron/Bohr van der Waals surface of benzoic acid methylthiol ester radical anion (the electrostatic potential decreases from the red to the blue pole of the spectrum, i.e., red is negative charge, green neutral, and blue positive charge). The surface is rendered transparent to show the backbone. (B) Map of the difference electron density between benzoic acid methylthiol ester radical anion and BC mapped onto the 0.002 electron/Bohr van der Waals surface of benzoic acid methylthiol ester radical anion.

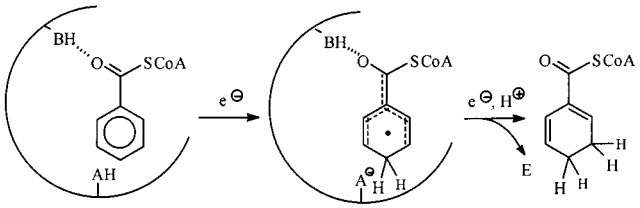


FIGURE 6: Modified, Birch-like mechanism of benzoyl-CoA reductase. Prior to reduction, the carbonyl oxygen is partially protonated by a weakly acidic proton. Simultaneously with the electron transfer to benzoyl-CoA, a bond to the para-attacking proton is formed. Finally, the cyclohexadienyl intermediate is reduced by a further electron and proton transfer.

## DISCUSSION

By virtue of its similarity in both geometry and energy, the benzoic acid methylthiol ester radical anion can be considered a good approximation of the transition state of the first step in the Birch-like mechanism of benzoyl-CoA reduction, the rate-limiting addition of an electron yielding a radical anion. Accordingly, the relative energies of the radical anions formed from different substrates are directly linked to their kinetic behavior. This model will also hold true if the transfer of the first electron is facilitated by a partial protonation at the carbonyl oxygen and/or if the first covalent protonation at the aromatic ring occurs more or less at the same time as the first electron transfer (see below and Figure 6). Although this would change the difference electron density, its pattern will be retained. In the following, we summarize how the kinetic data that we obtained support this model.

*Effect on $k_{cat}$.* A map of the electrostatic potential of benzoic acid methylthiol ester radical anion (Figure 5A)

shows that the electron density is larger in the ortho position than it is in the meta and para positions. Thus, the introduction of an electron-withdrawing group should have the greatest effect where maximal conjugation can be achieved, i.e., in the ortho position. Accordingly, these compounds should be reduced faster than benzoyl-CoA (vice versa for donor-substituted substrates) which is accordance with the kinetic data: donor substituents decrease the rate of reduction, whereas the two acceptor substituents increase it. While the model correctly predicts that the meta isomer of fluorobenzoyl-CoA is less reactive than the ortho isomer, one would expect it to be reduced at least as fast as benzoyl-CoA. In general, meta isomers of benzoyl-CoA seem to be less susceptible to the nature of the substituent (12% for 3-hydroxybenzoyl-CoA compared to 31% for 3-fluorobenzoyl-CoA), which can be understood in terms of the lower electron density. The lower reactivity of the meta and para isomers when compared with that of benzoyl-CoA might be due to the fact that this is the site where the protons appear in the product (*4*). In the case of a simultaneous protonation at these positions (see below), subsituents might decrease the overall rate.

For the configurational isomers of the thiol esters of these five-ring heteroaromatic compounds, an argument similar to that above applies. In their SOMO, the carbon atom adjacent to the heteroatom exhibits the highest electron density. Therefore, the electron-withdrawing effect of the thiol ester group exerts the greatest effect in the 2-isomers, which should be reduced faster than the 3-isomers.

*Effect on $K_m$.* Schramm and co-workers (*28*) have successfully related the binding constant of a substrate analogue to its electronic similarity with the transition state. The data obtained in this work provide evidence that this approach also suits benzoyl-CoA reductase: the substrate binding is dominated by electronic effects as demonstrated in a number of isosteric pairs of substrates. (The extremely slow turnover of benzoyl-CoA reductase allows us to interpret the $K_m$ essentially as a thermodynamic constant that reflects the binding of substrate.) Thus, electronic changes that make the neutral state of a substrate more similar to the electron distribution in the radical anion should enhance binding. As a consequence, introduction of an electron-donating group at the positions of the highest electron densities of the benzoic acid methylthiol ester radical anion (at the ortho and para positions; Figure 5B) renders the neutral substrate more like the radical anion and should enhance binding (vice versa for electron-withdrawing groups). According to the difference electron density, the effect should be greater in the para position than in the ortho position, whereas it should be less pronounced in the meta position. The effect of increased acceptor strength in the ortho-substituted benzoyl-CoA and the effect of the same substituent in position isomers on the $K_m$ are both in good accordance with this model.

*A Modified Hypothesis for the Mechanism of Substrate Reduction in Benzoyl-CoA Reductase Catalysis.* The Hammett plot shows that variations in $k_{cat}$ and $K_m$ can be ascribed to electronic changes of the alternative substrates of benzoyl-CoA reductase. While the effect is polar in the sense that increasing acceptor strength leads to an increase in $k_{cat}$ and $K_m$, the absolute effect is several orders of magnitude too small for the mechanism to involve a radical anion. In contrast, we propose an initial partial protonation of benzoyl-CoA at the carbonyl oxygen that in reducing the polarity of the reaction could explain the finding of the Hammett plot. In addition to this initial partial protonation, the transfer of the first electron and the covalent protonation of the ring should occur simultaneously (Figure 6). This would result in a kind of proton relay at the active site of benzoyl-CoA reductase which facilitates electron transfer by balancing the charge of the reaction. The Hammett plot suggests that the sequence of partial protonation, electron transfer, and covalent protonation might be altered for different types of substrates; the two slopes indicate that the polarity of the reaction mechanism for the three heteroaromatic substrates is inverted compared to that of the substituted aromatics. The electron rich heteroaromatic substrates allow a larger extent of protonation prior to electron transfer (e.g., the pyridine thiol esters could be protonated to yield a pyridinium cation). This assumption is supported by the KIE analysis. The KIE of benzoyl-CoA reductase for pyridine-2-carbonyl-CoA is in the range of a primary one, whereas the one for benzoyl-CoA is negligible. The latter finding might reflect an unsually high $\Delta pK_a$ in the covalent protonation of the aromatic ring. Small primary KIEs that are close to unity despite a proton transfer in the rate-limiting step have been reported for a number of NADH-dependent oxidoreductases (*29*), for which a simultaneous proton and single-electron transfer also is discussed.

The modified, Birch-like mechanism presented above circumvents the harshness of charge separation that was proposed in the previous mechanism and gives an improved idea of how the catalysis of benzoyl-CoA reduction can be achieved under physiological conditions.

## ACKNOWLEDGMENT

Georg Fuchs and Christoph Rüchardt are acknowledged for careful reading of the manuscript and valuable suggestions. We are grateful to Hans Dieter Beckhaus, Manfred Keller, and especially to Hendrik Zipse for their assistance with the calculations.

## REFERENCES

1. Birch, A. J., and Rao, S. (1972) *Adv. Org. Chem. 8*, 1−65.
2. Boll, M., and Fuchs, G. (1995) *Eur. J. Biochem. 234*, 921−933.
3. Boll, M., Albracht, S. J. P., and Fuchs, G. (1997) *Eur. J. Biochem. 244*, 840−851.
4. Boll, M., Laempe, D., Eisenreich, W., Bacher, A., Mittelberger, T., Heinze, J., and Fuchs, G. (2000) *J. Biol. Chem. 275*, 21889−21895.
5. Buckel, W., and Keese, R. (1995) *Angew. Chem. 107*, 1595−1598.
6. Heider, J., and Fuchs, G. (1997) *Eur. J. Biochem. 243*, 577−596.
7. Locher, K. P., Hans, M., Yeh, A. P., Schmid, B., Buckel, W., and Rees, D. C. (2001) *J. Mol. Biol. 307*, 297−308.
8. Boll, M., Fuchs, G., Meier, C., Trautwein, A., and Lowe, D. J. (2000) *J. Biol. Chem. 275*, 31857−31868.
9. Boll, M., Fuchs, G., and Lowe, D. J. (2002) *Biochemistry* (in press).
10. Smith, D. M., Golding, B. T., and Radom, L. (1999) *J. Am. Chem. Soc. 121*, 1383−1384.
11. Tschech, A., and Fuchs, G. (1987) *Arch. Microbiol. 148*, 213−217.
12. Schachter, D., and Taggert, J. V. (1976) *J. Biol. Chem. 203*, 925−933.

13. Gross, G. G., and Zenk, M. H. (1966) *Z. Naturforsch. 21b*, 683−690.
14. Aslam, H. M., Burden, A. G., Chapmann, N. B., Shorter, J., and Carton, M. (1981) *J. Chem. Soc., Perkin Trans. 2*, 500−508.
15. Fraisse, L., and Simon, H. (1988) *Arch. Microbiol. 150*, 381−386.
16. Schowen, R. L. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., and Northrop, D. B., Eds.) pp 64−99, University Park Press, Baltimore.
17. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
18. Stephens, P. J., Devlin, F. J., Chablowski, C. F., and Frisch, M. J. (1994) *J. Phys. Chem. 98*, 11623−11627.
19. Frisch, M. J., Trucks, G. W., Schlegel, H. B., Gill, P. M. W., Johnson, B. G., Robb, M. A., Cheeseman, J. R., Keith, T., Petersson, G. A., Montgomery, J. A., Raghavachari, K., Al-Laham, M. A., Zakrzewski, V. G., Ortiz, J. V., Foresman, J. B., Cioslowski, J., Stefanov, B. B., Nanayakkara, A., Challacombe, M., Peng, C. Y., Ayala, P. Y., Chen, W., Wong, M. W., Andres, J. L., Replogle, E. S., Gomperts, R., Martin, R. L., Fox, D. J., Binkley, J. S., Defrees, D. J., Baker, J., Stewart, J. P., Head-Gordon, M., Gonzalez, C., and Pople, J. A. (1995) *Gaussian 94*, revision E.2, Gaussian, Inc., Pittsburgh, PA.
20. Flükiger, P., Lüthi, H. P., Portmann, S., and Weber, J. (2000) *MOLEKEL*, version 4.0, Swiss Center for Scientific Computing, Manno, Switzerland.
21. Birch, A. L., and Slobbe, J. (1976) *Heterocycles 5*, 905−944.
22. Hermolin, J., Levin, M., Ikegami, Y., Sawayanagi, M., and Kosower, E. M. (1981) *J. Am. Chem. Soc. 103*, 4795−4813.
23. Kosower, E. M., Land, E. J., and Swallow, A. J. (1972) *J. Am. Chem. Soc. 94*, 986−987.
24. Feil, U. (1998) Ph.D. Thesis, University of Freiburg, Freiburg, Germany.
25. Dean, J. A., Ed. (1992) *Lange's Handbook of Chemistry*, 14th ed., Table 8.8, McGraw-Hill, New York.
26. Zlatkis, A., Lee, C. K., Wentworth, W. E., and Chen, E. C. M. (1983) *Anal. Chem. 55*, 1596.
27. Birch, A. J., Hinde, A. L., and Radom, L. (1980) *J. Am. Chem. Soc. 102*, 3370−3376.
28. Bagdassarian, C. K., Schramm, V. L., and Schwartz, S. D. (1996) *J. Am. Chem. Soc. 118*, 8825−8836.
29. Klinman, J. P. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., and Northrop, D. B., Eds.) pp 176−208, University Park Press, Baltimore.

BI0113770