Evidence for Quantum Mechanical Tunneling in the Coupled Cobalt−Carbon Bond Homolysis−Substrate Radical Generation Reaction Catalyzed by Methylmalonyl-CoA Mutase

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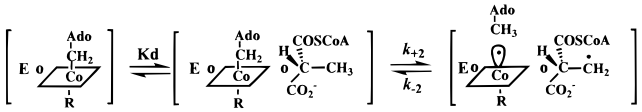
Methylmalonyl-CoA mutase catalyzes the coenzyme B_{12} or AdoCbl[1]-dependent rearrangement of methylmalonyl-CoA to succinyl-CoA.[2] In this and other AdoCbl-dependent isomerases, the role of the cofactor is to serve as a latent radical reservoir that is mobilized by homolysis of the Co−carbon bond during the catalytic cycle.[3] Previous stopped-flow studies from our laboratory on the homolytic cleavage step catalyzed by methylmalonlyl-CoA mutase revealed that it is kinetically coupled to hydrogen atom abstraction from the substrate (Scheme 1) and is

Scheme 1. Minimal Kinetic Scheme for Substrate-Induced Homolysis of the Co-C Bond by Methylmalonyl-CoA Mutase[a]



[a] The homolysis step is kinetically coupled to generation of substrate radical and is shown as one step here for simplicity. The kinetic measurements reported in this study do not permit distinction between the concerted and the (more likely) stepwise pathways as discussed previously.[6a]

characterized by an anomalously large primary deuterium isotope effect.[4] These studies were conducted at 25 °C where the very rapid homolysis rate precluded precise determination of its value, and an isotope effect of ≥20 was estimated. In this study, the magnitude of the primary deuterium isotope effect has been measured by conducting the stopped flow studies at lower temperatures. The deuterium isotope effect at 20 °C is 35.6. Analysis of the temperature dependence of k_H/k_D in an Arrhenius plot provides values for $E_{a_D} - E_{a_H}$ and the A_H/A_D ratio which are well outside the range for semiclassical behavior for traversing reaction barriers.[5] These data provide strong evidence for the involvement of hydrogen tunneling in the kinetically coupled homolysis−hydrogen atom abstraction step catalyzed by methylmalonyl-CoA mutase.

Presteady-state kinetic experiments were carried out under pseudo first-order conditions in an OLIS stopped-flow UV−visible spectrophotometer as described previously.[6a] These studies were conducted in the temperature ranges of 5−37 °C and 5−20 °C with the deuterated and protiated substrates respectively to determine the effect of substrate concentration on k_{obs}, the rate of decay of AdoCbl (Figure 1). Values for k_{+2}, the rate constant for Co−C bond homolysis, were obtained from nonlinear least-squares fits for the plots of k_{obs} versus substrate concentration using eq 1, where K_d and k_{+2} are described in Scheme 1. k_{+2} is a macroscopic rate constant describing the formation of cob(II)alamin and k_r is a macroscopic rate constant describing reformation of AdoCbl from cob(II)alamin.

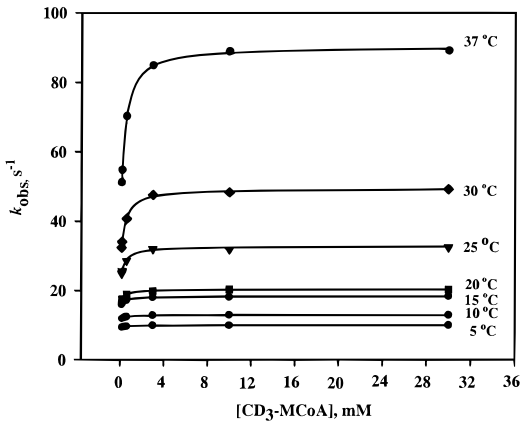


Figure 1. Substrate concentration dependence of k_{obs} as a function of temperature. Interpolated k_{obs} values were obtained from linear least-squares fits (with $r^2 \approx 0.999$) of the temperature dependence of k_{obs} at each substrate concentration using the Arrhenius equation as described previously.[6a] This procedure helps to minimize random errors in k_{obs} derived experimentally.[17] The lines represent nonlinear least-squares fits to eq 1 and provide values for k_{+2D} (Table 1).

$$k_{obs} = k_{+2}[S]/(K_d + [S]) + k_r \quad (1)$$

One of the criteria applied for detection of tunneling is measurement of the temperature dependence of the isotope effect.[5,7] The temperature dependence of the coupled Co-carbon bond homolysis rates in the presence of protiated (k_{+2H}) and deuterated (k_{+2D}) substrates were analyzed in an Arrhenius plot (Figure 2). The primary deuterium isotope effect increases from 35.6 at 20 °C to 49.9 at 5 °C (Table 1),[8] consistent with the inverse temperature dependence of isotope effects. The temperature dependence of the isotope effect (Figure 2, inset) was analyzed to obtain values

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(1) Abbreviations used: AdoCbl: 5′deoxyadenosylcobalamin, Co−carbon: cobalt−carbon, CoA: coenzyme A.

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(6) (a) Chowdhury, S.; Banerjee, R. *Biochemistry* **2000**, accepted for publication. Recombinant *Propionibacterium shermanii* methylmalonyl-CoA mutase was purified as described previously.[6b] The expression construct[6c] was provided by Dr. P. Leadlay at Cambridge. An external water bath was used to maintain the temperature (±0.5 °C) of the loading syringes in the mixing chamber. The syringes, tubing, and mixing chamber of the stopped flow apparatus were deoxygenated as described previously.[4] Holomethylmalonyl-CoA mutase (30 or 40 μM before mixing) in anaerobic 50 mM potassium phosphate buffer, pH 7.5, was mixed with an equal volume of the same reaction buffer containing variable amounts of (*R,S*)-[CD_3]-methylmalonyl-CoA (0.3 to 60 mM before mixing) and the change in absorbance at 525 nm was monitored. To approximate pseudo-first-order conditions, the concentration of the (*R*)-isomer was in 5- to 750-fold excess over enzyme. The rapid rate of AdoCbl disappearance with the protiated substrate precluded measurements above 20 °C. Experiments were conducted under reduced illumination. All experiments were run in duplicate with two independent preparations of enzyme. [CD_3]-methylmalonyl-CoA was synthesized as described previously[6d] and was >99.5 atom % enriched in deuterium. (b) Padmakumar, R.; Banerjee, R. *J. Biol. Chem.* **1995**, *270*, 9295−9300. (c) McKie, N.; Keep, N. H.; Patchett, M. L.; Leadlay, P. F. *Biochem. J.* **1990**, *269*, 293−298. (d) Padmakumar, R.; Padmakumar, R.; Banerjee, R. *Methods Enzymol.* **1997**, *279*, 220−224.

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(8) The deuterium isotope effects reported here may represent a lower limit. This is due to the possibility of a rapid equilibrium between AdoCbl and cob(II)alamin in the active site (Scheme 1) that would lead to a mixture of isotopomers at the 5′ position of deoxyadenosine and the substrate methyl group. In this case, k_{+2} would be a statistical average of the rates of protium and deuterium transfer coupled to Co−C bond homolysis. If, however, the forward commitment to catalysis is high and k_{-2} is small, then equilibration between the isotopomers would be less important.

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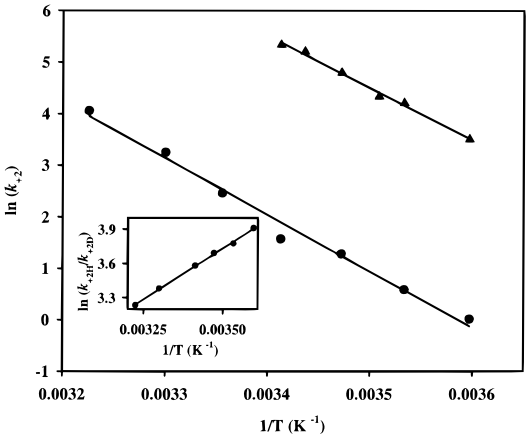

Figure 2. Arrhenius plot showing the temperature dependence of k_{+2H} and k_{+2D}. The values for k_{+2D} (●) were obtained in the present study, those for k_{+2H} (▲) had been determined previously.[6a] The lines represent linear least-squares fits to the Arrhenius equation and yielded values for k_{+2H} and k_{+2D} that are reported in Table 1 and were used to determine the primary deuterium isotope effect. (Inset) Arrhenius analysis of the temperature dependence of the kinetic isotope effect. The line represents a linear least-squares fit to eq 2 and yields values for A_H/A_D and $(E_{a_D} - E_{a_H})/R$ from the intercept and slope, respectively.

Table 1. Summary of the Kinetic Parameters for the Reaction Catalyzed by Methylmalonyl-CoA Mutase

temp. (°C)	k_{obs} (s^{-1})[a]	k_{+2H} (s^{-1})[b]	k_{+2D} (s^{-1})[b]	k_{+2H}/k_{+2D}
5	80 ± 4	44.4	0.89	49.9
10	116 ± 6	64.6	1.4	43.1
12	128 ± 9	82.8	1.9	42.7
15	180 ± 7	119.6	3.0	39.9
18	239 ± 6	171.5	4.6	37.2
20	267 ± 5	217.0	6.1	35.6

[a] k_{obs} values under V_{max} conditions (30 mM (*R*,*S*)-methylmalonyl-CoA after mixing, see Figure 1) are the average of four experiments. [b] These values were obtained from the respective best fit lines shown in Figure 2. The actual temperature ranges used for the studies with the protiated and deuterated substrates were 5−20 °C[6a] and 5−37 °C (this study), respectively.

for A_H/A_D and $E_{a_D} - E_{a_H}$ using eq 2, with the error analysis on these parameters being performed as described.[9]

$$\ln(k_{+2H}/k_{+2D}) = \ln(A_H/A_D) + (E_{a_D} - E_{a_H})/RT \qquad (2)$$

For semiclassical behavior, the ratio of the Arrhenius preexponential factors, A_H/A_D, is in the range of 0.7 to 1, and the difference in activation energies, $E_{a_D} - E_{a_H}$, is 1.15 kcal/mol.[5] Deviations from these limits such that A_H/A_D is <0.7 and $E_{a_D} - E_{a_H}$ is >1.15 kcal/mol (i.e., greater than the ground-state zero-point energy difference) are predicted for tunneling. For the coupled homolysis-hydrogen atom abstraction reaction catalyzed by methylmalonyl-CoA mutase, the values for A_H/A_D and $E_{a_D} - E_{a_H}$ are 0.078 ± 0.009 and 3.41 ± 0.07 kcal/mol, respectively. The difference in the activation enthalpies, $\Delta H^{\ddagger}_D$-$\Delta H^{\ddagger}_H$, is 5.9 kcal/mol, suggesting a role for protein dynamics in promoting hydrogen tunneling.[5b]

The first step in the reaction catalyzed by all AdoCbl-dependent isomerases, that is, homolysis of the Co−carbon bond, exhibits an ~10^{12}-fold rate enhancement with respect to the uncatalyzed reaction,[6,10] and its mechanism has been the subject of enduring debate. In methylmalonyl-CoA mutase,[4] glutamate mutase,[11] and ribonucleotide reductase,[12] strong kinetic evidence indicate that Co−carbon bond cleavage is kinetically coupled to formation of the next radical in the reaction pathway. In both methylmalonlyl-CoA mutase[4] and glutamate mutase,[11] anomalous primary deuterium isotope effects have been reported to be associated with this step. Very large isotope effects have also been observed for the hydrogen transfer from deoxyadenosine to the product radical in diol dehydratase[13] and ethanolamine ammonia lyase.[14] However, the basis for these large isotope effects have remained unknown although explanations involving branched pathways and protein-based radicals have been offered to account for the anomalies.[15] The temperature dependence of the kinetic isotope effect in the reaction catalyzed by methylmalonlyl-CoA mutase is clearly indicative of nonclassical behavior. The contribution of hydrogen tunneling to enzymatic reaction rates has been demonstrated in a limited number of cases.[16] To our knowledge, these data provide the first evidence for the involvement of quantum mechanical tunneling in a reaction catalyzed by an AdoCbl-dependent isomerase, and may be relevant to other AdoCbl-dependent isomerases in which large primary isotope effects have been measured.

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