When a Spectator Turns Killer: Suicidal Electron Transfer from Cobalamin in Methylmalonyl-CoA Mutase†

Monica D. Vlasie and Ruma Banerjee*

Department of Biochemistry, University of Nebraska, Lincoln, Nebraska 68588-0664

Received December 22, 2003; Revised Manuscript Received April 8, 2004

ABSTRACT: Methylmalonyl-CoA mutase belongs to the class of adenosylcobalamin (AdoCbl)-dependent carbon skeleton isomerases and catalyzes the rearrangement of methylmalonyl-CoA to succinyl-CoA. In this study, we have evaluated the contribution of the active site residue, R207, in the methylmalonyl-CoA mutase-catalyzed reaction. The R207Q mutation results in a 10^4-fold decrease in k_{cat} and >30-fold increase in the K_M for the substrate, methylmalonyl-CoA. R207 and the active site residue, Y89, are within hydrogen bonding distance to the carboxylate of the substrate. In the closely related isomerase, isobutyryl-CoA mutase the homologous residues are F80 and Q198, respectively. We therefore characterized the ability of the double mutant (Y89F/R207Q) of methylmalonyl-CoA mutase as well as of the single mutants (Y89F and R207Q) to catalyze the rearrangement of *n*-butyryl-CoA to isobutyryl-CoA. While none of the mutant enzymes is capable of isomerizing these substrates, the R207Q (single and double) mutants exhibited irreversible inactivation upon incubation with either *n*-butyryl-CoA or isobutyryl-CoA. The two products observed during inactivation under both aerobic and strictly anaerobic conditions were 5′-deoxyadenosine and hydroxocobalamin, which suggested internal electron transfer from cob(II)alamin to the substrate or the 5′-deoxyadenosyl radical. Deuterium transfer from substrate to deoxyadenosine demonstrated that the substrate radical is formed and is presumably the acceptor in the electron-transfer reaction from cob(II)alamin. These studies provide evidence for the critical role of active site residues in controlling radical reactivity and thereby suppressing inactivating side reactions.

Methylmalonyl-CoA mutase, the only member of the B_{12}-dependent isomerases found in both prokaryotes and in mammals (*1*−*3*), catalyzes the rearrangement of methylmalonyl-CoA to succinyl-CoA. This reaction represents an important route for converting catabolites of odd-chain fatty acids, branched-chain amino acids, and cholesterol to succinyl-CoA, which can then enter the Krebs cycle. In bacteria, the mutase is involved in the reverse metabolic direction, linking the production of propionate to succinate in a pathway first described by Wood and co-workers (*4*).

The closest relative of this enzyme, isobutyryl-CoA mutase, is involved in the rearrangement of isobutyryl-CoA to *n*-butyryl-CoA. A 42.9% identity and 65.3% similarity was reported between the α subunit of methylmalonyl-CoA mutase from *Propionibacterium shermanii* and the large subunit of isobutyryl-CoA mutase from *Streptomyces cinnamonensis* (*5*). Both enzymes catalyze the interchange between a hydrogen atom and the carbonyl-CoA on vicinal carbons (Figure 1).

Isobutyryl-CoA mutase is important in polyketide antibiotic-producing streptomycetes, like *S. cinnamonensis*, the main producer of the polyether antibiotic monesin A (*6*). Both butyryl-CoA isomers are involved in even-chain fatty acid and valine catabolism and can be converted to methylmalonyl-CoA (*7*, *8*). The biochemical pathways for interconversion of methylmalonyl-CoA isomers and butyryl-CoA isomers still remain to be discovered. The structural difference between methylmalonyl-CoA and isobutyryl-CoA is that the carboxylate in the former is replaced by a methyl group in the latter. This difference is reflected in the sequence of active site residues. Thus, sequence comparison reveals that most of the residues interacting with the substrate in methylmalonyl-CoA mutase are conserved in isobutyryl-CoA mutase, except for those located within hydrogen-bonding distance of the carboxylate portion of methylmalonyl-CoA (Figure 2).

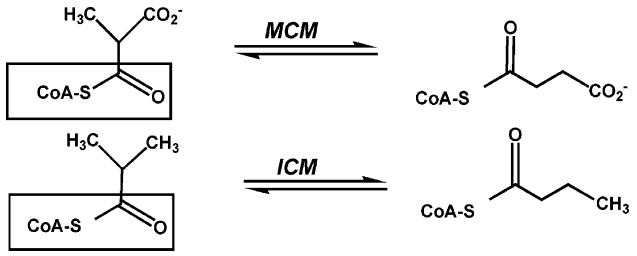


FIGURE 1: Reactions catalyzed by B_{12}-dependent methylmalonyl-CoA mutase and isobutyryl-CoA mutase.

The general reaction mechanism for methylmalonyl-CoA mutase (Scheme 1) involves an initial homolytic cleavage of the Co−C bond of the cofactor (step i) to form cob(II)-alamin and a highly reactive deoxyadenosyl radical. The latter abstracts a hydrogen atom from the substrate to form

† This work was supported by a grant from the National Institutes of Health (DK45776 and P20 RR17675). R.B. is an Established Investigator of the American Heart Association.

* Corresponding author. E-mail: rbanerjee1@unl.edu; tel: (402)-472-2941.

10.1021/bi036299q CCC: $27.50
Published on Web 06/08/2004

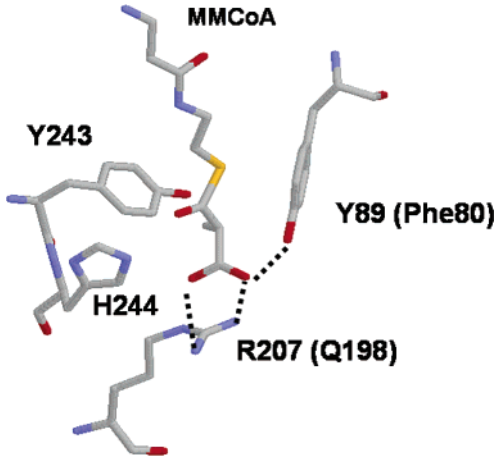
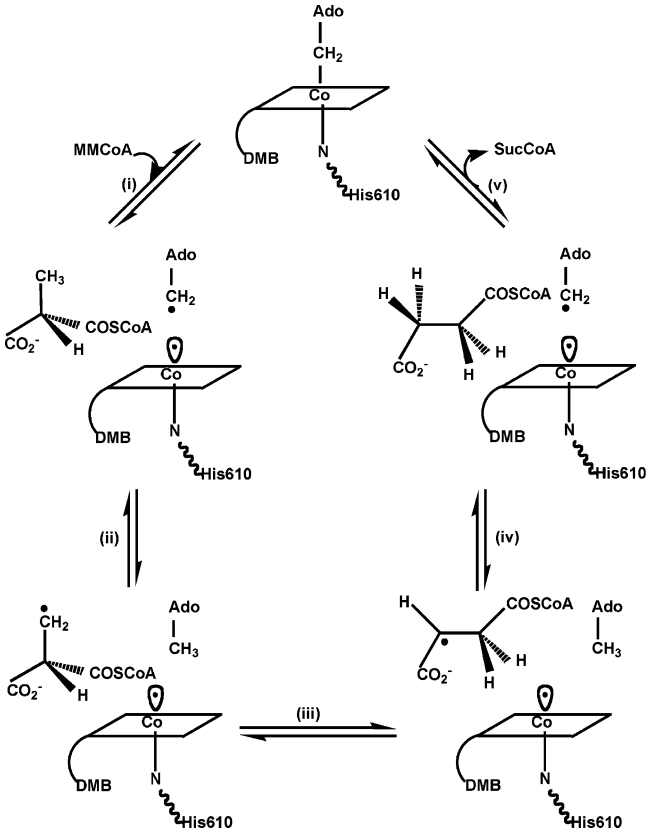


FIGURE 2: Active site residues interacting with the methylmalonate portion of the substrate in methylmalonyl-CoA mutase. In parentheses, are the corresponding residues that are different in isobutyryl-CoA mutase. The structure was drawn from the pdb file 4REQ.

Scheme 1



a substrate-centered radical (step ii), which then rearranges to a product-centered radical (step iii). A hydrogen atom is reabstracted from deoxyadenosine to form the product (step iv) followed by regeneration of the cofactor (step v). A key intermediate in this mechanism of action is the deoxyadenosyl radical, which shuttles radicals to and from the substrate and product, respectively. In contrast, cob(II)alamin formed in the initial step, is a "spectator" until the product is formed and recombines with the deoxyadenosyl radical at the end of the turnover cycle to regenerate the cofactor.

Two key issues in the mechanism of B_{12}-dependent isomerases are (i) controlling the timing of Co−C bond homolysis and (ii) controlling the reactivity of the radical intermediates formed during the reaction. Both these controls are important in preventing undesired side reactions which would lead to enzyme inactivation (*9*).

The crystal structure of methylmalonyl-CoA mutase in complex with the substrate (*10*, *11*) shows that the active site of this enzyme is deeply buried inside a TIM barrel structure, thereby shielding the highly reactive radical intermediates from solvent. Among the multiple interactions that the substrate makes with the enzyme, a few polar residues are involved in hydrogen bonds with the methymalonate moiety (Figure 2). Previous studies showed that mutation of H244, which interacts with the carbonyl-oxygen of the substrate thioester, makes the enzyme extremely sensitive to oxygen and lowers the protection of the reactive intermediates during catalysis (*12*, *13*). In contrast, mutation of Y89, which hydrogen bonds with the substrate-carboxylate, leads to an oxygen-stable enzyme. Furthermore, cob(II)alamin does not accumulate during turnover in these mutants rendering them less susceptible to oxidative side reactions (*14*).

The substrate carboxylate is held in the active site of the mutase by two hydrogen-bonding interactions with R207. Computational studies on this electrostatic interaction predict that a 3 kcal/mol destabilization of the succinyl-CoA radical and a 3 kcal/mol stabilization of the methylmalonyl-CoA radical would result from a negative charge on the carboxylate (*15*).

In this study, we report on the role of this active site residue in the methylmalonyl-CoA mutase-catalyzed reaction. R207Q has a large effect on the catalytic rate and on the affinity of the substrate, lowering the k_{cat} 10^4-fold and increasing the K_M for methylmalonyl-CoA >30-fold. Furthermore, the R207Q single mutant as well as the R207Q/Y89F double mutant designed to mimic the active site of isobutyryl-CoA mutase is susceptible to suicidal inactivation in the presence of isobutyryl-CoA or *n*-butyryl-CoA. A mechanism involving an internal electron transfer from cob(II)alamin to butyryl-CoA is proposed, which leads to irreversible inactivation in an oxygen-independent process.

EXPERIMENTAL PROCEDURES

Materials. Adenosylcobalamin (AdoCbl),[1] methylmalonyl-CoA, and *n*-butyryl-CoA were purchased from Sigma. [^{14}C]-CH_3-malonyl-CoA (56 Ci/mol) was purchased from New England Nuclear and butyric acid-d_7 was purchased from Aldrich. All other chemicals were reagent grade commercial products and were used without further purification.

Construction of Site-Specific Mutants. The plasmid vector pKOS116-95b containing the *P. shermanii* mutase gene was a gift from Kosan Bioscience (*16*). Site-directed mutants were created using the QuickChange kit (Stratagene) and the following sense primer for the R207Q: GGAGTTCATG-GTT*CAG*AACACCTACATCTACC. The mutagenic codon specifying Q is italicized. The antisense mutagenic primer had the complementary sequence. Following PCR amplification, a 1.6-Kb *Fse*I−*Apa*lI fragment containing the mutation was excised and ligated into pKOS116-95b digested with the same restriction enzymes. The entire 1.6-Kb fragment

[1] Abbreviations: AdH, adenine; dAdo, 5′-deoxyadenosine; AdoCbl, 5′-deoxyadenosyl adenosylcobalamin; *n*-BuCoA, *n*-butyryl coenzyme A; ICM, isobutyryl-CoA mutase; OHCbl, hydroxocobalamin.

was then sequenced to confirm the desired mutation. Construction of the Y89F mutant has been described previously (*14*). For construction of the Y89F/R207Q double mutant, the plasmids containing either the R207Q or Y89F mutations were digested with *Fse*I and *Hind*III. Following digestion, a 2-Kb and a 7.6-Kb fragment containing the R207Q and Y80F, respectively, were isolated and ligated back together. The presence of the two mutations was confirmed by nucleotide sequence determination.

Enzyme Expression and Purification. The tag-less mutant apoenzymes were purified using a modified procedure described previously for the purification of the wild-type enzyme (*17*). The *Escherichia coli* strain BL21(DE3) transformed with the pKOS plasmid (containing the desired mutation(s)) were grown at 37 °C in LB medium supplemented with 100 mg/L carbenicillin to an OD_{600} of 0.6. The culture was then induced at 27 °C with 1 mM IPTG and grown for an additional 5 h. The cells were harvested and then disrupted by sonication. The purification procedure preceding reconstitution with cofactor was the same as that described previously for the wild-type enzyme (*17*). Protein concentration was determined using the Bradford reagent (BioRad) with bovine serum albumin as a standard.

Enzyme Assays. Specific activity of methylmalonyl-CoA mutase was determined in the radiolabeled assay at 37 °C as described previously (*18*). 1 unit of activity catalyzes the formation of 1 μmol of succinyl-CoA min^{-1}. The concentration of the mutant enzymes was increased 1000- to 5000-fold in the standard assay with respect to the wild-type enzyme. The kinetic parameters for the mutants were determined by increasing the duration of the fixed timed assay from 3 to 10 min in the presence of varying concentrations of (*R*,*S*)-[^{14}C]-methylmalonyl-CoA (0.05−10 mM). The concentration of AdoCbl was kept constant in these assays and was always at least 10-fold in excess over the apoenzyme concentration.

Synthesis of Perdeuterated n-Butyryl-CoA. Perdeuterated *n*-butyryl-CoA was synthesized using a modification of the procedure described before for the synthesis of methylmalonyl-CoA (*19*). Briefly, the thiophenyl ester of [d_7]-butyric acid was prepared using dicyclohexylcarbodiimide as a condensing agent, followed by transesterification with coenzyme A. On the basis of HPLC analysis, >80% conversion of the coenzyme A to *n*-butyryl-CoA was observed. The product of this reaction was further purified by HPLC on a C_{18} reversed phase semipreparative column (250 × 10 mm) using a gradient of 0−32% acetonitrile in 0.1% trifluoroacetic acid. The purity of the product was assessed by ESI-MS in the positive ion mode. A single peak with *m*/*z* 845.1 was observed corresponding to *n*-[d_7]-butyryl-CoA.

UV−Visible Absorption Spectroscopy of Enzyme−Substrate Complexes. A solution of 30 μM AdoCbl-bound mutase in 50 mM potassium phosphate, pH 7.5, was made anaerobic by purging the sealed cuvette with oxygen-free argon, and the spectrum of the holoenzyme was recorded. In some experiments, sodium dithionite was added to a final concentration of 1.5 mM as an additional measure for ensuring anaerobiocity. Then, an anaerobic solution of methylmalonyl-CoA or *n*-butyryl-CoA or isobutyryl-CoA (to a final concentration of 4 mM) was added to initiate the reaction. The temperature was kept constant at 37 °C by a thermostated water circulator. The spectra were recorded at various times, with up to 4 h incubation of the enzyme with methylmalonyl-CoA and up to 2 h with *n*-butyryl-CoA or isobutyryl-CoA.

Determination of the Inactivation Rate Constant by UV−Visible Spectroscopy. The inactivation of R207Q and Y89F/R207Q mutant enzymes in the presence of *n*-butyryl-CoA was monitored by following the conversion of AdoCbl-bound enzyme to hydroxocobalamin (OHCbl). The reaction was monitored under aerobic or anaerobic conditions (as described above) at a constant temperature of 37 °C. The rate of inactivation was determined by plotting the absorbance at 351 nm versus time of incubation. The data were well fitted to a single-exponential growth function, $A = A_0 + a(1 - e^{-kt})$, where A is the absorbance at 351 nm, k is the rate constant for inactivation, and A_0 is the initial absorbance of the cofactor at 351 nm.

HPLC-Monitored Production of 5′-Deoxyadenosine and OHCbl. A solution containing 100 μM holoenzyme in potassium phosphate buffer, pH 7.5, was incubated with 4 mM *n*-butyryl-CoA at 37 °C in the dark. At various times ranging from 0 to 90 min, aliquots (10 μL) were removed and immediately quenched with 40 μL of 0.5% TFA. The quenched samples were centrifuged to remove precipitated protein. The decomposition products of AdoCbl during inactivation of the mutant enzymes 5′-deoxyadenosine and OHCbl were monitored by HPLC using a C_{18} reversed phase column with a gradient from 8 to 32% acetonitrile in 0.1% trifluoroacetic acid over 50 min at a flow rate of 1 mL/min. 5′-deoxyadenosine, OHCbl, and AdoCbl eluted at ∼10, ∼15, and ∼22% acetonitrile, respectively, under these conditions. Control reactions were performed in which either apoenzyme or *n*-butyryl-CoA was omitted from the reaction mixture. Elution was monitored at 254 and 350 nm, and the amount of each product formed was calculated from the area under the eluted peak relative to the area of known amounts of the respective standards. The data were well fitted with a single exponential-growth function for 5′-deoxyadenosine and OHCbl and a single exponential decay function for the AdoCbl cleavage, to obtain the rate constant for inactivation.

Attempts to Measure Rearrangement of n-Butyryl-CoA to Isobutyryl-CoA by the Mutant Enzymes. An anaerobic solution of 100 μM holoenzyme (wild-type, Y89F, R207Q, or Y89F/R207Q) was mixed with *n*-butyryl-CoA to a final concentration of 4 mM. The reaction mixture was incubated in the dark, at 37 °C for up to 2 h. Aliquots (50 μL) were withdrawn at various times and quenched with 100 μL of 0.5% trifluoroacetic acid. After centrifugation, the samples were analyzed by HPLC using a gradient ranging from 8 to 32% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 mL min^{-1}. Under these conditions, isobutyryl-CoA and *n*-butyryl-CoA standards eluted with retention times of 27 and 28 min, respectively.

Characterization of 5′-Deoxyadenosine and n-Butyryl-CoA during Inactivation of R207Q and Y89F/R207Q. A solution of 300 μM holoenzyme was mixed in the dark at 37 °C with 400 μM *n*-butyryl-CoA or *n*-[d_7]-butyryl-CoA in a total volume of 100 μL. After a 4 h incubation, the reaction was quenched with 200 μL of 0.5% TFA and centrifuged to remove the protein. The 5′-deoxyadenosine and *n*-butyryl-CoA were purified by HPLC on a C_{18} reversed phase column using the method described above. The fractions were pooled

Table 1: Comparison of the Kinetic Parameters for Wild Type versus Mutant Enzymes

	wild type	Y89F	R207Q	Y89F/R207Q
k_{cat} (min^{-1})	7200[a]	7.2 ± 1.2[a]	0.702 ± 0.08	0.45 ± 0.06
K_M,MMCoA (μM)	133 ± 37[a]	357 ± 83[a]	>4000	ND
k_{OHCbl} (min^{-1})			(0.027 ± 0.005)[b]	
spectra			0.026 ± 0.001	0.02 ± 0.002
HPLC			0.030 ± 0.003	0.013 ± 0.002
k_{Ado} (min^{-1})			0.036 ± 0.002	0.021 ± 0.001
k_{AdoCbl} (min^{-1})			0.034 ± 0.004	0.030 ± 0.002

[a] The values were taken from ref *14*. [b] The rate of OHCbl formation was measured spectrophotometrically under anaerobic conditions.

and dried by lyophilization and further characterized by ESI-MS in a positive ion mode.

Inactivation experiments in D_2O were performed under the same conditions as described above. To ensure that >80% of the exchangeable protons are replaced by deuterium, the enzyme stock solution was washed with 10 volumes of potassium phosphate buffer, pH 7.5 prepared in D_2O prior to the start of the reaction.

Stopped-Flow Scanning UV−Visible Spectroscopy. Pre-steady-state kinetic experiments were carried out in an OLIS stopped-flow UV−visible spectrophotometer equipped with a diode array detector (Applied Photophysics). An external water bath was used to maintain the temperature at 37 °C. Holoenzyme, (R207Q or Y89F/R207Q (100 μM before mixing)) in 50 mM potassium phosphate buffer, pH 7.5, was rapidly mixed with an equal volume of the same buffer containing either (*R*,*S*)-methylmalonyl-CoA (18 mM before mixing) or with *n*-butyryl-CoA (8 mM before mixing) and the spectral changes were recorded between 200 and 800 nm. Scanning was performed at a rate of 800 scans/s, and the reaction time was varied from 1 s to 10 min.

RESULTS

Steady-State Kinetic Properties of the Mutants. The mutant enzymes were expressed at levels comparable to that of wild-type protein and were purified to near homogeneity under the same conditions. Both mutations, Y89F and R207Q, have a profound effect on catalysis (Table 1). Thus, for the Y89F mutant, k_{cat} for the conversion of methylmalonyl-CoA to succinyl-CoA is decreased 1000-fold, whereas for the R207Q and Y89F/R207Q mutants the k_{cat} is further diminished 10 000- and 20 000-fold, respectively. Neither of the R207Q mutant enzymes appear to affect cofactor binding (data not shown) but the affinity of the substrate, methylmalonyl-CoA, is greatly reduced for the R207Q and Y89F/R207Q mutations ($K_M \geq 4$ mM) versus the wild-type enzyme (133 μM) or the single mutant, Y89F (357 μM).

UV−Visible Spectrum of the Mutant Enzymes in Complex with the Substrate and with n-Butyryl-CoA. The spectrum of the wild-type enzyme in the presence of methylmalonyl-CoA under steady-state turnover conditions shows accumulation of cob(II)alamin (*20*). Although all three mutants, Y89F, R207Q, and Y89F/R207Q exhibit isomerase activity, none showed any significant change in the spectrum in the presence of methylmalonyl-CoA under steady-state turnover conditions, even after prolonged incubation with the substrate

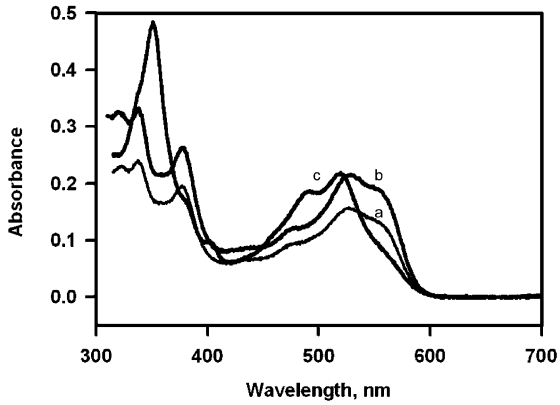


FIGURE 3: UV−visible spectra of Y89F (a), wild-type (b), and R207Q (c) enzyme in the presence of *n*-butyryl-CoA. The spectra were taken after a 1 h incubation of the enzymes (30 μM in 50 mM potassium phosphate buffer, pH 7.5) with 4 mM *n*-butyryl-CoA.

(up to 4 h) at 37 °C. Interestingly, in the presence of either *n*-butyryl-CoA or isobutyryl-CoA, the R207Q and Y89/R207Q mutants showed conversion of AdoCbl to OHCbl, signaling inactivation of the enzyme (Figure 3). When the same reactions were performed under strictly anaerobic conditions, i.e., by purging the solutions with oxygen-free argon and in the presence of the reducing agent, sodium dithionite as described under Experimental Procedures, the same spectral changes were observed with the R207 mutants. In contrast, no changes in the spectrum of the wild-type and Y89F mutant were observed in the presence of *n*-butyryl-CoA even after 4 h incubation (Figure 3). Thus, formation of OHCbl was observed only when R207 was mutated.

OHCbl represents an inactive cofactor form and does not support activity of the wild type or the mutant enzymes used in this study (not shown). Furthermore, OHCbl binds tightly to methylmalonyl-CoA mutase and does not exchange with free AdoCbl (present in the assay mixture) under the assay conditions. The R207Q mutant but not the wild-type enzyme lost activity when preincubated with butyryl-CoA (not shown), consistent with formation of OHCbl in the mutant.

Kinetics of OHCbl and 5′-Deoxyadenosine Formation. The time-dependent formation of OHCbl from AdoCbl in the R207Q mutant was followed by UV−visible spectroscopy (Figure 4) and is indicated by the increase in absorbance at 351 nm together with the blue-shift in the α/β bands for the cobalamin cofactor. OHCbl formation was measured under both aerobic and anaerobic conditions, and the rates were the same within experimental error (Table 1). The same rate of OHCbl formation was obtained when the R207Q mutant was incubated with 4 mM of isobutyryl-CoA (data not shown). Clear isosbestic points were observed during conversion of AdoCbl to OHCbl and indicate that no intermediate accumulates to detectable levels during inactivation.

The rate of inactivation of methylmalonyl-CoA mutase containing the R207Q mutation was also measured in the presence of perdeuterated butyryl-CoA and was shown to be ~7-fold slower than that in the presence of the protiated butyryl-CoA (Figure 5). The rate of OHCbl formation with butyryl-CoA was unchanged when the reaction was conducted in deuterated versus protiated buffer.

The other product formed during inactivation in the presence of *n*-butyryl-CoA was identified by HPLC analysis

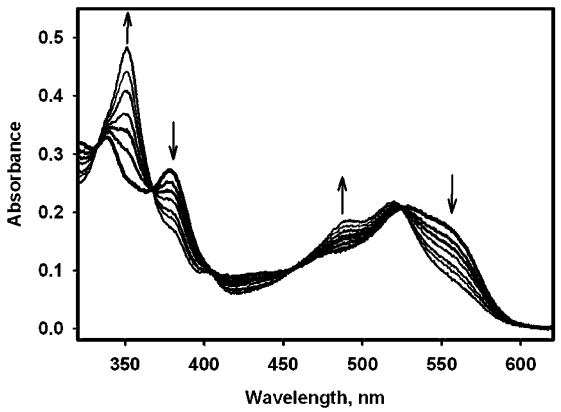


FIGURE 4: UV−visible spectral changes induced by the reaction of Y89F/R207Q with *n*-butyryl-CoA. 25 μM holoenzyme in 50 mM potassium phosphate buffer, pH 7.5 was incubated with 4 mM *n*-butyryl-CoA, in the dark, at 37 °C. The spectra were recorded between 5 and 60 min.

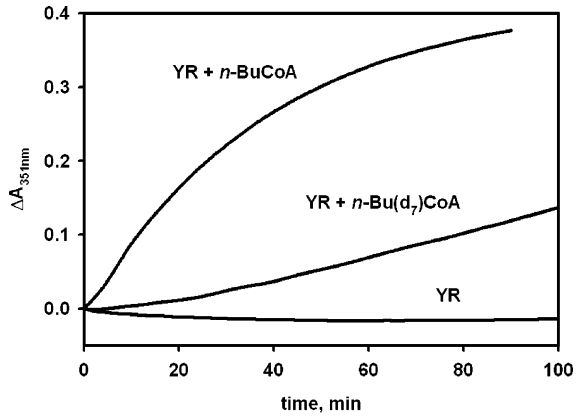


FIGURE 5: Isotope effect on OHCbl formation during inactivation of Y89F/R207Q in the presence of butyryl-CoA. Enzyme (30 μM in 50 mM potassium phosphate buffer, pH 7.5) was mixed with *n*-butyryl-CoA (4 mM) or perdeuterated *n*-butyryl-CoA (4 mM), at 37 °C and OHCbl formation was monitored by the increase in absorbance at 351 nm. A control experiment, in which the holoenzyme alone was monitored at the same wavelength, was also performed. A 7-fold difference was observed from the linear fits of the initial velocities in the presence of protiated vs deuterated butyryl-CoA.

to be 5′-deoxyadenosine. The rate of appearance of 5′-deoxyadenosine was similar to that of OHCbl formation (Figure 6).

Attempts to Measure Interconversion of n-Butyryl-CoA and Isobutyryl-CoA by Methylmalonyl-CoA Mutase. Previously, a gas chromatographic assay was reported to follow the interconversion of *n*-butyryl-CoA and isobutyryl-CoA by isobutyryl-CoA mutase (*5*, *21*). Here, we describe an alternative assay using reverse-phase HPLC. The assays conditions were such that production of isobutyryl-CoA would have been easily detected in a single turnover of the enzyme. However, no rearrangement of either isomer of butyryl-CoA was detected after a 2 h incubation for the R207Q mutants (during which time OHCbl formation was observed) or after 10 h for the wild-type and Y89F mutant enzymes. Thus, butyryl-CoA is not a substrate for wild-type methylmalonyl-CoA mutase or the Y89F/R207Q mutant (*5*, *21*).

Stopped-Flow Scanning Spectroscopy for the R207Q and Y89F/R207Q. In principle, the mutant enzymes could undergo inactivation leading to formation of OHCbl by two

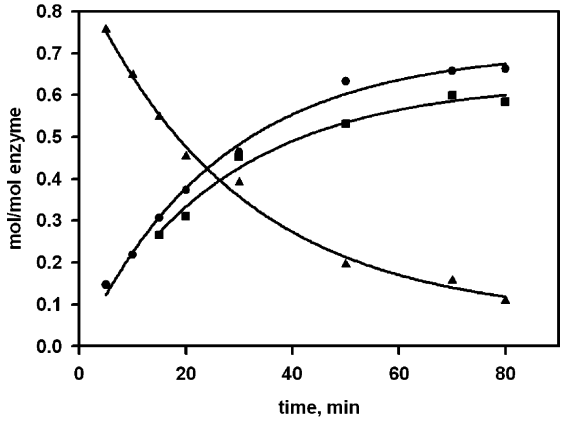


FIGURE 6: Formation of OHCbl and 5′-deoxyadenosine during suicide inactivation of R207Q in the presence of *n*-butyryl-CoA. The decay of AdoCbl (triangles) to OHCbl (squares) is accompanied by the formation of deoxyadenosine (circles). The kinetics of OHCbl and 5′-deoxyadenosine formation and AdoCbl decay were measured by HPLC as described under Experimental Methods. The rate constants from these data are listed in Table 1.

different pathways: the Co−carbon bond of the cofactor could be cleaved heterolytically or homolytically (Scheme 2). Our analysis reveals that the major products formed during cleavage of the cofactor are 5′-deoxyadenosine and OHCbl, thereby ruling out the heterolytic pathway as a plausible mechanism for the Co−C bond cleavage. In B_{12}-dependent lysine-5,6-aminomutase, Frey and co-workers also observed formation of OHCbl and 5′-deoxyadenosine during inactivation by a substrate analogue, consistent with an initial homolytic cleavage of the Co−carbon bond (*22*).

We attempted to measure cob(II)alamin formation by the R207Q mutants by rapid scanning stopped-flow spectroscopy in the presence of either *n*-butyryl-CoA or the true substrate, methylmalonyl-CoA. Although cob(II)alamin formation was observed over a 10-min time period, control experiments in which the enzyme was rapidly mixed with buffer showed the same spectral changes (not shown). Thus, we concluded that the formation of cob(II)alamin under these conditions is due to photolysis of the sample exposed to the high energy xenon lamp of the diode-array of the detector than due to homolysis of the Co−C bond during the reaction.

Reaction of the R207Q Mutants in D_2O with Protiated and Deuterated n-Butyryl-CoA. Following homolysis of the Co−carbon bond, an internal electron transfer could occur from cob(II)alamin to the deoxyadenosyl radical, leading to the formation of a carbanion which would be quenched by protonation (Scheme 2B, upper). Alternatively, a hydrogen atom could be abstracted from *n*-butyryl-CoA by the deoxyadenosyl radical to form a substrate radical which subsequently accepts an electron from cob(II)alamin to form a carbanion that is quenched by protonation (Scheme 2B, lower). Interception of the radical intermediates by oxygen can be ruled out since inactivation of the R207Q mutant enzymes occur under both aerobic and strictly anaerobic conditions at similar rates. To distinguish between electron transfer occurring to the deoxyadenosyl versus substrate radical, the reactions were conducted in deuterated buffer. The prediction was that deuterium incorporation would label the product (deoxyadenosine or substrate) to which electron transfer from cob(II)alamin had occurred. However, we were unable to detect by mass spectrometry incorporation of

Scheme 2

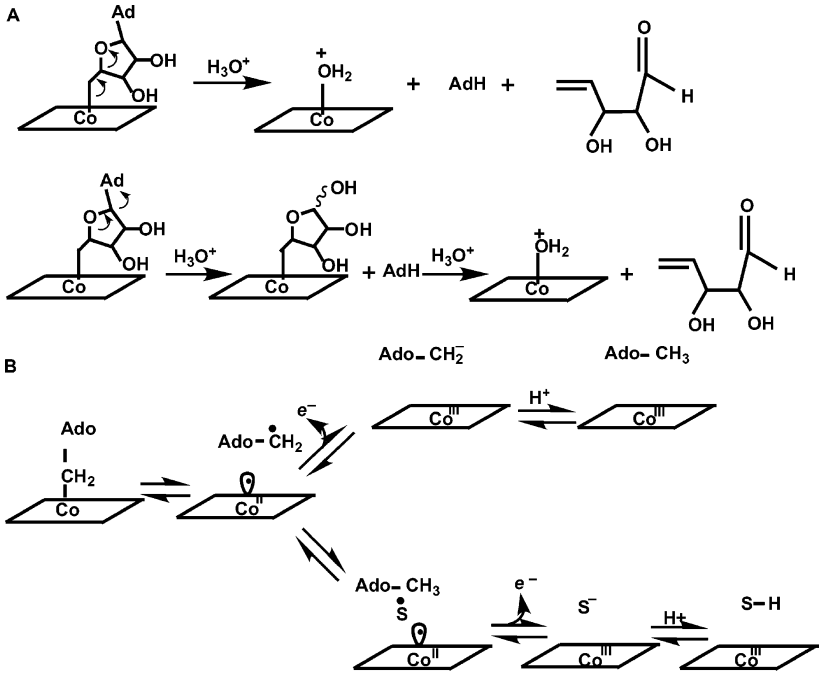


Table 2: Comparison of Masses of 5′-Deoxyadenosine and *n*-Butyryl-CoA Isolated during Inactivation of Y89F/R207Q with *n*-Butyryl-CoA in H_2O and D_2O

substrate	solvent	*m*/*z* of 5-dAdenosine	*m*/*z* of *n*-BuCoA
n-BuCoA	H_2O	252	837
	^2H_2O	252	837
n-[d_7]-BuCoA	H_2O	253; 254; 255	ND[a]
	^2H_2O	253; 254; 255	ND

[a] ND, not determined.

deuterium from solvent into either 5′-deoxyadenosine or to *n*-butyryl-CoA (Table 2).

One explanation for the failure to observe deuterium incorporation from the solvent is that the carbanion formed in the reaction could in principle abstract a proton from an amino acid side chain that is shielded in the active site and is slow to exchange, or from a nonacidic proton bonded to a carbon atom. The experimental results obtained in the reaction performed in D_2O therefore did not permit distinction between the two alternative acceptors for electron transfer from cob(II)alamin.

The crystal structure of the methylmalonyl-CoA mutase−substrate complex reveals an ~6 Å distance between the cobalt atom and the C2 carbon of the substrate from where the hydrogen atom is abstracted (*23*). Although the structure of Y89F/R207Q in complex with *n*-butyryl-CoA is not available, a similar distance could be expected for this substrate bound to the mutant enzyme. Therefore, the possibility of an electron transfer from cob(II)alamin to *n*-butyryl-CoA is plausible. Since the pathway involving electron transfer to *n*-butyryl-CoA radical involves a hydrogen atom transfer from the substrate analogue to the deoxyadenosyl radical, we next tried to detect incorporation of deuterium into deoxyadenosine from *n*-[d_7]-butyryl-CoA. ESI-MS revealed three major peaks with *m*/*z* 253.1, 254.1, and 255.1 corresponding to mono-, di-, and trideuterated 5′-deoxyadenosine (Figure 7). The amount of nondeuterated deoxyadenosine in this sample (*m*/*z* 252.1) was less than

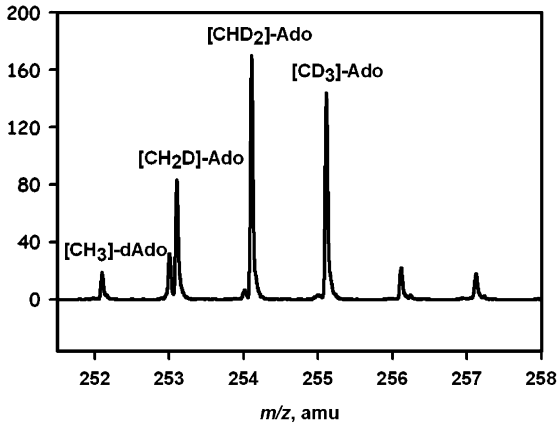


FIGURE 7: ESI-MS analysis for deuterium incorporation from *n*-[d_7]-butyryl-CoA to 5′-deoxyadenosine in the presence of the Y89F/R207Q mutant. The reaction conditions are described under Experimental Methods.

10%. In addition, MS-MS for these peaks confirmed the identity of 5′-deoxyadenosine by revealing the presence of the adenine moiety (*m*/*z* 136) (data not shown).

DISCUSSION

Effect of the R207Q Mutation on Catalysis. Previous investigations on the role of the Y89 residue in the mutase-catalyzed reaction revealed that it plays an important role in homolysis of the Co−carbon bond, presumably by destroying the deoxyadenosine binding site in the enzyme−substrate complex (*14*). The hydroxyl group in Y89 is involved in a hydrogen-bonding interaction with the carboxylate of the substrate. Loss of this interaction could differentially affect the stability of the radical intermediates (*24*). Computational studies predict that loss of this hydrogen-bonding interaction will preferentially destabilize the succinyl-CoA radical but have a small effect on the rearrangement barrier for the interconversion of the two radicals increasing it by 1 kcal/mol (*25*−*27*).

The substrate carboxylate is held in the active site of the mutase by two additional hydrogen-bonding interactions with R207. Theoretical studies on this electrostatic interaction predict a 3 kcal/mol destabilization of the succinyl-CoA radical would result from a negative charge on the carboxylate while the methylmalonyl-CoA radical would be stabilized by 3 kcal/mol (*15*).

Here we report the effects of the R207Q mutation on the mutase-catalyzed reaction. The R207Q mutation has a dramatic effect on catalysis and on substrate affinity. The observed 10^4-fold decrease in k_{cat} would account for an ~5 kcal mol^{-1} contribution of this residue to the overall energetics of the reaction provided that the effect of the R207Q change is restricted to the site of mutation. However, it is important to note that that the structure of the R207Q mutant is presently not available.

Suicide Inactivation of R207Q and Y89F/R207Q. Although most of the active site residues are conserved between methylmalonyl-CoA mutase and isobutyryl-CoA mutase, the two residues interacting with the carboxylate portion of the substrate in methylmalonyl-CoA mutase, Y89F and R207, are replaced in isobutyryl-CoA mutase by F80 and Q198, respectively. In this study, we have tested the possibility that these two residues are solely responsible for substrate recognition and catalysis by the sister mutases. However, butyryl-CoA proved not to be a substrate for either the wild-type or any of the other mutants generated in this study. Instead, the R207Q mutants in the presence of *n*-butyryl-CoA or isobutyryl-CoA undergo an undesired side reaction leading to irreversible inactivation of the enzyme. If the effects of the R207Q mutation are indeed restricted to the site of change, this would represent an excellent example of the role of a single residue in affecting "negative catalysis" as described by Retey (*28*) in which the reactivity of intermediates are finely tuned by multiple interactions with the protein so that unwanted side reactions are suppressed.

Our results on the mechanism of mutase inactivation show that it is accompanied by formation of 5′-deoxyadenosine and OHCbl, at similar rates, in an oxygen-independent process. The simplest routes by which this inactivation can occur are depicted in Scheme 2.

In principle, the Co−carbon bond of the cofactor could be cleaved heterolytically or homolytically. Two different acid-induced mechanisms for Co−carbon bond heterolysis have been previously described (*29*). One involves ring-opening and protonation of the ribofuranosyl oxygen (Scheme 2A, upper) and the other involves initial cleavage of the adenine moiety followed by hydrolysis of the Co−deoxyfuranosyl complex (Scheme 2A, lower). In both cases, heterolysis of the Co−carbon bond would lead to the production of adenine and dihydroxy-pentanal instead of 5′-deoxyadenosine.

The other possible mechanism for enzyme inactivation is via initial homolysis of the Co−carbon bond of the cofactor, which is the first step in the reaction mechanism by the mutase. This would lead to formation of cob(II)alamin and 5′-deoxyadenosine radical (Scheme 2B).

Although cob(II)alamin does not accumulate as an intermediate in the active site of the mutants, the identities of the products formed during inactivation rule out Co−carbon bond heterolysis as a likely mechanism. An internal electron transfer from cob(II)alamin to either the 5′-deoxyadenosyl radical or the substrate radical would lead to formation of OHCbl and a transient carbanion which is rapidly quenched by protonation. Electron transfer from cob(II)alamin to a carbon radical to generate a carbanion is thermodynamically unfavorable, since the cob(II)alamin/cob(III)alamin redox potential is high (~+0.2 V (*30*)), whereas the carbon radical/carbanion potential is expected to be quite low. Coupling of the electron transfer to a favorable proton transfer to quench the highly unstable carbanion intermediate would be critical for driving the overall reaction in the forward reaction.

Our results from the inactivation reaction in D_2O failed to distinguish between the two acceptor sites for the electron-transfer reaction. Neither 5′-deoxyadenosine nor butyryl-CoA exhibited deuterium incorporation from solvent. The simplest explanation for this observation is that protonation of the carbanion occurs from a proximal active site residue that is shielded from solvent or from a nonacidic proton bonded to a carbon atom.

When the R207Q mutants were incubated with *n*-[d_7]-butyryl-CoA, an isotopic effect of ~7 was measured and deuterium incorporation into deoxyadenosine was observed (Figure 5). Although there is no turnover of the enzyme in the presence of butyryl-CoA, a mixture of mono-, di-, and trideuterated deoxyadenosine was isolated from the inactivation reaction revealing multiple rapid rounds of hydrogen atom exchange between the two transient radicals, deoxyadenosyl and butyryl-CoA, before electron transfer from cob(II)alamin to butyryl-CoA, followed by quenching by protonation (Figure 7). These observations suggest that the observed isotope effect, since it involves multiple rounds of hydrogen transfer, is suppressed. Furthermore, the anomalously large deuterium isotope effect observed in wild-type methylmalonyl-CoA mutase (*31*) is not observed with the mutant, suggesting a change in the pathway for hydrogen atom transfer from quantum mechanical tunneling to a semiclassical over-the-barrier process. These results favor the route in Scheme 2B as the likely mechanism for inactivation of the mutant enzymes.

Suicide inactivation by internal electron transfer from cob(II)alamin has been reported in other B_{12}-dependent enzymes (*22*, *32*−*35*). Marsh and co-workers reported that glutamate mutase catalyzed the rapid formation of a Michael adduct between the deoxyadenosyl radical and the substrate analogue, 2-methyleneglutarate. This step was followed by an electron transfer from cob(II)alamin to the adduct and formation of OHCbl, leading to irreversible inactivation of the enzyme (*32*). Previous work done by Frey and co-workers on lysine 5,6-aminomutase showed that the enzyme underwent substrate-dependent inactivation with concomitant formation of OHCbl and 5′-deoxyadenosine. Furthermore, incorporation of a hydrogen atom from the labeled substrate into deoxyadenosine was observed, suggesting a mechanism for inactivation that is similar to that described in Scheme 2B.

The work described herein is the first example in which the B_{12}-dependent enzyme, methylmalonyl-CoA mutase, misfires by an internal electron transfer. Our results illustrate that the protein not only enhances the reaction rate as it guides a radical-mediated rearrangement, but that it also exercises exquisite control over the reactivities of intermediates, thereby suppressing suicidal side reactions.

ACKNOWLEDGMENT

We would like to acknowledge Dr. Haiteng Deng at the Metabolomics/Proteomics Core Facility (University of Nebraska, Lincoln) for his assistance with the mass spectrometric analyses.

REFERENCES

1. Banerjee, R. (1997) The yin-yang of cobalamin biochemistry, *Chem. Biol. 4*, 175−186.
2. Banerjee, R. (2003) Radical carbon skeleton rearrangements: catalysis by coenzyme B12-dependent mutases, *Chem. Rev. 103*, 2083−2094.
3. Banerjee, R., and Ragsdale, S. W. (2003) The many faces of vitamin B12: Catalysis by cobalamin-dependent enzymes, *Ann. Rev. Biochem. 72*, 209−247.
4. Allen, S. H. G., Kellermeyer, R. W., Stjernholm, R., and Wood, H. G. (1964) Purification and properties of enzymes involved in the propionic acid fermentation, *J. Bacteriol. 87*, 171−187.
5. Zerbe-Burkhardt, K., Ratnatilleke, A., Philippon, N., Birch, A., Leiser, A., Vrijbloed, J. W., Hess, D., Hunziker, P., and Robinson, J. A. (1998) Cloning, sequencing, expression, and insertional inactivation of the gene for the large subunit of the coenzyme B12-dependent isobutyryl-CoA mutase from Streptomyces cinnamonensis, *J. Biol. Chem. 273*, 6508−6517.
6. Moore, B. S., Eisenberg, R., Weber, C., Bridges, A., Nanz, D., and Robinson, J. A. (1995) On the sterospecificity of the coenzyme B12-dependent isobutyryl-CoA mutase reaction, *J. Am. Chem. Soc. 117*, 11285−11291.
7. Dorman, D. E., Paschal, J. W., Nakatsukasa, W. M., Huckstep, L. L., and Neuss, N. (1976) The use of 13C-NMR. Spectroscopy in biosynthetic studies. II[1]. Biosynthesis of narasin, a new polyether ionophore from fermentation of Streptomyces aureofaciens [2], *Helv. Chim. Acta 59*, 2625−2634.
8. Vrijbloed, J. W., Zerbe-Burkhardt, K., Ratnatilleke, A., Grubelnik-Leiser, A., and Robinson, J. A. (1999) Insertional inactivation of methylmalonyl coenzyme A (CoA) mutase and isobutyryl-CoA mutase genes in Streptomyces cinnamonensis: influence on polyketide antibiotic biosynthesis, *J. Bacteriol. 181*, 5600−5605.
9. Banerjee, R., and Vlasie, M. (2002) Controlling the reactivity of radical intermediates by coenzyme B(12)-dependent methylmalonyl-CoA mutase, *Biochem. Soc. Trans. 30*, 621−624.
10. Mancia, F., and Evans, P. (1998) Conformational changes on substrate binding to methylmalonyl CoA mutase and new insights into the free radical mechanism, *Structure 6*, 711−720.
11. Mancia, F., Keep, N. H., Nakagawa, A., Leadlay, P. F., McSweeney, S., Rasmussen, B., Bösecke, P., Diat, O., and Evans, P. R. (1996) How coenzyme B12 radicals are generated: The crystal structure of methylmalonyl-coenzyme A mutase at 2 Å resolution, *Structure 4*, 339−350.
12. Maiti, N., Widjaja, L., and Banerjee, R. (1999) Proton transfer from Histidine244 may facilitate the 1,2 rearrangement reaction in coenzyme B12-dependent methylmalonyl-CoA mutase, *J. Biol. Chem. 274*, 32733−32737.
13. Thoma, N. H., Evans, P. R., and Leadlay, P. F. (2000) Protection of radical intermediates at the active site of adenosylcobalamin-dependent methylmalonyl-CoA mutase, *Biochemistry 39*, 9213−9221.
14. Vlasie, M. D., and Banerjee, R. (2003) Tyrosine 89 accelerates Co-carbon bond homolysis in methylmalonyl-CoA mutase, *J. Am. Chem. Soc. 125*, 5431−5436.
15. Dybala-Defratyka, A., and Paneth, P. (2001) Theoretical evaluation of the hydrogen kinetic isotope effect on the first step of the methylmalonyl-CoA mutase reaction, *J. Inorg. Biochem. 86*, 681−689.
16. Dayem, L. C., Carney, J. R., Santi, D. V., Pfeifer, B. A., Khosla, C., and Kealey, J. T. (2002) Metabolic engineering of a methylmalonyl-CoA mutase-epimerase pathway for complex polyketide biosynthesis in Escherichia coli, *Biochemistry 41*, 5193−5201.
17. Padmakumar, R., and Banerjee, R. (1995) Evidence from EPR spectroscopy of the participation of radical intermediates in the reaction catalyzed by methylmalonyl-CoA mutase, *J. Biol. Chem. 270*, 9295−9300.
18. Taoka, S., Padmakumar, R., Lai, M.-t., Liu, H.-w., and Banerjee, R. (1994) Inhibition of Human Methylmalonyl-CoA by Various CoA-esters, *J. Biol. Chem. 269*, 31630−31634.
19. Padmakumar, R., Gantla, S., and Banerjee, R. (1993) A Rapid Method for the synthesis of methylmalonyl-Coenzyme A and other CoA-esters, *Anal. Biochem. 214*, 318−320.
20. Padmakumar, R., Padmakumar, R., and Banerjee, R. (1997) Evidence that cobalt-carbon bond homolysis is coupled to hydrogen atom abstraction from substrate in methylmalonyl-CoA mutase, *Biochemistry 36*, 3713−3718.
21. Ratnatilleke, A., Vrijbloed, J. W., and Robinson, J. A. (1999) Cloning and sequencing of the coenzyme B(12)-binding domain of isobutyryl-CoA mutase from Streptomyces cinnamonensis, reconstitution of mutase activity, and characterization of the recombinant enzyme produced in Escherichia coli, *J. Biol. Chem. 274*, 31679−31685.
22. Tang, K. H., Chang, C. H., and Frey, P. A. (2001) Electron transfer in the substrate-dependent suicide inactivation of lysine 5,6-aminomutase, *Biochemistry* 40, 5190−5199.
23. Mancia, F., Smith, G. A., and Evans, P. R. (1999) Crystal structure of substrate complexes of methylmalonyl-CoA mutase, *Biochemistry 38*, 7999−8005.
24. Thomä, N. H., Meier, T. W., Evans, P. R., and Leadlay, P. F. (1998) Stabilization of intermediates by an active site tyrosine residue in methylmalonyl-CoA mutase, *Biochemistry 37*, 14386−14393.
25. Smith, D. M., Golding, B. T., and Radom, L. (1999) The facilitation of enzyme-catalyzed reactions by partial proton transfer: Application to coenzyme B12- dependent methylmalonyl-CoA mutase, *J. Am. Chem. Soc. 121*, 1383−1384.
26. Smith, D. M., Golding, B. T., and Radom, L. (1999) Understanding the mechanism of B12-dependent methylmalonyl-CoA mutase: Partial proton transfer in action, *J. Am. Chem. Soc. 121*, 9388−9399.
27. Loferer, M. J., Webb, B. M., Grant, G. H., and Liedl, K. R. (2003) Energetic and stereochemical effects of the protein environment on substrate: A theoretical study of methylmalonyl-CoA mutase, *J. Am. Chem. Soc. 125*, 1072−1078.
28. Retey, J. (1990) Enzymic reaction selectivity by negative catalysis or how do enzymesdeal with highly reactive intermediates, *Angew. Chem. Int. Ed. Engl. 29*, 355−361.
29. Jensen, M. P., and Halpern, J. (1999) Dealkylation of Coenzyme B12 and Related Organocobalamins: Ligand Structural Effects on Rates and Mechanisms of Hydrolysis, *J. Am. Chem. Soc. 121*, 2181−2192.
30. Lexa, D., and Saveant, J.-M. (1983) The electrochemistry of vitamin B12, *Acc. Chem. Res. 16*, 235−243.
31. Chowdhury, S., and Banerjee, R. (2000) Evidence for quantum mechanical tunneling in the coupled cobalt carbon bond homolysis-substrate radical generation reaction catalyzed by methylmalonyl-CoA mutase, *J. Am. Chem. Soc. 122*, 5417−5418.
32. Huhta, M. S., Ciceri, D., Golding, B. T., and Marsh, E. N. (2002) A novel reaction between adenosylcobalamin and 2-methyleneglutarate catalyzed by glutamate mutase, *Biochemistry 41*, 3200−3206.
33. Magnusson, O. T., and Frey, P. A. (2002) Interactions of diol dehydrase and 3′,4′-anhydroadenosylcobalamin: suicide inactivation by electron transfer, *Biochemistry 41*, 1695−1702.
34. Toraya, T., and Mori, K. (1999) A reactivating factor for coenzyme B12-dependent diol dehydratase, *J. Biol. Chem. 274*, 3372−3377.
35. Abend, A., Bandarian, V., Nitsche, R., Stupperich, E., Retey, J., and Reed, G. H. (1999) Ethanolamine ammonia-lyase has a base-on binding mode for coenzyme B12, *Arch. Biochem. Biophys. 370*, 138−141.

BI036299Q