Protection of Radical Intermediates at the Active Site of Adenosylcobalamin-Dependent Methylmalonyl-CoA Mutase[†,‡]

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Received February 24, 2000; Revised Manuscript Received May 4, 2000

ABSTRACT: Adenosylcobalamin-dependent methylmalonyl-CoA mutase catalyzes the interconversion of methylmalonyl-CoA and succinyl-CoA via radical intermediates generated by substrate-induced homolysis of the coenzyme carbon−cobalt bond. From the structure of methylmalonyl-CoA mutase it is evident that the deeply buried active site is completely shielded from solvent with only a few polar contacts made between the protein and the substrate. Site-directed mutants of amino acid His244, a residue close to the inferred site of radical chemistry, were engineered to investigate its role in catalysis. Two mutants, His244Ala and His244Gln, were characterized using kinetic and spectroscopic techniques. These results confirmed that His244 is not an essential residue. However, compared with that of the wild type, k_{cat} was lowered by 10^2- and 10^3-fold for the His244Gln and His244Ala mutants, respectively, while the K_m for succinyl-CoA was essentially unchanged in both cases. The primary kinetic tritium isotope effect (k_H/k_T) for the His244Gln mutant was 1.5 ± 0.3, and tritium partitioning was now found to be dependent on the substrate used to initiate the reaction, indicating that the rearrangement of the substrate radical to the product radical was extremely slow. The His244Ala mutant underwent inactivation under aerobic conditions at a rate between 1 and 10% of the initial rate of turnover. The crystal structure of the His244Ala mutant, determined at 2.6 Å resolution, indicated that the mutant enzyme is unaltered except for a cavity in the active site which is occupied by an ordered water molecule. Molecular oxygen reaching this cavity may lead directly to inactivation. These results indicate that His244 assists directly in the unusual carbon skeleton rearrangement and that alterations in this residue substantially lower the protection of reactive radical intermediates during catalysis.

Methylmalonyl-CoA mutase catalyzes the adenosylcobalamin (AdoCbl,[1] coenzyme B_{12})-dependent interconversion of 2(*R*)-methylmalonyl-CoA (MMCoA) and succinyl-CoA (SuCoA) (*1*−*3*). The enzyme from the Gram-positive bacterium *Propionibacterium shermanii* is an $\alpha\beta$ heterodimer containing an α chain (80 kDa) and a β chain (70 kDa) (*4*, *5*) with only one active site, associated with the α subunit. The AdoCbl-dependent mutases utilize the unusual cobalt−carbon bond of the coenzyme as a radical initiator (*2*). Substrate binding to methylmalonyl-CoA mutase triggers cobalt−carbon bond breakage (Figure 1, **I**), generating a Co(II) species and the highly reactive 5′-deoxyadenosyl radical which is capable of abstracting a hydrogen from substrate to yield substrate radical **II**. Kinetic evidence suggests that carbon−cobalt bond cleavage and hydrogen abstraction are tightly coupled so that the steady-state concentration of the 5′-deoxyadenosyl radical is always very low (*6*, *7*). The substrate radical then undergoes intramo-

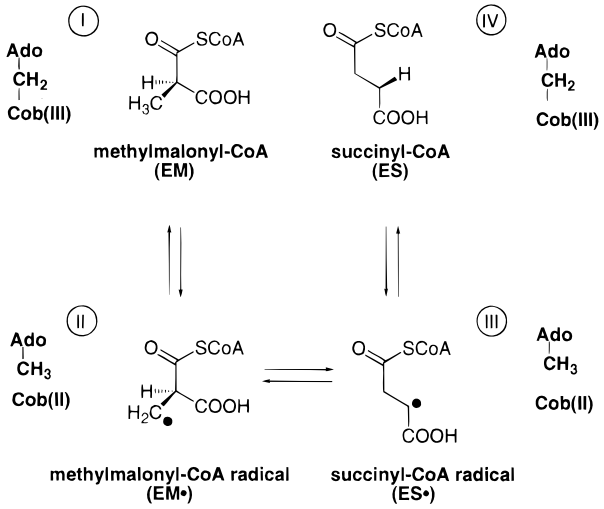

FIGURE 1: Proposed mechanistic scheme for the mutase-catalyzed rearrangement.

[†] This work was supported by Grant G8806378CB from the United Kingdom Medical Research Council.

[‡] The Protein Data Bank (PDB) accession code for the atomic coordinates of the His244Ala mutant of methylmalonyl-CoA mutase is 1e1c.

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[1] Abbreviations: AdoCbl, adenosylcobalamin; HO-Cbl, hydroxocobalamin; MMCoA, methylmalonylcoenyzme A; SuCoA, succinyl-CoA; CoA, coenzyme A; TFA, trifluoroacetic acid; Tyr89, tyrosine A89 of the mutase α subunit; His244, histidine A244 of the mutase α subunit.

10.1021/bi0004302 CCC: $19.00
Published on Web 07/08/2000

lecular 1,2-transfer of the thioester moiety to form a product radical **III**. The migrating thioester moiety is subsequently replaced by a hydrogen atom, with retention of configuration (*8*) to yield product **IV**. Two plausible alternative mechanisms have been proposed for the 1,2-transfer step, an associative model proceeding via a cyclopropyloxy radical intermediate (*9*) and a mechanism involving fragmentation to a formyl-CoA radical and acrylate (*10*).

A poorly understood aspect of catalysis by such AdoCbl-dependent enzymes has been the extent to which stabilization of radical intermediates, and the protection of such intermediates from attack by oxygen or adventitious nucleophiles, might be mediated by specific amino acid residues. It has previously been suggested that in enzymes such as methylmalonyl-CoA mutase, the protein might play an important passive role by protecting such potentially highly reactive radical intermediates from external attack (*11*). In agreement with this, the X-ray crystal structure of the *P. shermanii* methylmalonyl-CoA mutase has revealed that the radical rearrangement takes place in a buried active site offering no evident access to solvent (*5*, *12*, *13*). However, evidence has recently been obtained which shows that tyrosine 89, which is involved in binding the carboxylate anion of the substrate, plays a direct role in promoting the rearrangement. In a Tyr89Phe mutant, the rate of hydrogen transfer and the rate of interconversion of substrate and product radicals are both significantly lowered (*14*). In preliminary experiments, it has also been reported (*15*) that specific mutagenesis of histidine 244, a residue hydrogen bonding to the thioester moiety of the substrate, rendered the enzyme labile to attack by oxygen. His244 is of particular significance because it is in contact with the substrate and likely to interact with radical intermediates irrespective of whether an associative or a dissociative mechanism operates. We have therefore undertaken a detailed study of the kinetic and structural consequences of the specific mutagenesis of His244. The results strongly suggest that the side chain of His244 not only stabilizes radical intermediates [perhaps by partial proton donation as recently proposed (*16*)] but also protects them from attack by molecular oxygen.

MATERIALS AND METHODS

Materials. The purification of *P. shermanii* methylmalonyl-CoA mutase from a recombinant *Escherichia coli* strain has been described previously (*17*). [$^2H_4$]Succinyl-CoA was synthesized according to the method of Simon and Shemin (*18*). AdoCbl and HO-Cbl were purchased from Sigma Chemical Co. The sources of other materials have been described previously (*3*, *14*).

Production of His244Gln and His244Ala Mutants Using PCR Mutagenesis. His244Gln and His244Ala mutants were constructed using a PCR-based megapriming strategy (*19*). The methylmalonyl-CoA mutase expression vector pMEX2 (*17*) contains two *Xho*I restriction enzyme sites flanking the codon for His244. Construction of the mutants was carried out in two stages. A 0.9 kbp megaprimer was assembled harboring one *Xho*I site at the 5′-region and the desired mutation at the 3′-end. This megaprimer was used in the second stage to assemble the entire mutagenic 2.0 kb *Xho*I fragment. The primers were 5′-GTG TTC GAG CAG CTG ATG GAT CGC-3′ positioned on pMEX2 5′ further upstream of the *Xho*I site and 5′-GTT CTT CAC TTC CTT CGA GTA CA-3′ positioned further downstream of the *Xho*I site in pMEX2. The mutagenic primers were 5′-CAT TTC CGG CTA Cgc CAT GCA GGA AGC-3′ for His244Ala and 5′-TTC CGG CTA CCA gAT GCA GGA AGC C-3′ for His244Gln. PCR conditions were identical to those described previously (*14*). The final PCR product with flanking *Xho*I sites and containing the desired mutation was subcloned blunt-ended into dephosphorylated *Sma*I-digested pUC18 (Pharmacia, Uppsala, Sweden). The entire insert was fully sequenced to confirm the intended mutation and exclude PCR-induced errors. The mutagenic *Xho*I fragment was then substituted for the wild-type *Xho*I fragment, giving rise to plasmids pMex-H244Q and pMex-H244A.

Expression of His244Ala and His244Gln Mutants. The two mutant methylmalonyl-CoA mutases were expressed in *E. coli* and purified to homogeneity as previously described (*14*, *20*). The expression levels of the His244Gln and His244Ala mutant proteins were similar to that observed for wild-type methylmalonyl-CoA mutase.

Determination of Michaelis−Menten Parameters. K_m and k_{cat} were determined by measuring the initial reaction rate using various concentrations of succinyl-CoA (*21*, *22*). The results of these measurements were fitted to a linear Hanes plot of s/v versus s (*23*). The primary kinetic deuterium isotope effect on conversion of succinyl-CoA to methylmalonyl-CoA was also measured using the spectrophotometric assay. Experiments were carried out under noncompetitive conditions varying the concentration of [$^2H_4$]succinyl-CoA at 25 °C. The primary kinetic deuterium isotope effect on V_{max} for [$^2H_4$]succinyl-CoA was determined from initial rate measurements over a range of substrate concentrations (20 μM to 1 mM).

Tritium Partitioning and Isotope Effect Measurements. The experimental arrangement used to study tritium release from [^{3}H]AdoCbl bound to the His244Gln mutant was as described previously (*14*, *20*). Quenched-flow experiments were performed at 25 °C using a BioLogic Instrument (BioLogic). Stock solutions were made up in 50 mM Tris-HCl buffer (pH 7.5). Stock solution A contained 6 μM methylmalonyl-CoA mutase containing bound [^{3}H]AdoCbl (specific activity of 12 000 dpm/mol); stock solution B contained 2 mM substrate, either (*RS*)-methylmalonyl-CoA or succinyl-CoA, and the stock solution for quenching contained 100 g/L trifluoroacetic acid. Reactions were started by mixing 40 μL of substrate with 40 μL of enzyme over the course of 110 ms, and the reaction mixture was allowed to age for a variable period of time (0−8 s). The reaction was terminated by adding 35 μL of quench solution and 20 μL of MilliQ water added to 80 μL of the reaction mixture over a time period of 35 ms. Quenched samples were transferred into opaque Eppendorf tubes, immediately frozen on dry ice, and stored at −20 °C before HPLC analysis. HPLC analysis was performed as described previously (*14*).

UV−Visible Spectroscopic Experiments. Spectral changes upon substrate addition under aerobic conditions were monitored using a dual-beam spectrophotometer, model 9100 (Shimadzu). One cuvette contained the His244Ala mutant (12 μM) and succinyl-CoA (400 μM), while the reference cuvette contained 12 μM enzyme only. All experiments were carried out in 50 mM Tris-HCl at pH 7.5 and 25 °C.

Recording of UV−Vis Spectra under Anaerobic Conditions. Samples containing substrate or enzyme were made anaerobic by repeated evacuation followed by purging with oxygen-free argon. Spectra were recorded using a 300 μL cuvette, sealed with a septum, that had been purged with argon for 2 min prior to the addition of enzyme and substrate. Enzyme and substrate were transferred to cuvettes using airtight syringes.

Stopped-Flow Experiments. Experiments were carried out using a thermostated Applied Photophysics SX.18MV stopped-flow apparatus (Leatherhead, U.K.) fitted with an SX F81 diode array, which gave a separation per photodiode of 2.17 nm and a readout time of 4 ms.

Data Collection and Refinement of the His244Ala Mutant. A 97.2% complete data set to 2.62 Å resolution, consisting of 99 776 unique reflections, was collected on a rotating anode X-ray source. The crystal was suspended directly from the hanging drop in a thin film of liquid and frozen at 95 K with a 600 Series Cryostream Cooler (Oxford Cryosystems, Oxford, U.K.). Data were collected with a 300 mm diameter MAR research image plate (Hamburg, Germany) and integrated with MOSFLM. Scaling and processing were performed using the CCP4 suite of programs (*24*). Data were merged with an R_{merge} of 0.08 with a multiplicity of 3.4 and an I/σ of 11.4 (3.2 in the outer shell, 2.3−2.2 Å), and the *B*-factor derived from the Wilson plot was 39.5 Å^2. The structure was determined by molecular replacement using one $\alpha\beta$ heterodimer from a previously determined structure of desulfo-CoA (*5*). Calculations were carried out at 4 Å resolution using the program AmoRe (*25*). Two heterodimeric molecules were found in the asymmetric unit, in an arrangement very similar to that seen in the wild type (*5*). The molecular replacement solution after rigid-body refinement at 4 Å resolution had a correlation coefficient of 83% and an *R*-factor of 30%. The model was refined using refmac (*26*) and rebuilt using the program O (*27*). Noncrystallographic symmetry restraints were applied to keep the two molecules in the asymmetric unit in a similar conformation. The model has an *R*-factor of 20% for 95% of the data to 2.6 Å resolution, with an R_{free} for the remaining 5% of 29%.

RESULTS

Steady-State Measurements on the His244Gln Mutant. Determination of the steady-state parameters was used for initial characterization of the His244Gln mutant. The mutant enzyme has a K_m of 85 ± 15 μM, not significantly changed from that of the wild type (96 ± 5 μM). However, k_{cat} is lowered 72-fold, from 48 ± 0.5 to 0.66 ± 0.04 s^{-1}. The mutant enzyme exhibits a kinetic deuterium isotope effect (k_H/k_D = 2.8 ± 0.3) slightly lower than that observed for the wild type (k_H/k_D = 3.8 ± 0.4) in previous work (*14*, *28*).

Tritium Partitioning Measurements on the His244Gln Mutant. Preliminary experiments indicated that more than 90% of the tritium was washed out from AdoCbl to the coenzyme A esters within the first 3 s of the reaction. Subsequent kinetic measurements were therefore performed using a quenched-flow apparatus (BioLogic). The results of tritium partitioning when methylmalonyl-CoA was used as the substrate are shown in Figure 2. Tritium was released from AdoCbl into succinyl-CoA with a first-order rate constant of 2.5 ± 0.4 s^{-1} and into methylmalonyl-CoA with a rate constant of 0.88 ± 0.14 s^{-1}. The distribution of tritium between the two CoA esters was 1:3 in favor of succinyl-CoA, and this ratio did not change within the first 8 s of the reaction. The experiment was repeated using succinyl-CoA as substrate, and the results are also shown in Figure 2. Tritium was released from AdoCbl back into succinyl-CoA with a first-order rate constant of 2.6 ± 0.3 s^{-1}. In this experiment, the distribution of the released tritium was 97:3 in favor of succinyl-CoA over methylmalonyl-CoA. This ratio remained constant for the first 8 s of the reaction.

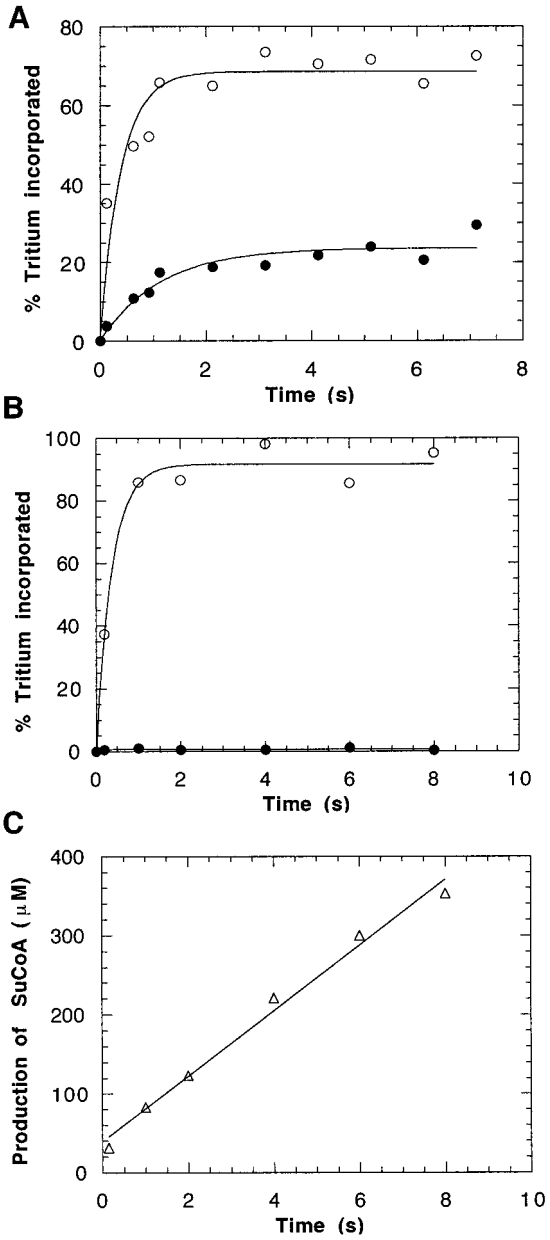

FIGURE 2: (A) Appearance of tritium in methylmalonyl-CoA (●) and in succinyl-CoA (○) when methylmalonyl-CoA was added as the substrate to the His244Gln mutant of methylmalonyl-CoA mutase. (B) As for panel A but with succinyl-CoA added as the substrate. (C) Formation of succinyl-CoA from methylmalonyl-CoA. Production of succinyl-CoA was linear for the first 8 s.

Tritium Isotope Effect Measurements on the His244Gln Mutant. Turnover was linear for the first 8 s, and the calculated turnover number for the mutant enzyme (k_H) was 7.6 ± 0.05 s^{-1} (Figure 2). From the partitioning experiments whose results are depicted in Figure 2, the release of tritium

in the forward direction was fitted as $k_{Tf} = 2.5 \pm 0.4\ s^{-1}$. If a rapid rotation of the methyl group of enzyme-bound deoxyadenosine is assumed, a statistical factor of 2 must be taken into consideration (*29*). The primary tritium isotope effect ($k_H/2k_{Tf}$) was determined to be $1.5 \pm 0.3\ s^{-1}$, a highly suppressed kinetic tritium isotope effect.

Stopped-Flow Measurements on the His244Gln Mutant. Stopped-flow experiments were carried out to determine the rate of cobalt−carbon bond breakage in the His244Gln mutant. The design of these measurements was based on stopped-flow studies by Banerjee and co-workers (*6*). Substrate binding to methylmalonyl-CoA mutase causes homolysis of the cobalt−carbon bond with concomitant reduction of cobalt from cobalt(III) to cobalt(II) (Figure 1). The observed rates for the reaction of the wild type and His244Gln with methylmalonyl-CoA were found to be independent of the substrate concentrations used, indicating a rapid pre-equilibrium preceding cobalt−carbon bond homolysis. The observed rates in both experiments lie in the range of 800 s^{-1} and are indistinguishable from each other within experimental error (data not shown). An observed rate of greater than 800 s^{-1} has previously been reported for the wild type (*6*).

UV−Visible Spectrometry of the His244Ala Mutant. The His244Ala mutant was reconstituted with AdoCbl and purified as the holoenzyme. In the absence of substrate, the UV−visible spectrum of the reconstituted holoenzyme was indistinguishable from that of the wild type (data not shown). In the presence of substrate, however, and under aerobic conditions, the formation of a spectroscopically distinct species with a characteristic absorption maximum at 352 nm could be monitored (Figure 3A). The spectrum recorded for the His244Ala mutant in the presence of substrate is that of hydroxocobalamin (HO-Cbl), a decomposition product of AdoCbl. Under anaerobic conditions, however, no significant amount of HO-Cbl was formed within 20 min (Figure 3B). Similar observations have been made previously for the His244Gly mutant (*16*).

The increase in absorbance at 352 nm after addition of substrate in the presence of oxygen was used to determine the rate of production of HO-Cbl (data not shown). The substrate succinyl-CoA was present at the near-saturating concentration of 400 μM. The increase in absorbance at 352 nm was fitted to a single-exponential curve ($A_{352} = Ce^{-kt}$), from which the rate constant for production of HO-Cbl (k_{HO-Cbl}) was found to be $3.6 \times 10^{-3} \pm 7 \times 10^{-6}\ s^{-1}$.

Pre-Steady-State Characterization of the His244Ala Mutant under Aerobic Conditions. Rapid scanning stopped-flow spectroscopy under aerobic conditions was used to investigate early intermediates in the reaction catalyzed by the His244Ala mutant. In this experiment, 100 μM His244Ala methylmalonyl-CoA mutase was mixed with 2.5 mM methylmalonyl-CoA. The spectra obtained (data not shown) indicate that the extent of cobalt−carbon bond breakage (reflecting the balance between cleavage and re-forming of the bond) reaches a plateau after 4 ms which is maintained for approximately 200 ms. This plateau represents 3% of the AdoCbl converted to cob(II)alamin, using the molar extinction coefficient for cob(II)alamin suggested by Banerjee and co-workers (*6*). After this lag period, a rapid increase in the production of HO-Cbl was observed. Single-wavelength

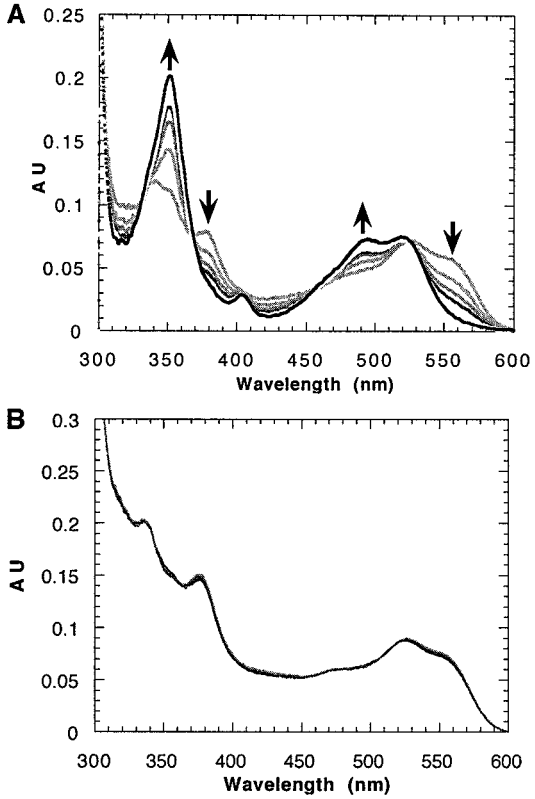

FIGURE 3: (A) Formation of a spectroscopically distinct species (black) after addition of substrate (400 μM succinyl-CoA) to a solution of 12 μM His244Ala mutant (gray) at 25 °C. Spectra were recorded (from gray to black) after 2, 6, 10, 15, and 27 min. Arrows indicate whether peaks decreased or increased with time. (B) As for panel A but under anaerobic conditions.

stopped-flow experiments under identical conditions, in which changes in absorbance at 525 nm were observed, were used to confirm this observation (data not shown).

Steady-State Characterization of the His244Ala Mutant. On the basis of the spectrophotometric assay and HPLC analysis, the His244Ala mutant was initially active under aerobic conditions, but the enzymatic activity then decayed. HPLC experiments indicated that about 20−30 mol of substrate was produced per mole of enzyme before the His244Ala mutant was completely inactivated. A typical trace obtained in the coupled spectrophotometric assay was linear for the first 40−60 s, and then flattened off. The initial slope of the spectrophotometric assay was used to derive steady-state parameters without rigorous exclusion of oxygen. k_{cat} was determined to be $0.038 \pm 0.009\ s^{-1}$, while K_m was 82.5 ± 15 μM. It appears that the mutation mainly affects k_{cat}, while K_m remains largely unchanged. When the spectrophotometric assay was performed under anaerobic conditions, there was no enzyme inactivation and the linear trace that was obtained had a slope identical to the initial slope in the presence of oxygen.

Lability of Wild-Type Methylmalonyl-CoA Mutase during Aerobic Turnover. A similar UV−visible scanning experiment showed that wild-type methylmalonyl-CoA mutase is also subject to inactivation during turnover, albeit at a much slower rate. The experiment was performed using 12 μM wild-type methylmalonyl-CoA mutase in the presence of 400 μM succinyl-CoA (Figure 4). The half-life for the inactivation was ~170 min ($k_{HO-Cbl} \approx 7 \times 10^{-5}\ s^{-1}$).

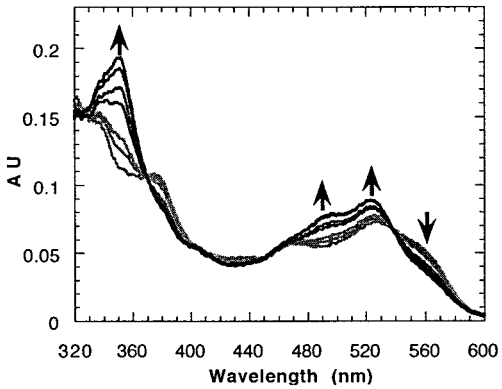

FIGURE 4: Wild-type methylmalonyl-CoA mutase under aerobic conditions in the presence of 400 μM succinyl-CoA. Spectra were taken after 1, 20, 42, 120. 150, 180, and 250 min.

Crystal Structure of the His244Ala Mutant. Initial attempts to crystallize the His244Ala mutant in the presence of succinyl-CoA, desulfo-CoA, or succinyl(carbadethia)-CoA under conditions similar to those used for the wild type (*13*) were not successful. Crystallization was achieved, however, using a much higher AdoCbl concentration. Crystals were obtained using a solution of 14.4% PEG 4000, 20% (v/v) glycerol, and 12 mM desulfo-CoA in 100 mM Tris-HCl (pH 7.5), to which AdoCbl had been added as solid, to a final concentration of 16 mM.

The structure of the His244Ala mutant could be superimposed on that determined for wild-type mutase crystallized in the presence of desulfo-CoA. The only major difference is that at position His244 in the mutant structure (at 2.6 Å resolution) there is a cavity in the place formerly occupied by the imidazole ring of the histidine residue which is now filled by a ordered water molecule, as shown in Figure 5. A second, more distal, water molecule found in both the wild type and the mutant is known to be displaced upon substrate binding (*13*). As seen in previous structures for methylmalonyl-CoA mutase (*5*), the cobalt appears mainly as a five-coordinate cob(II)alamin. No electron density is found on the upper face of the corrin which can safely be assigned to the 5′-deoxyadenosine group. The cobalamin species found in the crystals most likely represents a mixture of cob(II)alamin with minor contributions from HO-Cbl and AdoCbl.

DISCUSSION

Two mutant proteins have been constructed, expressed, and purified to homogeneity, in which histidine 244 of the α subunit of methylmalonyl-CoA mutase was changed to glutamine and alanine. The mutant enzyme produced by specific substitution of histidine 244 with glutamine gave rise to a functional enzyme, exhibiting a drop in k_{cat} for succinyl-CoA of approximately 72-fold compared to that of the wild type, while the K_m remained unchanged. The specificity constant (k_{cat}/K_m) dropped from 500 M^{-1} s^{-1} in the wild type to 8 M^{-1} s^{-1} in the His244Gln mutant, consistent with the mutation having an effect on the stabilization of the transition state, rather than an effect on substrate binding or product release. Determination of the primary kinetic deuterium isotope effect on V_{max} revealed a slightly lower isotope effect ($k_H/k_D = 2.8 \pm 0.3$) in the mutant than in the wild type ($k_H/k_D = 3.8 \pm 0.4$) (*20*). This suggests that, compared to the wild type, enzymatic steps not involving the transfer of hydrogen contribute more to overall rate limitation in the His244Gln mutant. Since these kinetic isotope effect measurements were carried out over a time

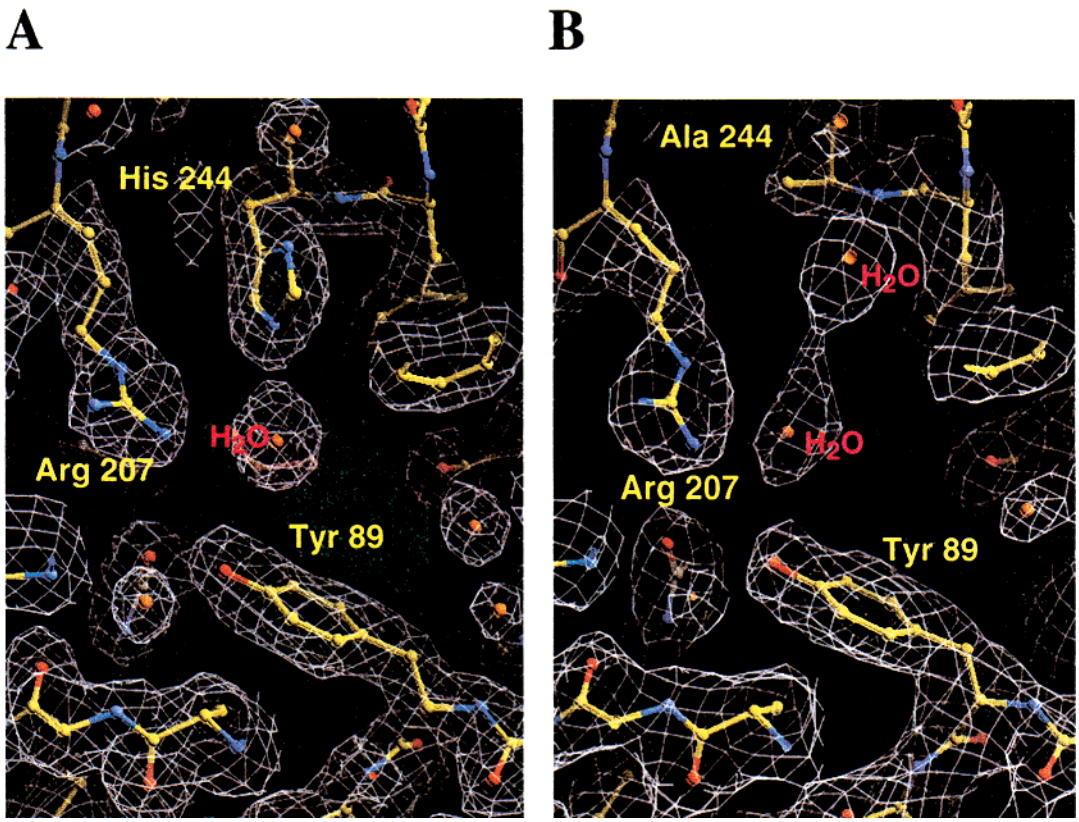

FIGURE 5: Section of the $2F_{obs} - F_{calc}$ SIGMAA-weighted electron density map of the active site of wild-type (A) and His244Gln (B) methylmalonyl-CoA mutase containing bound substrates at 2.6 Å.

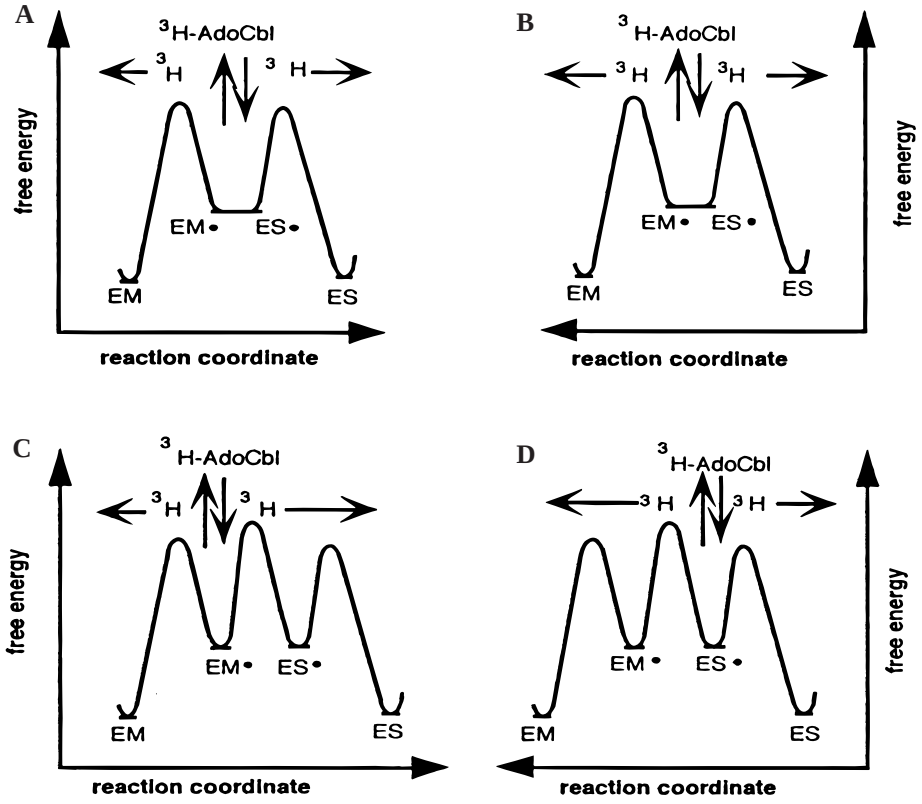

FIGURE 6: Alternative qualitative free energy profiles for tritium release from AdoCbl. (A) ES• and EM• in rapid equilibrium. Reaction initiated by addition of methylmalonyl-CoA (M). (B) As for panel A but reaction initiated by addition of succinyl-CoA (S). The partitioning of released tritium between M and S will be identical in panels A and B. (C) Slow EM• and ES• interconversion. Reaction initiated by addition of methylmalonyl-CoA (M). (D) As for panel C but reaction initiated by addition of succinyl-CoA (S). The partitioning of released tritium between S and M will be different in panels C and D.

period (10−40 s) during which AdoCbl is fully deuterated, the only steps which do not involve direct transfer of hydrogen, and may therefore have become more rate-limiting in this mutant, are substrate binding and release, and the reversible interconversion of the substrate radical ES• and the product radical EM• (Figure 1).

Tritium partitioning experiments were used to examine whether the rate of interconversion of substrate and product radicals is significantly decreased in the mutant and to what extent this step is now rate-determining. In wild-type mutase, tritium partitions identically, 75:25 in favor of succinyl-CoA over methylmalonyl-CoA, irrespective of which CoA ester is used to initiate the reaction, under conditions where the reverse reaction can be neglected (*20*). This indicates that in wild-type mutase, the species EM• and ES• must be in rapid equilibrium with each other so that they are kinetically indistinguishable on the time scale of the experiment (Figure 6a,b). This is not true for the His244Gln mutant, where the partitioning ratio was found to differ depending on the CoA ester used as the substrate (Figure 6c,d). When methylmalonyl-CoA was used to initiate the reaction, the tritium released from AdoCbl was found to partition 75:25 in favor of succinyl-CoA, that is, in a partitioning ratio identical to that for the wild type. However, when succinyl-CoA was used, a different partitioning ratio was observed; the alteration in the partitioning ratio was rather less in favor of the product, with only some 3% of the tritium released being found in methylmalonyl-CoA and 97% being found in succinyl-CoA. The dependence of the partitioning ratio upon the substrate used to initiate the reaction demonstrates that the species EM• and ES• are in this case clearly kinetically distinguishable; it appears that, as shown in Figure 6 (c and d), the rate of interconversion of the free radical species EM• and ES• has been sufficiently lowered in the H244Q mutant that the percentage of tritium found in the starting substrate is crucially dependent on the substrate that is used. Therefore, as has been observed previously in the Tyr89Phe mutant (*14*), the interconversion of substrate to product radical has become slow in the His244Gln mutant.

The measurement of the kinetic isotope effect for tritium release from AdoCbl to succinyl-CoA provides additional insight into the extent to which the hydrogen reabstraction from the cofactor is rate-limiting in the mutant. For the wild-type enzyme using methylmalonyl-CoA as the substrate, the primary kinetic isotope effect (k_H/k_T) was determined to be 4.9 ± 0.2, which is considerably smaller than a full normal primary tritium kinetic isotope effect, which typically lies in the range of 20−30 (*30*). This suppressed effect was interpreted to mean that the energetics of the wild-type mutase are balanced, with steps other than hydrogen transfer (such as product release, breakage of the cobalt−carbon bond, and radical rearrangement) contributing partially to the overall rate limitation. In contrast, for the His244Gln mutant enzyme, the tritium isotope effect using methylmalonyl-CoA as the substrate (k_H/k_T) was found to be 1.5 ± 0.3. This isotope effect, which is even more suppressed than in the wild type, shows that the hydrogen reabstraction from AdoCbl by the product radical is now even less rate-determining. In parallel, stopped-flow studies were used to test whether the rate of cobalt−carbon bond breakage is

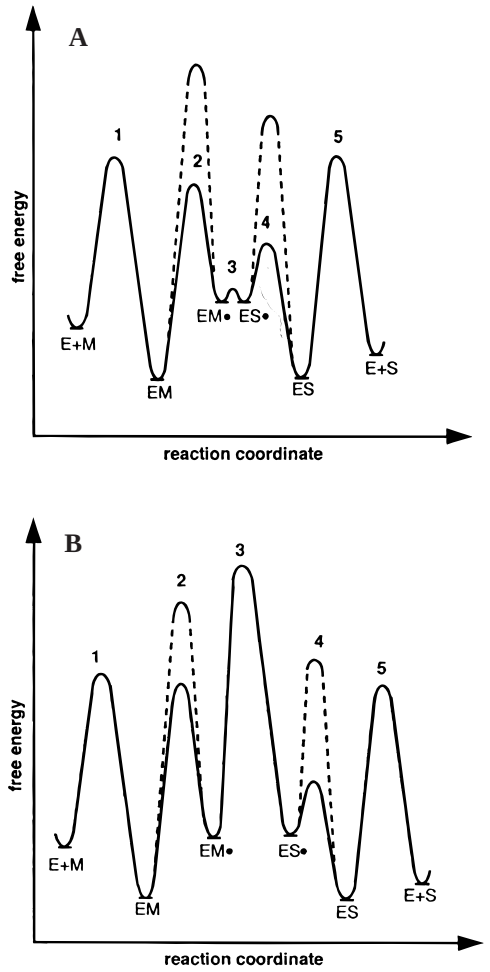

FIGURE 7: Qualitative free energy profile of the reaction catalyzed by (A) the wild type and (B) the His244Gln mutant of methylmalonyl-CoA mutase. The substrate-based radicals derived from methylmalonyl-CoA (M) and succinyl-CoA (S) are designated EM• and ES•, respectively. The solid line represents the energy profile for hydrogen and the dashed line the energy profile for tritium.

affected by the mutation. The observed rate for homolysis in the His244Gln mutant was found to be identical, within experimental error, to that of the wild type.

These measured tritium kinetic isotope effects appear to be smaller than the deuterium kinetic isotope effects measured for the mutase-catalyzed reaction, which would be extremely unusual. However, several contributing effects make up the observed overall primary deuterium kinetic isotope effect on V_{max}: a secondary effect on cobalt−carbon bond breakage, a primary effect on breakage of the carbon−hydrogen bond, and another primary effect on the reabstraction of hydrogen from the cofactor to yield product. In contrast, the tritium kinetic isotope effects discussed here only affect the step of hydrogen reabstraction from cofactor.

A qualitative free energy profile derived from the kinetic data obtained in this study is shown in Figure 7, where it is compared with the free energy profile previously deduced for the wild-type enzyme (*20*). The rate of cobalt−carbon bond breakage is shown at a comparable height in wild-type and mutant enzymes, based on stopped-flow studies measuring the rate of conversion of EM to EM• (and by inference of ES to ES•) (steps 2 and 4). The major difference between the free energy profiles for the His244Gln mutant and the wild type is that the interconversion of product and substrate radical is slow in the His244Gln mutant, as shown by the uneven partitioning observed, depending on the substrate used to initiate the reaction. There must be a higher free energy barrier between EM• and EM (step 2) than between ES• and ES (step 4). The differences in the ground state between free methylmalonyl-CoA and succinyl-CoA are drawn to reflect the equilibrium constant for the mutase-catalyzed reaction, which is 1:20 in favor of succinyl-CoA (*21*). In the absence of further data, the energy minima for bound substrates ES and EM are set at the same level. The tritium effects are shown for clarity as effects on the transition state, although they actually affect the ground state.

The kinetics of this mutant enzyme provide further support for the proposal that the protein is directly interacting with and stabilizing substrate and product radicals during the course of the rearrangement. In contrast to the effect observed with the Tyr89Phe mutation (*14*), the His244Gln mutation seems to exert its effect mainly on the interconversion of substrate and product radical rather than on the hydrogen transfer steps.

The His244Ala mutant was expressed and purified using a procedure identical to that of wild-type methylmalonyl-CoA mutase. Although initially active, the His244Ala mutant underwent inactivation in the presence of substrate under aerobic conditions. Having obtained an estimate of the turnover number and the rate of the production of HO-Cbl under aerobic conditions, in both cases using succinyl-CoA as the substrate, we found it possible to estimate the probability of suicide inactivation per turnover. Dividing k_{cat} by the measured rate of formation of HO-Cbl under aerobic conditions (k_{cat}/k_{HO-Cbl}) gave 10.5 (0.038 s^{-1}/0.0036 s^{-1}), implying that one in approximately 10 turnovers results in inactivation. This value is likely to represent a lower limit; k_{HO-Cbl} was determined under conditions where the reaction starts to approach equilibrium and therefore, unlike k_{cat}, contains contributions from the forward and reverse reactions. The effect of the oxygen concentration on k_{HO-Cbl} is also unknown. HPLC analysis of the amount of product produced during inactivation gave a similar estimate, in which one turnover in 10−100 turnovers would lead to inactivation.

The decomposition product of the cofactor AdoCbl was identified, on the basis of its spectroscopic properties, as HO-Cbl. Investigation of the spectroscopic properties of the enzyme in the presence of substrate, but under anaerobic conditions, does not result in either production of HO-Cbl or inactivation. The enzyme-bound AdoCbl is perfectly stable in the absence of substrate, and degradation only occurs upon substrate-induced cleavage of the cobalt−carbon bond. Apparently, the formation of a cobalt(II) intermediate leads to both turnover and suicide inactivation. Quenching of any of the intermediate radical species would result in a failure to reform AdoCbl, so that cob(II)alamin is then oxidized to HO-Cbl. The integrity of the enzyme-bound cofactor when oxygen is excluded suggests that oxygen functions as a potential radical quencher. On the basis of the results of the rapid scanning stopped-flow experiments, it appears that under aerobic conditions cobalt−carbon bond breakage is very fast and HO-Cbl is subsequently produced after a lag phase of approximately 200 ms. The mechanism for the production of HO-Cbl after cobalt−carbon bond cleavage remains obscure, and attempts to identify the fate of

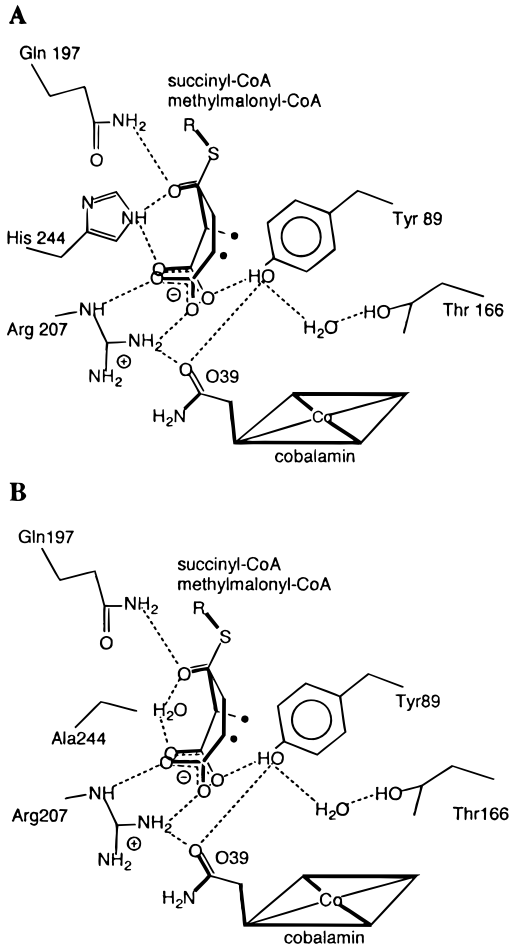

FIGURE 8: Schematic drawing of the active site of (A) the wild type and (B) the His244Ala mutant of methylmalonyl-CoA mutase. The two substrate-derived radicals are indicated by a dot (●).

coenzyme B_{12} after oxygen-dependent inactivation were unsuccessful.

The rapid inactivation of enzyme-bound AdoCbl to HO-Cbl was not observed in the Tyr89Phe mutant (*14*) for which k_{cat} (0.086 s^{-1}) and K_m (64 μM) are similar to the values for the His244Ala mutant. Therefore, slow turnover, which implies in the case of the Tyr89Phe mutant that the enzyme spends a longer time in its radical form (Figure 1, **II** and **III**), does not automatically lead to an increased rate of oxygen-mediated inactivation.

Further clues to the oxygen lability of the His244Ala mutant were provided by the crystal structure of this mutant in the presence of the substrate analogue desulfo-CoA. In initial attempts to crystallize the mutant, HO-Cbl was produced during the crystallization trials. Crystals could only be obtained using the substrate analogue desulfo-CoA in the presence of high coenzyme B_{12} concentrations. The positions of the side chains in the active site of the mutant and in the remainder of the structure were found to be indistinguishable from those in the wild type (*5*) within experimental error. The only significant structural difference is the presence of a water molecule (Figure 8), in a cavity formerly occupied by the imidazole ring of His244 in the wild type. There is clearly a specific protective effect exercised by the side chain of His244, by obstructing the close approach of molecular oxygen.

This finding prompted an investigation of the inactivation of the wild-type enzyme under aerobic conditions. Inactivation was indeed observed, with a half-life 80 times slower than that observed in the His244Ala mutant. Since the k_{cat} for the wild type is (2×10^3)-fold greater, the rate of oxidative inactivation in the wild type, although detectable, is some (1.6×10^5)-fold lower than for the mutant. In the His244Ala mutant, the direct juxtaposition of molecular oxygen to the radicals in the active site seems to drastically increase the rate of inactivation.

This view of the role of His244 has received some recent corroboration from Banerjee and colleagues, who have shown that the His244Gly mutant of methylmalonyl-CoA mutase is also oxygen labile (*16*). Although they could not exclude a deep-seated structural change in this mutant, the results presented here make it rather more likely that the oxygen sensitivity seen in His244Gly is also caused specifically by the loss of the His244 side chain.

CONCLUSIONS

The kinetic experiments described here, together with the X-ray crystallographic determination of the structure of the His244Ala mutant, provide insight into the dual role of a key amino acid residue at the active site of methylmalonyl-CoA mutase. For the His244Gln mutant, the results have allowed the construction of a qualititative free energy profile which, when contrasted with that previously deduced for the wild type, points to a direct role for His244 in enhancing the rate of the radical rearrangement step. The striking oxygen lability observed for the His244Ala mutant has led in turn to the recognition that the same oxygen sensitivity is present at a low level in wild-type mutase. The additional cavity at the active site of the His244Ala mutant apparently permits molecular oxygen far greater access to the reactive radical intermediates at the active site.

ACKNOWLEDGMENT

Financial support was provided by the Studienstiftung des deutschen Volkes to N.H.T. We thank Dr. Filippo Mancia for help with data collection and Jèrôme Huppertz for help with the preparation of the figures.

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BI0004302