Proton Transfer from Histidine 244 May Facilitate the 1,2 Rearrangement Reaction in Coenzyme B$_{12}$-dependent Methylmalonyl-CoA Mutase*

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Methylmalonyl-CoA mutase is an adenosylcobalamin-dependent enzyme that catalyzes the 1,2 rearrangement of methylmalonyl-CoA to succinyl-CoA. This reaction results in the interchange of a carboxyl-CoA group and a hydrogen atom on vicinal carbons. The crystal structure of the enzyme reveals the presence of an aromatic cluster of residues in the active site that includes His-244, Tyr-243, and Tyr-89 in the large subunit. Of these, His-244 is within hydrogen bonding distance to the carbonyl oxygen of the carboxyl-CoA moiety of the substrate. The location of these aromatic residues suggests a possible role for them in catalysis either in radical stabilization and/or by direct participation in one or more steps in the reaction. The mechanism by which the initially formed substrate radical isomerizes to the product radical during the rearrangement of methylmalonyl-CoA to succinyl-CoA is unknown. 

ab initio molecular orbital theory calculations predict that partial proton transfer can contribute significantly to the lowering of the barrier for the rearrangement reaction. In this study, we report the kinetic characterization of the H244G mutant, which results in an acute sensitivity of the enzyme to oxygen, indicating the important role of this residue in radical stabilization. Mutation of His-244 leads to an ~300-fold lowering in the catalytic efficiency of the enzyme and loss of one of the two titratable pK$_a$ values that govern the activity of the wild type enzyme. These data suggest that protonation of His-244 increases the reaction rate in wild type enzyme and provides experimental support for ab initio molecular orbital theory calculations that predict enhancement of the rearrangement reaction by the interaction of the migrating group with a general acid. However, the magnitude of the rate enhancement is significantly lower than that predicted by the theoretical studies.

Methylmalonyl-CoA mutase catalyzes the 1,2 rearrangement of methylmalonyl-CoA to succinyl-CoA and is the only coenzyme B$_{12}$-dependent enzyme that is present in both microbial and animal kingdoms (1, 2). The role of the B$_{12}$ cofactor or adenosylcobalamin (AdoCbl)$^3$ is to function as a free radical reservoir responsible for the controlled generation of a substrate radical at the initiation of the reaction cycle (3). The first common step in AdoCbl-dependent reactions is the homolytic cleavage of the cobalt-carbon bond to generate a radical pair (Scheme I). Stopped-flow kinetic studies indicate that the homolysis reaction is coupled to the subsequent step, in which a hydrogen atom is abstracted from the methyl group of the substrate to generate a substrate-centered radical (4). Evidence for kinetic coupling has also been obtained for the related glutamate mutase-catalyzed reaction (5). In ribonucleotide reductase, homolysis of the cobalt-carbon bond appears to be coupled to the generation of a protein-derived thyl radical (6). Electron paramagnetic resonance studies (7–10) provide evidence for the presence of both metal and carbon-centered radicals in the methylmalonyl-CoA mutase-catalyzed reaction. In the next step, an interchange of the carboxyl-CoA group and a hydrogen atom occurs between vicinal carbons. The precise mechanism by which the substrate radical isomerizes to the product radical is unresolved, with pathways involving free radical intermediates (3), fragmentation products (11), carboxylations (12), carbanions (13), and organocobalt adducts (14, 15), each having been proposed (Scheme II). The final step involves reabstraction of a hydrogen atom from deoxyadenosine to generate product and the intact cofactor, AdoCbl.

In the methylmalonyl-CoA mutase-catalyzed reaction, a cyclopropoxy radical is predicted for a pathway involving direct radical rearrangement (Schemes II, pathway c). Ab initio molecular orbital calculations predict that full or partial protonation of the substrate radical can facilitate the rearrangement reaction (12, 16–18). Since the conjugate acid of the thioester carbonyl oxygen has a pK$_a$ of ~6–19 (19), this precludes complete protonation of the carbonyl oxygen of methylmalonyl-CoA by any active-site residue. However, recent high level ab initio molecular orbital theory-based calculations predict that even partial proton transfer can contribute substantially to lowering the transition state barrier for the putative cyclopropoxy radical (18).

The crystal structure of methylmalonyl-CoA mutase has been solved recently (20–22). It reveals a buried active site in the α subunit, which opens to the surface via a long tunnel that is plugged by the CoA tail of the substrate. The carbonyl group of the substrate is in hydrogen-bonding distance to an active-site residue, His-244 (Scheme III), which is involved in a hydrogen-bonding network that includes the carboxylate of the substrate as well as Arg-207 (23). His-244 is thus well positioned to function as a general acid that could promote the rearrangement reaction by hydrogen bonding to the carboxyl-CoA group in the ground state and/or in the transition state. Mutation of the aromatic residue Tyr-89 to phenylalanine results in altered energetics with respect to the wild type enzyme, which is consistent with an increase in the barrier to interconversion between the substrate and product radicals (24).

To test the hypothesis that His-244 facilitates the rearrangement reaction by serving as a general acid, we have employed

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The abbreviations used are: AdoCbl, 5′-deoxyadenosylcobalamin; DEPC, diethyl pyrocarbonate; OHbl, hydroxocobalamin.
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site-directed mutagenesis. Mutation of this residue to glycine results in an acute sensitivity of the mutase-catalyzed reaction to oxygen, in contrast to the relative stability of the wild type enzyme to oxygen, and results from quenching of the radical intermediates. The catalytic efficiency of the H244G mutant is ~300-fold lower than that of the wild type enzyme under anaerobic conditions. The pH dependence of the wild type and mutant enzymes under maximal velocity conditions are distinct and suggests that one of the two kinetic pKₐ values associated with the wild type reaction is due to His-244. Our data provide experimental support for a modest rate enhancement of the rearrangement reaction by His-244, in contrast to the large effects predicted by the molecular orbital theory calculations, and reveal the importance of the aromatic cluster residue, His-244, in radical shielding.

EXPERIMENTAL PROCEDURES

Materials—Methylmalonyl-CoA and DEPC were purchased from Sigma. Thiokinase was purchased from Roche Molecular Biochemicals. Radioisotopic [14C]CH₃-malonyl-CoA (56.4 Ci/mole) was purchased from Amersham. Thiokinase was purchased from Roche Molecular Biochemicals.

Activity of the enzyme and the pH dependence of the reaction were determined in the fixed-time radiolabeled assay at 37 °C using the same conditions described previously for the native enzyme (9). Under these conditions, the spectrum of the enzyme was obtained under steady-state turnover conditions and revealed the presence of the aromatic cluster residue, His-244, in radical shielding.

Enzyme Purification—The enzyme was purified by gel filtration on a Superose 12 column (Pharmacia). The enzyme had a specific activity of 0.44 units/mg of protein at 37 °C under V_max conditions in the anaerobic assay. The thermal stabilities of the H244G mutant and wild type enzymes were compared by preincubation of the enzymes for varying lengths of time at 37 °C before their use in the standard assay and were found to be indistinguishable (data not shown).

Spectrum of H244G under Steady-state Turnover Conditions—The spectrum of the enzyme was obtained under steady-state turnover conditions and revealed the predominance of AdoCbl (Fig. 1A, spectrum 2). It is marked by a decrease in absorption across much of the spectral range. Similar spectral changes have been reported for the wild type enzyme under equilibrium conditions (9). In addition, similar spectral changes were noted in the reverse direction, i.e. when H244G was mixed with succinyl-CoA under anaerobic conditions (data not shown). In contrast, the spectrum of wild type enzyme mixed with oxygen (data not shown) was identical under aerobic and anaerobic conditions (Fig. 1B).

Since the H244G mutant is significantly more labile to oxygen than the native enzyme, it was of interest to determine whether or not the mutation had altered the stability of AdoCbl in the ground state, i.e. in the absence of substrate. Both H244G and wild type enzyme stabilize bound concentration at different pH values. That the enzymes were operating under V_max conditions was confirmed by Michaelis-Menten analysis of the substrate dependence of the reaction velocity at each pH, with substrate concentrations ranging from 0.5× to 10× K_m. The pH profiles obtained from both data sets were comparable. Due to the acute lability of H244G to oxygen, the standard assay had to be modified to make measurements under anaerobic conditions. For this, all reagents were bubbled with N₂ for 3 h before use. The enzyme solution was deoxygenated by gently blowing a stream of N₂ over its surface for 10 min at 4 °C. One unit of enzyme activity produces 1 μmol of succinyl-CoA/min at 37 °C.

RESULTS

Purification and Expression of the Mutant Protein—The mutase of His-244 to glycine does not adversely affect the expression level of the recombinant protein. Since the chromatographic behavior of the mutant was indistinguishable from that of wild type enzyme, it permitted its purification using the previously described protocol (10). The purified enzyme had a specific activity of 0.44 units/mg of protein at 37 °C under V_max conditions in the anaerobic assay. The thermal stabilities of the H244G mutant and wild type enzymes were compared by preincubation of the enzymes for varying lengths of time at 37 °C. The enzyme was found to be indistinguishable (data not shown).

Aerobic Photolysis of Holomethylmalonyl-CoA Mutase—The spectrum of AdoCbl was measured under anaerobic assay conditions in the radiolabeled assay described above. The final concentration of DEPC was 200 μm, and 500 μm hydroxylamine was employed to reverse the reaction as described previously (26).
mutant and is similar to p-site: His-244 and His-610 (20). The latter serves as the lower CoA mutase reveals the presence of two histidines in the active activity (Scheme III).

The presence of the cofactor, AdoCbl, completely protects against inhibition in both wild type and mutant enzymes. Since the susceptibility of the second histidine mutant, H610A, to DEPC is similar to that of H244G,2 these results indicate DEPC inhibition of wild type enzyme results from alkylation at His-244, the effect of DEPC on the activity of H244G was examined (Table II). In contrast to the wild type enzyme, H244G is more resistant to 200 μM DEPC retaining ~30% of its activity versus only 6% for wild type after preincubation of the enzyme with DEPC.

The carbon skeleton rearrangements catalyzed by a subgroup of AdoCbl-dependent enzymes represent chemically challenging reactions, and competing hypotheses have been advanced to explain the mechanism of this step (Scheme II). Of these, mechanisms involving carboxylation (pathway a) or carbanion (pathway b) intermediates seem unlikely to be important since they would be accompanied by the reduction or oxidation, respectively, of the homolysis product, cob(II)alamin. The absorption spectra of cobalamins containing cobalt in the 3+, 2+, and 1+ oxidation states are distinct, and spectral changes indicative of oxidation state changes have not been observed during enzyme-monitored turnover in either methylmalonyl-CoA mutase (4) or glutamate mutase (5). Based on the crystal structures of methylmalonyl-CoA mutase-containing substrate analogs, the pathway involving an organocobalt adduct (pathway e) appears to be precluded by the distance between the cobalt and the vicinal substrate carbon atoms at which the rearrangement reaction occurs (20, 21, 23).

The dissociative mechanism (pathway d) predicts the generation of acrylate and a formyl-CoA radical as intermediates (11). To account for the observed retenive substitution at both carbon centers (30), rotation of the acrylate around the C1–C2 bond has to be invoked (11). The driving force for this conformational rotation is not clear. Furthermore, formyl-CoA and acrylate inhibit methylmalonyl-CoA mutase with a very high Kf, which is >100-fold larger than the Km for succinyl-CoA (31). Recently ab initio calculations indicate that the fragmentation-
reassociation pathway requires significantly greater energy (93.2 kJ mol\(^{-1}\) versus 46.9 kJ mol\(^{-1}\) for direct rearrangement). Thus, there are no compelling reasons at present to consider this mechanism for the methylmalonyl-CoA mutase-catalyzed reaction.

Although there is insufficient evidence to rigorously dismiss the alternative pathways, the most likely mechanism for methylmalonyl-CoA mutase based on the available information, is direct rearrangement (Scheme II, pathway c). This intramolecular rearrangement reaction is estimated to have a barrier of 46.9 kJ mol\(^{-1}\) (18). Although complete protonation is predicted to lower the barrier to migration by 36.9 kJ/mol (18) versus 536 in Scheme I), an incremental lowering of this barrier is predicted by partial protonation of the carbonyl group with acids of intermediate strengths (18). The crystal structure of methylmalonyl-CoA mutase indicates that His-244 in the large subunit is within hydrogen-bonding distance to the carbonyl oxygen of the carbonyl-CoA moiety (20, 21). Its location is, thus, suggestive of a potential role in facilitation of the rearrangement reaction.

The mutation of His-244 to glycine represents a nonconservative change, and the interpretation of the kinetic results presented here must therefore be treated with caution. However, a preliminary report on the mutation of His-244 to alanine indicates that replacement of the imidazole ring results in the occupancy of a water molecule between the bound substrate and the alanine in the crystal structure determined at a resolution of 2.8 Å (32). The kinetic properties of the H244A and H244G mutants are similar, and they are both susceptible to oxidation under turnover conditions (32). This engenders confi-
idence in the pH-dependent changes associated with the H244G mutation do not result from unwanted conformational changes elsewhere in the protein.

One of the remarkable features of methylmalonyl-CoA mutase is that it catalyzes a reaction involving radical intermediates under aerobic conditions. This raises the question of how the enzyme shields the reactive organic and metallic radicals. His-244 is involved in a hydrogen-bonding interaction with the carbonyl group of the CoA moiety of the substrate. The addition of substrate to an aerobic solution of wild type holomethylmalonyl-CoA mutase rapidly leads to an equilibrium mixture of substrate and product in which succinyl-CoA is favored by a factor of 23 (33). Under these conditions the spectrum of the enzyme resembles that of the starting enzyme but displays lower absorbance across the entire wavelength range (Fig. 1B). In contrast, addition of substrate to H244G leads to the rapid appearance of OHChl, an oxidation product of the intermediate, cob(II)alamin (Fig. 1A). This can be prevented by the exclusion of oxygen from the solution. The sensitivity of H244G to oxygen is mirrored by the difference in its activity under aerobic (specific activity = 0.02 units/mg) and anaerobic (specific activity = 0.44 units/mg) conditions. Thus in H244G, cob(II)alamin is intercepted by oxygen once every 22 turnovers under aerobic conditions. In contrast, the same specific activity is measured for the wild type enzyme whether or not oxygen is present in the assay mixture (data not shown). Hence, His-244 buried in the active site, as revealed by the static crystal structures (20–22), nevertheless plays an important role in radical stabilization in the aerobic milieu that the enzyme operates in. The mutation does not, however, alter the susceptibility of H244G to photolysis by oxygen. In addition, they furnish support for the participation of His-244 as a general acid in the mutase reaction. To our knowledge, these data provide the first experimental support for the ab initio molecular orbital theory-based calculations that partial proton transfer may facilitate the rearrangement reaction in this AdoCbl-dependent enzyme.

In summary, this study demonstrates that a major role of His-244 is in shielding the reactive radical intermediates formed during the rearrangement reaction from interception by oxygen. In addition, they furnish support for the participation of His-244 as a general acid in the mutase reaction. To our knowledge, these data provide the first experimental support for the ab initio molecular orbital theory-based calculations that partial proton transfer may facilitate the rearrangement reaction in this AdoCbl-dependent enzyme.

## REFERENCES

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