Crystal Structure of Substrate Complexes of Methylmalonyl-CoA Mutase

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ABSTRACT: X-ray crystal structures of methylmalonyl-CoA mutase in complexes with substrate methylmalonyl-CoA and inhibitors 2-carboxypropyl-CoA and 3-carboxypropyl-CoA (substrate and product analogues) show that the enzyme−substrate interactions change little during the course of the rearrangement reaction, in contrast to the large conformational change on substrate binding. The substrate complex shows a 5′-deoxyadenine molecule in the active site, bound weakly and not attached to the cobalt atom of coenzyme B_{12}, rotated and shifted from its position in the substrate-free adenosylcobalamin complex. The position of Tyrα89 close to the substrate explains the stereochemical selectivity of the enzyme for (2*R*)-methylmalonyl-CoA.

Methylmalonyl-CoA mutase belongs to a group of enzymes catalyzing unusual 1,2-rearrangements which use adenosylcobalamin (AdoCbl)[1] to create radical intermediates in the reaction (for a recent review see ref *1*). It catalyzes the reversible rearrangement between (2*R*)-methylmalonyl-CoA and succinyl-CoA and is the only member of this class found in both bacteria and animals. Binding of substrate induces the enzyme-catalyzed homolytic cleavage of the Co−C bond of the cofactor to form cob(II)alamin and a 5′-deoxyadenosyl radical. This radical then abstracts a hydrogen atom from the substrate to form the substrate radical, in a step which is closely linked kinetically to the first (*2*, *3*). The substrate radical then rearranges to the product radical, in a step which is the least understood, and finally the product is formed by reabstraction of a hydrogen atom from the cofactor.

There are three major roles of the protein in the reaction: (a) catalysis of the homolysis of the Co−C bond when the substrate binds (*4*); (b) protection of the reactive radical intermediates from unwanted side reactions; (c) catalysis of the rearrangement step itself. The structure of *Propionibacterium shermanii* methylmalonyl-CoA mutase was initally determined as a complex with the truncated substrate desulfo-CoA (i.e., coenzyme A lacking the terminal thiol group) (*5*), showing that the active site is deeply buried between the two major domains of the protein (Figure 1A). This provides the required protection of radical species. The structure of the enzyme in the absence of substrate (*6*) showed an open substrate binding site, allowing easy access for the substrate. The large conformational change on substrate binding closes up the active site and destroys the site for the cobalt-linked adenosyl group: it is this conformational change which drives the initial formation of the adenosyl radical, coupling the energy of substrate binding to the breaking of the Co−C bond.

This paper reports structures of the complexes with the true substrate/product mixture and with two inhibitors. The substrate complex shows a 5′-deoxyadenosine molecule in the active site, alongside the substrate but not attached to the cobalt atom. These structures show the details of the interactions in the active site and account for the observed specificity and stereochemistry of the reaction, as the incorrect stereoisomer of methylmalonyl-CoA would not fit into the binding site.

MATERIALS AND METHODS

Materials. Recombinant *P. shermanii* was expressed in *Escherichia coli* and purified as described (*7*). 3-Carboxypropyl-CoA was synthesized as described (*8*).

Synthesis of 2-Carboxy(R)-propyl-CoA. The availability of methyl 3-bromo-2-methyl(*R*)-propionate led us to consider the preparation of the required CoA derivative by a procedure analogous to that used for the preparation of 3-carboxypropyl-CoA (*8*). The nucleophilic displacement of the bromide proceeded efficiently. The main consideration was the change in ester used, from *tert*-butyl to methyl. TFA which readily hydrolyzes the *tert*-butyl group is not useful for the methyl ester. Strong base hydrolysis was unsuccessful. Porcine esterase is specific for esters of fatty acids and favors those with α-methyl-substitution. The α-methyl-substituted ethylthiopropionyl moiety would appear sufficiently like a fatty acid ester for this esterase to cleave the methyl ester, and this was found to be the case, although the slow rate of hydrolysis required the use of a very high concentration of esterase.

Coenzyme A (100 mg) was dissolved in potassium carbonate buffer (pH 8.5, 1 M, 2 mL) and ethanol (3 mL). (+)-Methyl(*R*) 3-bromo-2-methylpropionate (Fluka, 100 mg) was added and the flask purged with nitrogen, closed, and left at room temperature for 2 days. TLC (silica in chloroform−methanol−water−diisopropylethylamine, 45:35:8:2)

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[1] Abbreviations: CoA, coenzyme A; Cbl, cobalamin; AdoCbl, adenosylcobalamin (coenzyme B_{12}).

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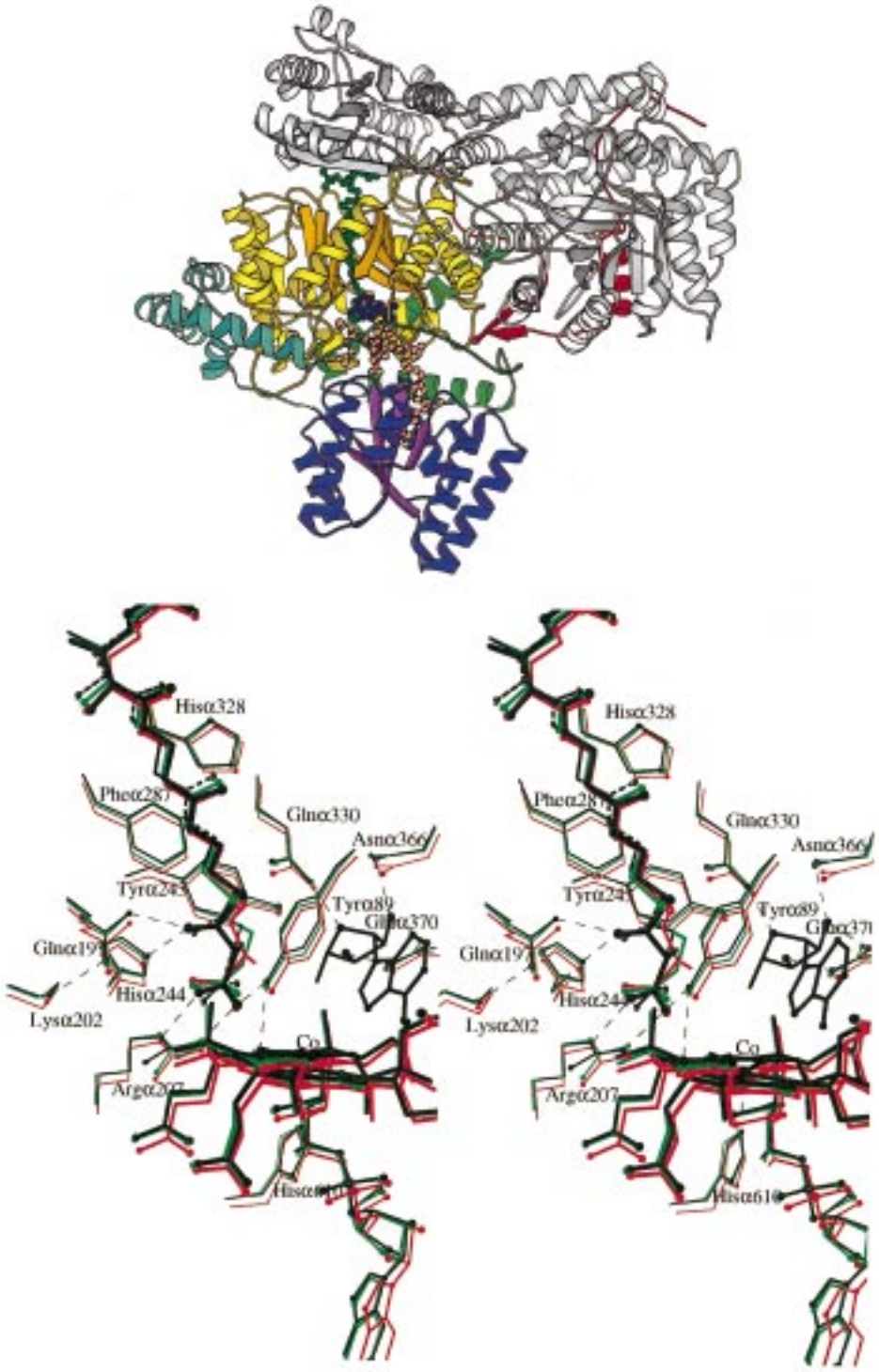

FIGURE 1: (A, top) Schematic view of methylmalonyl-CoA mutase with bound substrate. The active α-chain is colored by structural domains: red, N-terminal arm; yellow, TIM barrel substrate binding domain; green, linker regions; blue, C-terminal B_{12} binding domain. Cobalamin is colored pink, 5′-deoxyadeosine dark blue, and succinyl-CoA green. (B, bottom) Stereoview of the active site region showing the similarity of the three complexes: black, substrate/product complex; red, 2-carboxypropyl-CoA complex; green, 3-carboxypropyl-CoA complex.

showed (by UV absorption at 254 nm) no remaining CoA (R_f 0.6) and a single eluted spot (R_f 0.65) with some baseline. The solution was washed with diethyl ether, acidified by the addition of Dowex 50 H^+ to pH 1, and filtered and the resin washed with 2 volumes of water. The filtrate was neutralized by the addition of diisopropylethylamine and then lyophilized. The product was dissolved in water (200 mL) and applied to a DEAE-Sephacel column (50 mL in 5 mM triethylammonium bicarbonate, pH 7). Gradient elution gave the methyl ester of the required product as the major UV-absorbing fraction, which was collected and lyophilized.

The ester was dissolved in triethylammonium carbonate buffer (20 mM, 50 mL, pH 7.8) containing $CaCl_2$ (200 μM), treated with porcine liver esterase (Sigma, 4000 units), and stirred at room temperature overnight. TLC as above showed two major products (R_f 0.6 and 0.4). The solution was

Table 1

	substrate complex	2-carboxypropyl-CoA	3-carboxypropyl-CoA
data collection[a]			
unit cell dimensions			
a, *b*, *c* (Å)	120.1, 160.9, 88.5	122.5, 161.4, 87.0	119.9, 160.4, 88.4
β (deg)	104.6	104.8	105.0
resolution range (Å)	20−2.2	18−2.2	16−2.2
unique reflections	164053	160294	161729
multiplicity	4.3 (3.5)	4.5 (3.2)	4.4 (3.6)
completeness (%)	99.9 (99.9)	97.0 (93.8)	98.9 (98.6)
$\langle I\rangle/\sigma(\langle I\rangle)$	5.3 (1.6)	11.0 (6.0)	17.1 (8.9)
R_{merge}[b] (%)	10.3 (37.9)	10.7 (16.5)	6.5 (13.2)
B (Wilson plot) (Å^2)	40	36	36
refinement			
$\langle B\rangle$	38	26	26
R-factor[c] (R_{free})	0.222 (0.277)	0.195 (0.229)	0.216 (0.263)

[a] Values in parentheses apply to the high-resolution shell. [b] $R_{merge} = \Sigma\Sigma_i|I_h - I_{hi}|/\Sigma\Sigma_i I_h$, where I_h is the mean intensity for reflection *h*. [c] $R = \Sigma|F_P - F_c|/\Sigma F_P$.

lyophilized to remove the buffer salts, dissolved in water, and applied to a DEAE-Sephacel column (100 mL in triethylammonium carbonate, 5 mM). Elution with a gradient of triethylammonium carbonate (up to 1 M) gave the required 2-carboxy(*R*)-propyl-CoA as the last major UV-absorbing fraction eluted, which was collected and lyophilized (30 mg).

Mass spectrum, M − H 852.6 (FAB, negative ion). ^{1}H NMR [500 MHz, $2H_2O$, Na^+ salt, ∂ (ppm, water 4.80)] 0.73 (3H, s), 0.88 (3H, s), 1.14 (3H, d, $J = 6.9$ Hz), 2.47 (2H, t, $J = 6.7$ Hz), 2.51 (1H, ddq, $J = 6.0, 8.6, 6.9$ Hz), 2.58 (1H, dd, $J = 6.0, 13$ Hz), 2.67 (2H, t, $J = 6.7$ Hz), 2.72 (1H, dd, $J = 8.6, 13$ Hz), 3.35 (2H, t, $J = 6.7$ Hz), 3.44 (1H, dt, $J = 13.7, 6.7$ Hz), 3.49 (1H, dt, $J = 13.7, 6.7$ Hz), 3.55 (1H, dd, $J = 4.9, 9.7$ Hz), 3.82 (1H, dd, $J = 4.9, 9.7$ Hz), 4.01 (?H, s), 4.24 (?H, vbrt,), 4.58 (?H, br, $J = 6.7$ Hz), 6.18 (1H, d, $J = 6.8$ Hz), 8.28 (1H, s), 8.56 (1H, s). This spectrum was essentially the same as that for CoA except for the addition of the resonances at 1.14, 2.51, 2.58, and 2.72 ppm, which are those expected for the 2-methyl, 2 proton, and the distinct two protons of the methylene of the added 2-carboxypropyl group. The only other major change is the appearance of one of the methylene group triplets in CoA as a pair of distinct ab-coupled protons at 3.44 and 3.49 ppm. Small changes in shift were also observed but could have arisen from inaccurate setting of the p^{2}H.

Crystallization and Structure Determination. Crystals were grown essentially as for the desulfo-CoA complex, by vapor diffusion at 23 °C. The protein solution contained 20 mg mL^{-1} protein, 1 mM AdoCbl, 1 mM DTT, and 2−10 mM succinyl-CoA or inhibitor (2- or 3-carboxypropyl-CoA), in 100 mM Tris-HCl, pH 7.5. The reservoir solution was 17−18% PEG 4000, in 100 mM Tris-HCl, pH 7.5, and 0.01% NaN_3, diluted by 20% v/v glycerol. Equal 10 μL volumes of protein and reservoir solution were mixed to form a hanging drop, and the vessel was flushed with argon before sealing.

All X-ray data were collected to 2.2 Å resolution at Elettra (Trieste, Italy), at a wavelength of 1.24 Å (10 keV) on crystals cooled to 95 K. Intensities were integrated with Mosflm (*9*), and other calculations were done with the CCP4 suite (*10*). Statistics are given in Table 1. Because the crystals of the different complexes were not strictly isomorphous, the structures were solved by molecular replacement using the program Amore (*11*) at 4 Å resolution, followed by rigid-body and restrained refinement with Refmac at 2.2 Å resolution (*12*). The two molecules in the asymmetric unit were restrained to have similar structures. Coordinates and structure factors have been deposited in the Protein Data Bank with accession numbers 4REQ (substrate complex), 6REQ (3-carboxypropyl-CoA complex), and 7REQ (2-carboxypropyl-CoA complex).

RESULTS AND DISCUSSION

Overall Crystal Structure and Substrate Binding Site. The two inhibitors resemble the substrate and product but lack the carbonyl oxygen of the thioester which is essential for the rearrangement: 3-carboxylpropyl-CoA mimics succinyl-CoA, and (2*R*)-carboxypropyl-CoA mimics methylmalonyl-CoA. Crystals grown with succinyl-CoA or with either of the inhibitors were approximately isomorphous to crystals of the desulfo-CoA complex originally solved (*5*) and to each other but sufficiently nonisomorphous that the initial solution was by molecular replacement, followed by rigid-body refinement, before the final refinement (see Materials and Methods). The refined structures are closely similar to each other and to the desulfo-CoA complex, even around the active site, apart from differences arising directly from the different ligands (see Figure 1B), demonstrating that there is no significant conformational change during the rearrangement reaction itself.

Electron density in the active site region shows clearly the interactions between the ligands and the protein. For the 2-carboxypropyl-CoA complex, maps show clear density for the (2*R*)-methyl group and for the carboxyl group hydrogen-bonded to the guanidinium group of Argα207. 3-Carboxypropyl-CoA, however, is linear rather than branched, with an additional torsion angle between the 2- and 3-carbon atoms, and interpretation of the density is harder: models with the C2,C3 torsion either ~+60° or ~−60° both fit the density well (see Figure 2A). The latter seems to be correct, as refinement starting with the angle at −60° leaves it approximately unchanged (angles −45° and −59° for the two molecules in the asymmetric unit), while refinement starting from +60° moves the angle toward −60° (angles 0 and +6°).

The electron density from the crystals grown with succinyl-CoA showed that the active site contained methylmalonyl-CoA: clear density could be seen for the methyl group

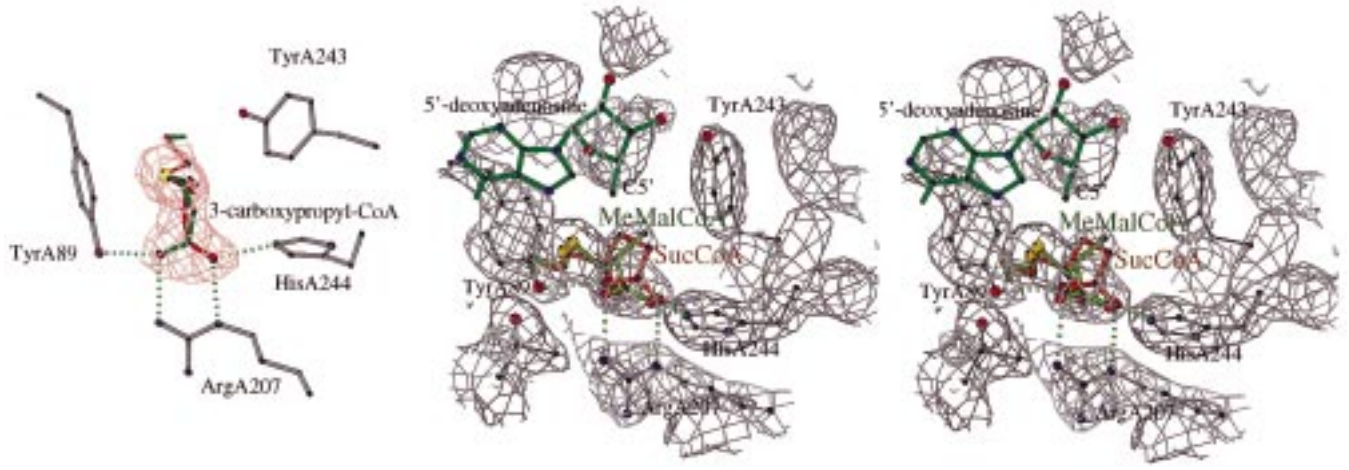

FIGURE 2: Electron density maps of ligands. (A, left) Difference density (Σ_a-weighted $mF_o - DF_c$ omit map) for 3-carboxypropyl-CoA showing alternative interpretations with different C2,C3 torsion angles: green, correct −60° conformation; red, incorrect +60° conformation. (B, right) Stereoview of the substrate/product complex ($2mF_o - DF_c$ map) showing clear density for the methyl group of methylmalonyl-CoA and its interactions with Hisα244, Tyrα89, and Argα207.

(Figure 2B). Examination of difference electron density after refinement with a methylmalonyl-CoA model suggested that succinyl-CoA was probably also present in the crystals. The model was refined with an equal 0.5:0.5 mixture of the substrate and product: the *B*-factors refined to equivalent values, suggesting that the equal half-occupancy is plausible. The equilibrium constant for the reaction favors succinyl-CoA by a factor of about 20, but it is common for the equilibrium between bound substrates to be much more equal, as seems to be the case here, since we see methylmalonyl-CoA in the electron density map. However, because any succinyl-CoA present is hidden underneath the methylmalonyl-CoA, the torsion angles of the succinyl group cannot be determined at this resolution. It was modeled in a similar way to the 3-carboxypropyl group, and the 2,3 torsion angle was refined to −46° and −33° in the two molecules in the asymmetric unit. The stereochemical consequences of the conformation of the succinyl group are discussed below.

Adenosyl Group. As in the desulfo-CoA complex, none of these complexes showed any electron density for an adenosyl group linked to the cobalt atom. All crystals grown with any CoA derivative seem principally to contain reduced five-coordinate cob(II)alamin. This presumably arises from loss of 5′-deoxyadenosine during the crystallization process. However, during the course of refinement it became apparent that, for the substrate complex, electron density in the active cavity could reasonably be interpreted as a 5′-deoxyadenosine molecule, though at less than full occupancy and not bonded to the cobalt (Figure 3A). Detailed interpretation is made more difficult because the cavity contains a number of water molecules, visible in the other structures, which confuse the difference maps. Density for the adenine ring seems particularly convincing: the model places adenine nitrogens and ribose hydroxyls close to hydrogen-bonding groups on the protein or waters and places the C5′ carbon close to the substrate, in a suitable position for hydrogen abstraction by a adenosyl radical (the species seen in the crystal is presumably not a radical). The exact details of the adenosine binding cannot, however, be identified with certainty.

Comparing the position of the adenosine molecule with that seen attached to the cobalt atom in the substrate-free crystals (*6*), which corresponds to the initial structure before the substrate binds, we note that the adenosine has moved away from the cobalt but still overlaps with its initial position (Figure 3B). It fits into the only open space in the active site cavity, with the adenine ring almost perpendicular to the corrin ring.

Catalysis and Substrate Specificity. The species visualized in the crystals are not true intermediates on the reaction pathway, as they lack the radicals, but it is reasonable to assume that they represent structures very closely similar to the substrate and product radical complexes. The most notable observation is that there is no significant conformational change between substrate and product complexes, in contrast to the enormous change on substrate binding. This is consistent with the observation that the rearrangement step is fast compared to other steps of the reaction (*13*): the rate-limiting step seems to be product release.

Exactly how the enzyme catalyzes the rearrangement reaction remains unclear. Just holding the substrate radical in the correct orientation may be a major part of the catalysis. However, we can identify certain amino acid residues as important in interactions with substrate, product, and transition state (Figure 5). Argα207 holds the carboxyl of the substrate in the correct position [it is interesting that in isobutyryl-CoA mutase, which catalyzes a very similar reaction on a substrate lacking the carboxyl group, this arginine is replaced by glutamine (*14*)]; Hisα244 contributes to the catalysis and stabilizes the intermediate, since mutation to Gln or Ala reduces k_{cat} without altering K_M (*15*); and Tyrα89 is clearly important; it guides the stereochemistry of the reaction and also contributes to the catalysis, in addition to its role in driving the adenosyl group off the cobalt (*6*). Mutation to Phe reduces k_{cat} by 580, without affecting K_M (*16*), and this mutation slows down the rearrangement step itself.

The enzyme is fairly specific for its true substrates, but the sulfur atom of the thioester may be replaced by a methylene group, and succinyl-(carbadethia)-CoA is an

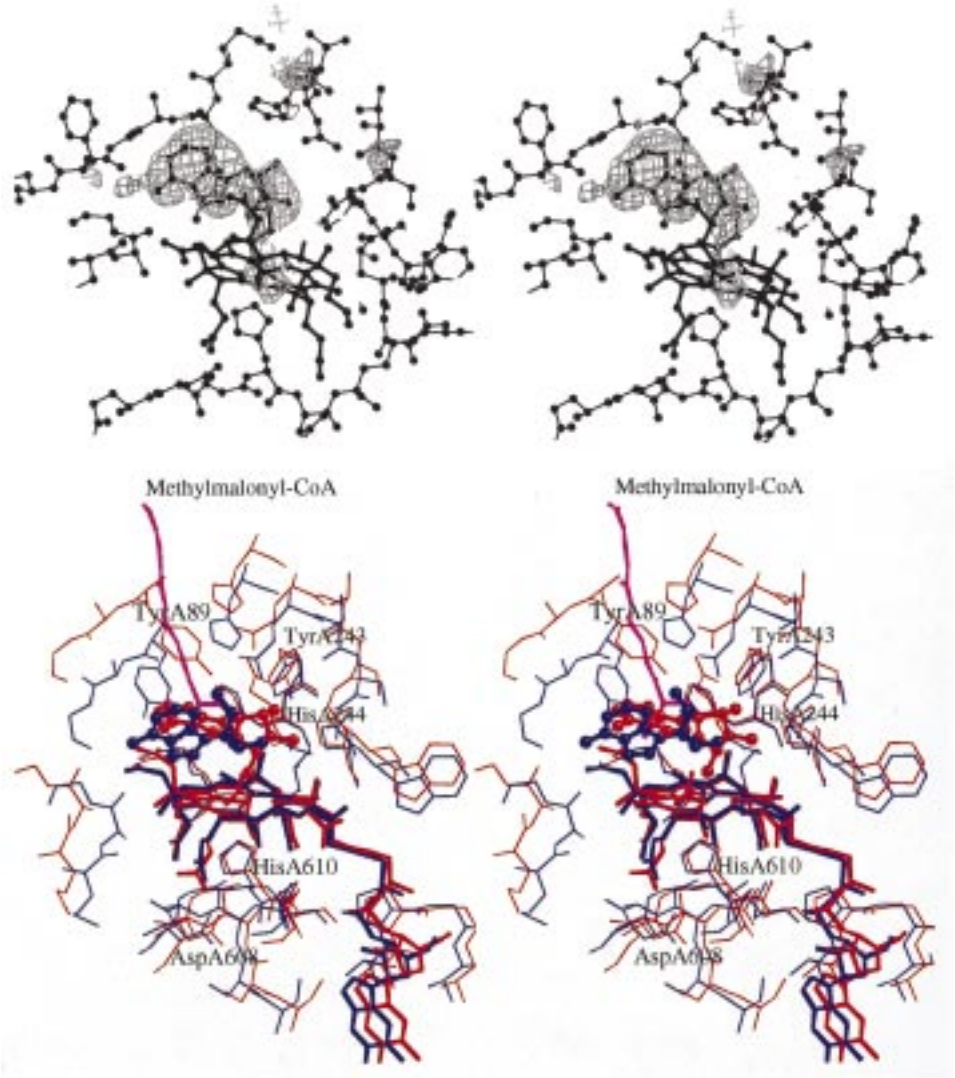

FIGURE 3: Stereoviews of 5′-deoxyadenosine binding. (A, top) Difference density ($mF_o - DF_c$ omit map) for the 5′-deoxyadenosine molecule; this is weak density so the exact placing of the molecule is not certain. (B, bottom) Comparison of the 5′-deoxyadenosine positions in the substrate-free conformation, attached to the cobalt atom (red), and in the substrate complex, rotated away from the corrin ring (blue). 5′-Deoxyadenosine molecules are shown with balls on the atoms, and the substrate methylmalonyl-CoA is colored magenta.

excellent substrate: the sulfur atom is thus not involved in the reaction, and there are no specific interactions with it. Ethylmalonyl-CoA is a substrate, though a poor one (*17*): examination of the structure shows that there is space for the extra methyl group. Other analogues which have the wrong distance between the CoA part and the terminal carboxyl group are poorer inhibitors than those with the correct distance (*18*): the optimum distance is 2 or 3 carbons between the sulfur and the carboxyl, as in the true substrate and product, corresponding to the distance between the CoA binding site and Argα207 which binds the carboxyl.

Stereochemistry of the Reaction. There are two aspects of the stereochemistry of the rearrangement reaction which the structure can explain: the preferential abstraction of the 3-H_{Re} hydrogen atom in the formation of the radical from succinyl-CoA and the specificity for (2*R*)-methylmalonyl-CoA. Isotope labeling experiments (*19, 20*) imply that the H_{Re} hydrogen atom attached to the 3-carbon is usually but not always removed in the formation of the succinyl-CoA radical. On the basis of the structure of the 3-carboxypropyl-CoA complex, the succinyl group is likely to be mostly in the C2,C3 −60° conformation but perhaps spend some of the time in the C2,C3 +60° conformation (see Figure 4, species **I** and **II**). In each case, the H_{Re} hydrogen atom is closer to the adenosyl radical, but in the C2,C3 −60° conformation there is less difference, which could perhaps occasionally lead to the H_{Si} atom being removed. These two conformations are likely to be not very different in energy, as it is their similarity which makes it hard to distinguish them in the structure.

The succinyl-CoA radical has an sp^2 carbon at the 3 position and is likely to have a C2,C3 torsion angle of close to 0° (Figure 4, **III**), held by its carboxyl group to Argα207. In the rearrangement reaction, a new chiral center is formed at this carbon atom. In principle, either stereoisomer could be formed, but modeling of intermediate **VI** or the incorrect (2*S*)-methylmalonyl-CoA product shows that the methyl carbon atom of the product (equivalent to C2 of succinyl-CoA) would clash with the ring of Tyrα89 (see Figure 2B). It is therefore this tyrosine residue which provides the stereospecificity of the reaction. Figure 4 shows one possible mechanism for the rearrangement, an associative pathway

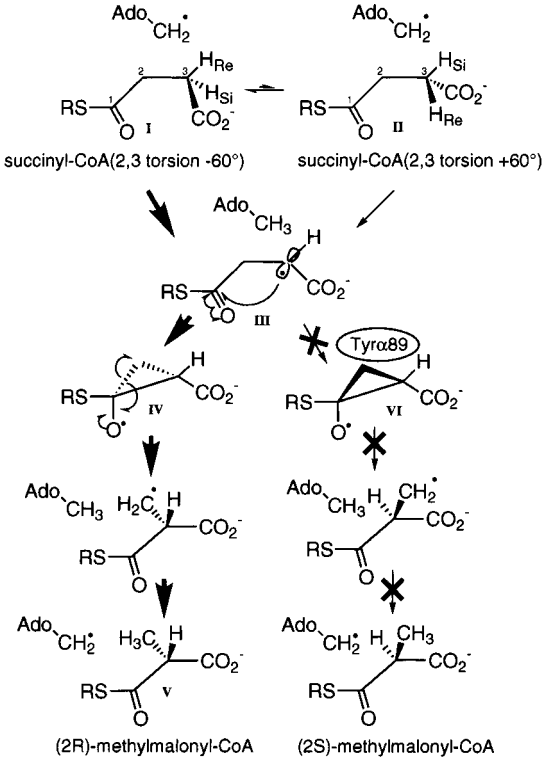

FIGURE 4: Stereochemical course of the reaction starting from succinyl-CoA. In the first stage, the H_{Re} hydrogen is closer to the adenosyl radical than the H_{Si}. (2*S*)-Methylmalonyl-CoA and intermediates leading to it would clash with Tyrα89; thus this tyrosine controls the stereochemical fate of the reaction.

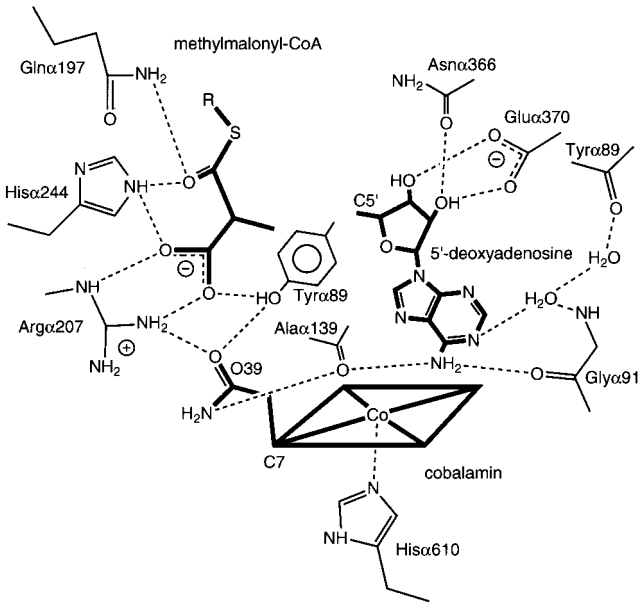

FIGURE 5: Schematic view of interactions in the active site. Note that the exact positioning of 5′-deoxyadenosine is unclear, so the interactions cannot be certain (see text).

via a cyclopropinyl radical: the stereochemical course of the alternative dissociative pathway via acrylate and the formyl-CoA radical would be very similar, but with the stereochemical discrimination being the appropriate positioning of acrylate. There is no reason to invoke rotation of the acrylate intermediate proposed in ref *21* as long as the succinyl radical (species **III**) has a C2,C3 torsion angle close to 0°.

CONCLUSIONS

Putting together the structure of the substrate complex with the previously reported "open" structure of the enzyme in the absence of substrate (*6*), we can delineate some key steps in the structural course of the reaction. The starting point is the enzyme with adenosylcobalamin bound and with the substrate-binding TIM barrel domain broken open and the active site accessible to the bulky substrate. When the substrate binds, the active site closes down on the substrate, and this conformational change drives the adenosyl group off the cobalt, by steric interference with Tyrα89, forming the adenosyl radical. Kinetic information suggests that the lifetime of the adenosyl radical is very short (*2*, *3*) and that the substrate radical is formed immediately. The structure we see of the substrate complex represents the intermediate stages of the reaction, with 5′-deoxyadenosine in a weakly bound site and the substrate radical rearranging to the product radical. When the adenosyl radical is re-formed, it can react with the Co^{II} atom, to regenerate adenosylcobalamin, favoring the opening up of the structure to release the product. The rate-limiting step (at least in wild-type enzyme) appears to be product release, consistent with the large conformational change involved. The rearrangement step itself involves no significant conformational change, consistent with its relatively high speed (*13*).

A curious feature of the enzyme is its apparent lack of a tight binding site for the intermediate 5′-deoxyadenosine, which has proved rather elusive in the crystallographic experiments and is only weakly visualized here. Fortunately for the enzyme (or perhaps by design), the adenosyl radical has only a very short life during the reaction, since a loosely bound radical could be very destructive. The longer lived substrate radical is presumably tightly bound, like the substrate and product themselves. A major role of the enzyme is to prevent side reactions of the reactive radical intermediates, presumably by preventing too much free motion in the active site.

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