Protein Science (1996), 5:1922–1927. Cambridge University Press. Printed in the USA.

FOR THE RECORD

Homology modeling of human methylmalonyl-CoA mutase: A structural basis for point mutations causing methylmalonic aciduria

NICOLAS H. THOMÄ AND PETER F. LEADLAY

Cambridge Centre for Molecular Recognition and Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, United Kingdom

(RECEIVED May 28, 1996; ACCEPTED June 21, 1996)

Abstract: Point mutations in the human gene encoding coenzyme B_{12} (adenosylcobalamin)-dependent methylmalonyl-CoA mutase give rise to an inherited disorder of propionic acid metabolism termed *mut* methylmalonic aciduria. Almost all such mutations alter amino acids in the homodimeric human enzyme that are identical to residues in the catalytic α-subunit of the heterodimeric methylmalonyl-CoA mutase from the bacterium *Propionibacterium shermanii*, to which the mature human enzyme shows an overall 65% sequence identity. To explore how specific mutations might cause the observed clinical phenotype, 12 known mutations were mapped onto a three-dimensional homology model of the subunit of the human enzyme, generated using the program MODELLER on the basis of the recently published 2.0 Å X-ray crystal structure of the *P. shermanii* methylmalonyl-CoA mutase. Eight mutations are found in the C-terminal B_{12}-binding domain, of which 4 (G623R, G626C, G630E, G703R) are in direct contact with the corrin and are clustered around the histidine ligand (H627) provided by the protein to coordinate the cobalt atom of the B_{12} cofactor. Introduction of a side chain, particularly one that is charged, at any of these positions is expected to disrupt the flavodoxin-like fold and severely impair its binding of B_{12}. Mutation at either of two other highly conserved glycine residues in this domain (G648D, G717V) also disrupts critical elements in the fold as would the introduction of an additional positive charge in the mutation H678R. Mutation of an arginine in a solvent-exposed loop to a hydrophobic residue (R694W) is also pathogenic. The remaining mutations have been mapped to the N-terminal region of the mutase, two of which introduce a buried, uncompensated charge, either near the subunit interface (A377E), or near the narrow channel through which acyl-CoA esters gain access to the active site (W105R). The extreme N-terminus of methylmalonyl-CoA mutase is predicted to make extensive contacts with the other subunit, and a mutant in this region (R93H) may prevent the correct assembly of the dimer.

Reprint requests to: P.F. Leadlay, Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, United Kingdom; e-mail: pfl10@mole.bio.cam.ac.uk.

Keywords: adenosylcobalamin; homology modeling; methylmalonic aciduria; MODELLER

Adenosylcobalamin or coenzyme B_{12} (AdoCbl) is an essential cofactor for the ribonucleotide reductase of *Lactobacillus* (Booker & Stubbe, 1993) and for a number of prokaryotic enzymes that catalyze 1,2-rearrangements, including unusual carbon skeletal rearrangements (Halpern, 1985). The primary role of AdoCbl is in the generation of a free radical that initiates catalysis (Marsh, 1995). One of the most intensively studied of these rearrangements is the interconversion of (2R)-methylmalonyl-CoA and succinyl-CoA, catalyzed by methylmalonyl-CoA mutase (MCM, EC 5.4.99.2) (Rétey, 1990). In the prokaryote *Propionibacterium shermanii*, MCM functions in the terminal pathway for fermentation of succinyl-CoA into propionate. The *P. shermanii* enzyme is a heterodimer in which only the α-subunit (80 kDa, 728 amino acids) binds AdoCbl and substrates, whereas the role of the homologous β-subunit (70 kDa, 638 amino acids) remains unclear. In humans, MCM is required for the metabolism of propionate, derived for example from odd-chain fatty acids, into succinyl-CoA (Willard & Rosenberg, 1980). Human MCM is targeted to the mitochondrial matrix, where, after the cleavage of an 32-residue N-terminal signal sequence, it is found as a homodimer of 78.5 kDa per subunit (718 amino acids) which binds 2 mol AdoCbl per dimer. The cloning and sequence analysis of both the human MCM (Ledley et al., 1988; Jansen et al., 1989) and the MCM from *P. shermanii* (Marsh et al., 1989) has revealed the very high amino acid sequence homology (65% identity) between the mature human enzyme and the α-subunit of the *P. shermanii* enzyme, despite their differences in quaternary structure.

Inborn genetic defects in the gene encoding human MCM give rise to a serious disorder of propionic acid and methylmalonic acid metabolism termed *mut* methylmalonic aciduria (*mut* MMA; McKusick, 1990). Two classes of mutations have been distinguished (Willard & Rosenberg, 1977) using cultured primary fibroblasts from patients with *mut* MMA. Mutations are designated

mut° if the cultured cells show no MCM activity and no metabolism of administered propionate. Other mutations, in which there are low residual levels of MCM activity and propionate metabolism, which can be increased by addition of hydroxycobalamin, are designated *mut*⁻.

At least 12 pathogenic *mut*° and *mut*⁻ mutations have now been identified and characterized (Jansen & Ledley, 1990; Raff et al., 1991; Crane et al., 1992; Qureshi et al., 1994) in which the lesion is a point mutation leading to alteration of an amino acid in the MCM apoenzyme. In particular, a cluster of such mutations was noted near the C-terminus of the protein (Crane & Ledley, 1994), which is now recognized as the common cobalamin-binding domain of many B_{12}-binding enzymes (Marsh & Holloway, 1992; Drennan et al., 1994; Mancia et al., 1996). Intriguingly studies with somatic cell hybrids have demonstrated that a number of these mutations show interallelic complementation with a mutation (R93H) in the N-terminal region of MCM. We report here on the use of the recently solved crystal structure of *P. shermanii* MCM (Mancia et al., 1996) to derive a molecular model of the human MCM, which in turn has provided the first insight into the molecular basis for pathogenic mutations in the MCM enzyme.

Results: *Evaluation of the model*: The human MCM model and that of the *P. shermanii* MCM α-subunit are essentially identical in backbone structure except for two short deletions in surface loops and the loss of a 3(10) helix in the human enzyme (Fig. 1). The homology model of the human MCM subunit that was derived using MODELLER (Sali & Blundell, 1993) fulfilled the criteria implemented in the program PROCHECK (Laskowski et al., 1993), including Ramachandran plot, planarity of peptide bonds, tetrahedral distortion at Cα atoms, nonbonded interactions, and hydrogen bonding energies, as required for a structure at 2 Å resolution. For example, 94% of residues reside in the most favored areas of the Ramachandran plot (90% for structures solved at 2 Å resolution or better) and the overall G-factor, which measures stereochemical quality, is 0.09 (better than the values of −0.7 to −0.1 for structures solved at 2 Å resolution). This favorable outcome reflects the high sequence identity (65%) between the human MCM and the template, and the fact that no insertions needed to be modeled. The N-terminal extended segment (residues 32–87), which interacts with the other subunit, shows a lower sequence identity (40%) between human and *P. shermanii* MCM than does the rest of the enzyme and was therefore modeled with lower confidence.

Each subunit of the model consists of essentially two domains (Fig. 2A,B). An N-terminal eight-stranded β/α barrel is preceded by an extended segment wrapping around the other subunit. A long linker region encloses the eight-stranded β/α barrel and connects it to the C-terminal β/α domain containing five parallel β-sheet strands. The C-terminal domain packs on one end of the eight-stranded β/α barrel, sandwiching the corrin ring of the cobalamin and, forming the active side cavity. The two identical subunits are related by a local two fold symmetry with helices Iα6 and Iα7 from each subunit packed around the dyad (Fig. 2A).

Mutations in the adenosylcobalamin-binding domain: Twelve pathogenic mutations that arise from single amino acid substitutions in the human MCM apoenzyme are listed in Table 1. Eight of these affect highly conserved amino acid residues in the C-terminal β/α flavodoxin-like domain (residues 578–750) that binds adenosylcobalamin (Fig. 3). The structure of the cobalamin-binding domain was first recognized for methionine synthase (Drennan et al., 1994)

Table 1. *Mutations in the human methylmalonyl-CoA mutase gene*

Mutation	Phenotype	Location in the model structure
G623R	*mut*°	B_{12} binding fold, in direct proximity to B_{12}
G626C	*mut*⁻	B_{12} binding fold, in direct proximity to B_{12}
G630E	*mut*°	B_{12} binding fold, in direct proximity to B_{12}
G703R	*mut*°	B_{12} binding fold, in direct proximity to B_{12}
G648D	*mut*⁻	B_{12} binding fold
G717V	*mut*⁻	B_{12} binding fold
R694W	*mut*⁻	B_{12} binding fold
R369H	*mut*⁻	$(\alpha/\beta)_8$ barrel, dimer interface
A377E	*mut*°	$(\alpha/\beta)_8$ barrel, dimer interface
W105R	*mut*°	$(\alpha/\beta)_8$ barrel, substrate channel
R93H	*mut*°	N-terminal extension

where it binds methylcobalamin, but the MCM from *P. shermanii* (Mancia et al., 1996) and the human MCM model show a near-identical three-dimensional fold. The core is formed by five parallel β strands (IIβ1–IIβ5) that alternate with α helices (IIα1–IIα5). The dimethylbenzimidazole portion of the cobalamin is accommodated in a deeply buried pocket flanked on one side by strands IIβ3 and IIβ4 and on the other side by side chains from helices IIα1 and IIα5. The corrin ring binds on one end of the domain against the loops connecting the C-termini of the β-strands with the N-termini of the following α-helices. In particular, the loop between IIβ1 and IIα1 contains His 627, which provides the ligand to cobalt in these enzymes (Fig. 2C). His 627, Asp 625, and Lys 621 form part of a hydrogen bonded triad of residues that is proposed to modulate the reactivity at cobalt either by acting as a proton relay (Drennan et al., 1994) or by poising the histidine ligand at an unusually long distance from the cobalt atom (Mancia et al., 1996).

The critical importance of the histidine containing loop is underscored by the finding that all three conserved glycine residues in this loop are essential for MCM activity (Fig. 2C). Mutations G623R and G630E are *mut*° mutations, whereas G626C shows a *mut*⁻ phenotype. Gly 623 is positioned near the start of the loop in a tight turn, whose main-chain torsion angles are incompatible with substitution by arginine. Gly 626 (corresponding to Gly A-609 in *P. shermanii* MCM) makes direct hydrogen bonding contact between its main-chain NH group and the propionamide side chain at C-8 of the corrin ring. This contact would be disrupted by the cysteine side chain in G626C, which would also make an unsatisfactory overlap with the main chain at Val 619 and Asp 620. This mutant is only marginally active in the presence of added hydroxycobalamin (Crane & Ledley, 1994). Gly 630 (Gly A-613 in *P. shermanii* MCM) also occupies a critical position in this loop, at the start of helix IIα2. A glycine at this position is essential to help create the dimethylbenzimidazole-binding pocket, and the C_α of Gly 630 is within 3 Å of one oxygen of the phosphodiester group in the cofactor "tail," whereas the backbone NH is probably hydrogen bonded to it via a well-ordered water molecule, as found in *P. shermanii* X-ray crystal structure (Mancia et al., 1996). The mutation G630D would not be accommodated in this arrangement because of the increased bulk and charge of the side chain.

Gly 703 on the inner face of strand IIβ4 (residue Gly A-686 in *P. shermanii* MCM) is another conserved residue essential to create the dimethylbenzimidazole-binding pocket (Drennan et al., 1994). The main-chain NH of Gly 703 is hydrogen bonded to the

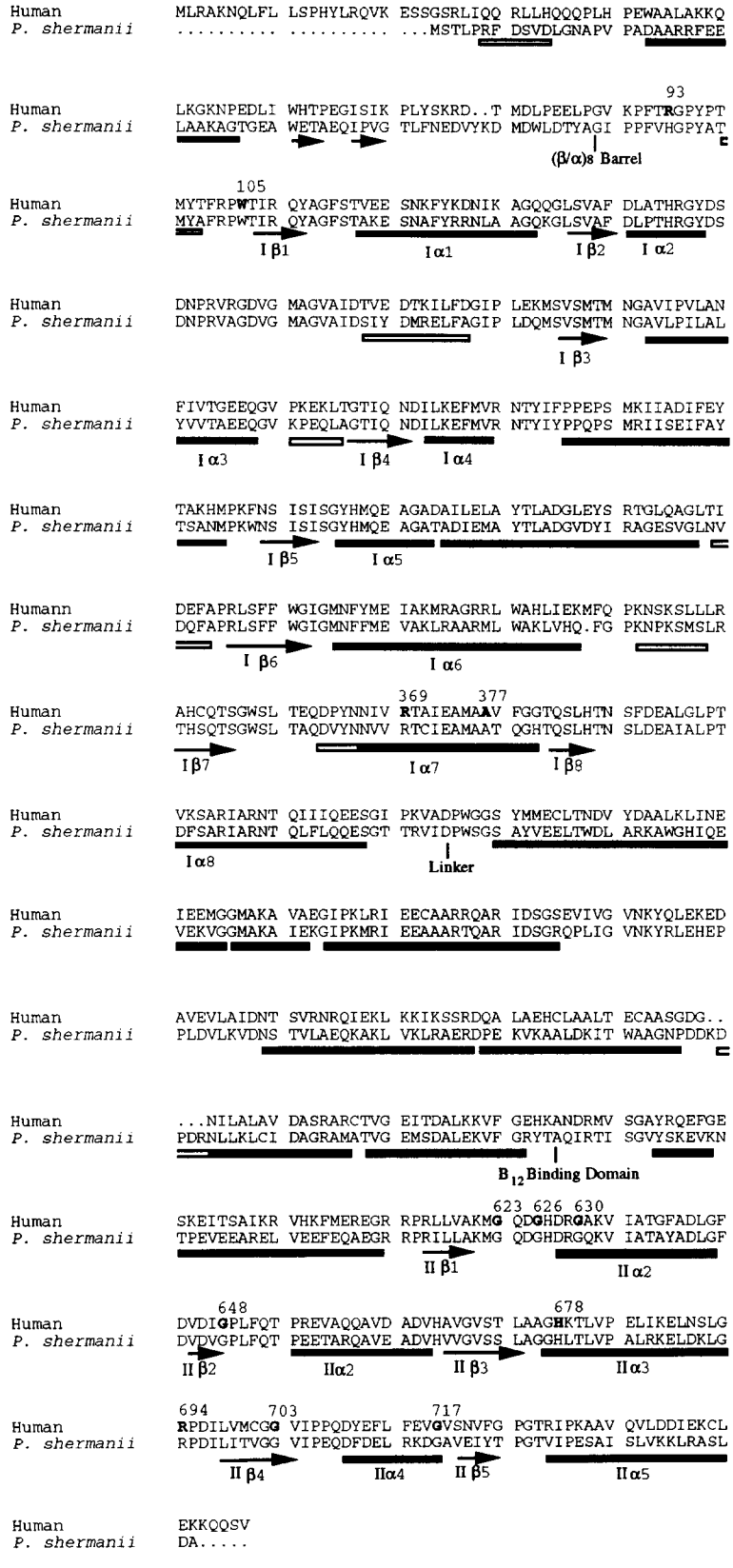

Fig. 1. Sequence alignment of the mature human methylmalonyl-CoA mutase with the α subunit of the *P. shermanii* enzyme. Secondary structure elements as derived from the *P. shermanii* MCM crystal structure are indicated; closed and open boxes represent α helices and 3(10) helices respectively, arrows are used for β-strands. The α helices and β sheets involved directly in the formation of the $(\beta/\alpha)_8$ barrel have the prefix I; helices and β strands comprising the $(\beta/\alpha)_5$ barrel responsible for B_{12} binding have the prefix II. Starting points of the $(\beta/\alpha)_8$ domain, the domain linker, and the B_{12} binding domain are indicated. The positions of individual mutations in human MCM are indicated in bold with the residue number printed above.

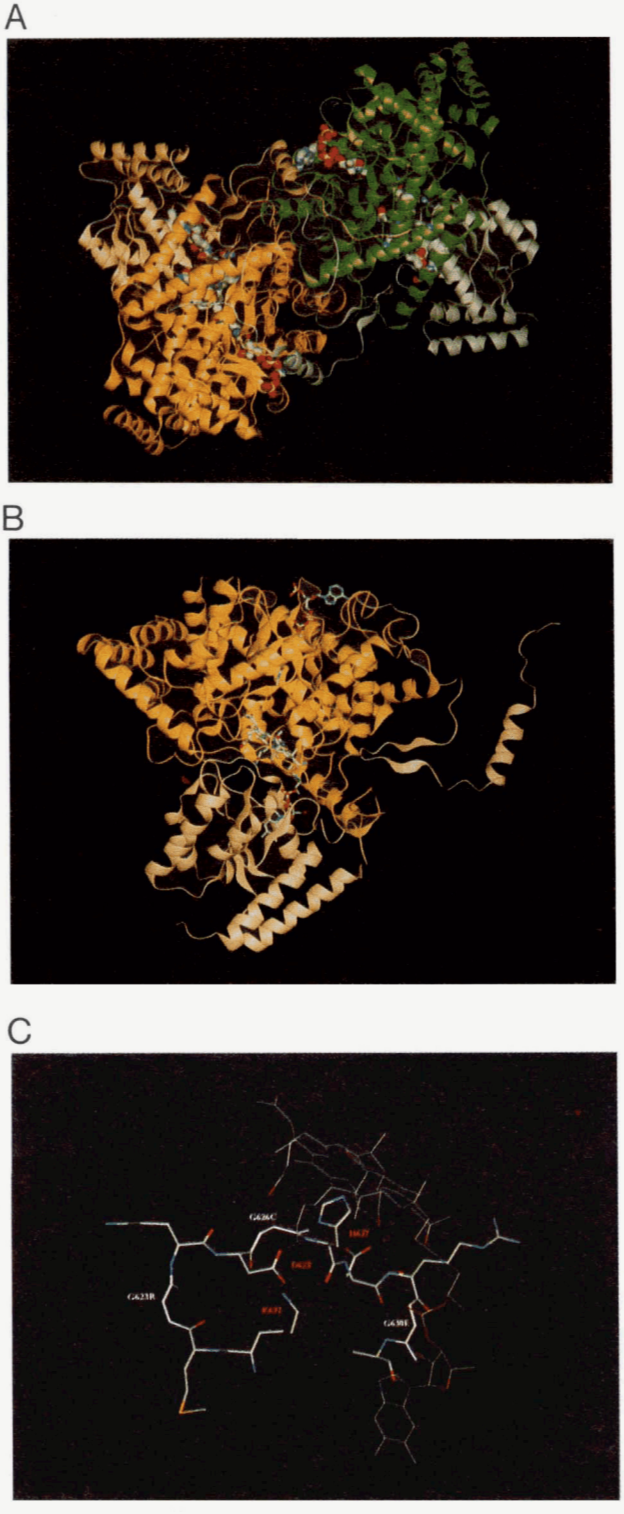

Fig. 2. A: Model of the human methylmalonyl-CoA mutase homodimer. The two subunits are shown in yellow and green. In each subunit the darker coloring indicates the $(\beta/\alpha)_8$ barrel. Coenzyme-A and the cobalamin are shown in space-filled mode. **B:** Model of the individual subunit of the human enzyme. The $(\beta/\alpha)_8$ domain is shown in dark yellow, the N-terminal extension and the B_{12} domain are shown in light yellow. The coenzyme-A derivative threads through the $(\beta/\alpha)_8$ barrel to reach the deeply buried active site. The corrin ring of B_{12} is sandwiched between the $(\beta/\alpha)_8$ barrel and the B_{12} domain. **C:** Cobalamin and the protein-derived loop providing His 627. The proposed regulatory triad consisting of His 627, Asp 625, and Lys 621 is indicated. Glycines 623, 626, and 630, each of which are sites for mutations in the human enzyme, are shown in white.

2′-hydroxy group of the ribose ring in the pseudo-nucleotide tail, and the mutation G703R (*mut*°) would clearly lead to severe steric clashes and the introduction of a positive charge into a buried environment.

The loop at the C-terminus of strand IIβ2 is also initiated by a conserved glycine residue, and mutation here (G648D) leads to a *mut*$^-$ phenotype, presumably because the turn is disrupted, and there would be steric interference with the packing of the domain, through unfavorable nonbonded contacts with the strand IIβ1. Residue His 678 lies at the end of another such loop on the corrin-binding face of the β/α domain, at the start of helix α3. In the mutation H678R, the increased size of the side chain is likely to lead to unfavorable contact with side chains Asp 709 and Pro 706, and to unfavorable charge-charge interaction with Lys 679, although His 678 is not in direct contact with the corrin.

Other *mut*$^-$ mutations mapped within the β/α cobalamin-binding domain are likely to exert their effects by disruption of the fold rather than by direct effects on adenosylcobalamin binding (Fig. 3). Thus, the conserved residue Gly 717 is found in a sharp turn at the C-terminal end of helix IIα4 and mutation G717V would be incompatible with this geometry. The conserved residue Arg 694 is positioned on the loop at the C-terminal end of helix α3, with its side chain predicted to form an electrostatic interaction with those of conserved residues Asp 648 and Asp 696. Mutation R694W would introduce a hydrophobic group on the surface of the domain, which is energetically unfavorable and would also disrupt these favorable interactions unless the domain were significantly distorted.

Mutations in the N-terminus and β/α-barrel domain: Three mutations (R369H, A378E, and W105R) have been mapped to the β/α-(TIM-) barrel domain (Fig. 3). The mutation R369H (N. Kogekar & W.A. Fenton, unpubl. results, quoted by Qureshi et al., 1994) affects a residue in helix Iα7 of the barrel domain, on one of the two α-helices that form the dimer interface. Arg 369 is salt bridged to amino acid Glu 415 provided by the other subunit. Although the mutation could effect dimerization, no simple explanation can be offered on the basis of the homology model for the *mut*$^-$ phenotype, because there is no experimental evidence for the relation between dimerization and adenosylcobalamin binding. The other two mutations introduce charged side chains into the interior of the fold. Ala 377 is also located on the interface helix Iα7 in the β/α-(TIM-) barrel domain, pointing in toward the β-strands of the barrel in a very hydrophobic environment. Therefore, in the mutation A377E, the introduction of charge and added bulk would cause substantial disruption of the packing of the β/α domain and possibly also disruption of the subunit interface. Similarly, residue Trp 105 is found near the N-terminus of the first β-strand of the barrel, which lines the access channel for CoA ester substrates. The tryptophan side chain is in a hydrophobic pocket pointing away from neighboring residues Arg 103 and Arg 108, which bind the ADP-ribosyl moiety of the CoA ester substrate at the entrance to the substrate channel along the central axis of the TIM-barrel. The mutation W105R would therefore be expected to distort the packing of these residues and interfere with the fold and with substrate binding.

The residue Arg-93 lies near the N-terminal extension that precedes the barrel domain. This region of the human MCM model is predicted with least confidence but, by inspection of the *P. shermanii* crystal structure, it is thought that Arg B-95, the equivalent residue in the β subunit, makes strong contacts with the α subunit. Although the R93H mutation gives rise to a *mut*° phenotype, co-

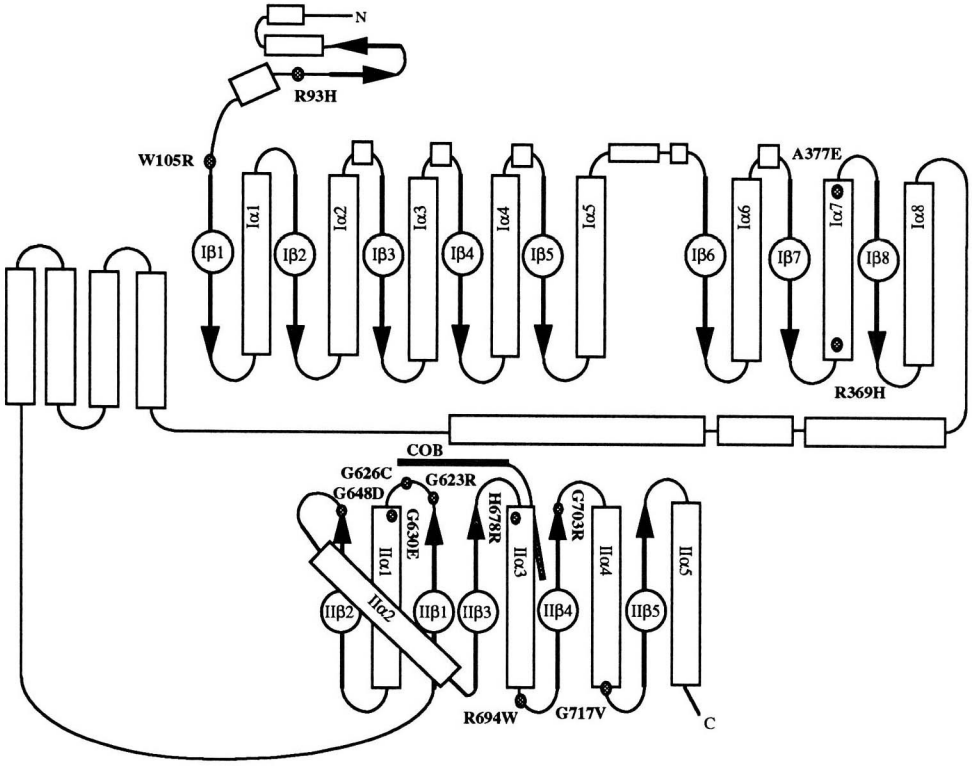

Fig. 3. Topology diagram of the human methylmalonyl-CoA mutase (modified from Drennan et al., 1994). The position of each mutation in human MCM is indicated by filled circles. COB represents cobalamin.

transfection of a gene bearing this mutation with a gene bearing a mutation in the cobalamin-binding domain has been shown to stimulate propionate uptake in *mut*° cell line (Raff et al., 1991; Crane & Ledley, 1994; Qureshi et al, 1994). This interallelic complementation has been noted between R93H and both *mut*$^-$ mutations (G626C, G648D, R694W, G717V, G703R) and *mut*° mutations (G630E, G623R). These findings can at least be rationalized on the basis of the homology model. The cobalamin-binding domain of the R93H mutations is predicted to be intact and functional, and this residue does not contribute to either the binding of substrate or to the active site. A possible (and testable) explanation is that dimerization is required for activity and that the R93H mutation may exert its effect by interfering with homo dimerization. If the R93H mutation does not prevent at least partial formation of heterodimers, then the MCM activity of such heterodimers would account for the observed complementation.

Discussion: The very high amino acid sequence similarity (65% identity) between the mature human MCM and the α-subunit of *P. shermanii* MCM has allowed the construction of a useful homology model for the human enzyme, using the program MODELLER, which operates to satisfy spatial constraints. The human MCM differs in being a homodimer rather than a $\alpha\beta$ heterodimer, and it binds 2 mol adenosylcobalamin per dimer rather than 1. The interface region in *P. shermanii* MCM is composed of two α-helices of the $(\beta/\alpha)_8$-barrel domain, drawn from each subunit, and a good fit of the residues in the human dimer could be modeled by rigid body fitting. The only exceptions are the first 8 N-terminal amino acids of the homodimer involved in steric clashes due to obvious structural differences in this region between the *P. shermanii* α subunit, used as the modeling template for the human MCM subunit, and the *P. shermanii* β subunit used for rigid body fitting to generate a human homodimer. Further work will be needed to define the interface interactions in detail, and the potential role of the N-terminal region in the dimer formation.

Meanwhile, the homology model for human MCM has already provided fresh insight into the spatial relationships of a number of the natural mutations which cause *mut* methylmalonic aciduria, and into their possible effects on protein folding and catalysis. The model also provides a structural basis for interpretation of the effects of mutation discovered in the future, and for prediction of the effect of mutations introduced into cloned MCM genes by site-specific mutagenesis.

Materials and methods: The homology model for the human methylmalonyl-CoA mutase was generated using the computer program MODELLER (Sali & Blundell, 1993). The input to the program is an alignment of the target sequence with the related three-dimensional structure. The output is a three-dimensional model of the target sequence including all main-chain and side-chain heavy atoms. The initial alignment was prepared including all available methylmalonyl-CoA mutase α subunits using the program PILEUP as provided by the GCG package (Genetics Computer Group, Wisconsin). The PILEUP output was used to edit the final alignment between the mature human methylmalonyl-CoA (residues 33–728) and the *P. shermanii* α subunit (residues 1–728) (Fig. 1), used as input for MODELLER. The two sequences share an overall 65% sequence identity.

MODELLER first derives distances and dihedral restraints from the known *P. shermanii* MCM 2-Å crystal structure (Mancia et al.,

1996) and imposes them onto the sequence of the human enzyme guided by the externally supplied sequence alignment. These spatial restraints, in combination with energy terms enforcing proper stereochemistry, are combined into an objective function, which is subsequently optimized in Cartesian space by the use of the variable target function method and molecular dynamics with simulated annealing. The strength of the restraints derived is critically dependent on the sequence identity of the two proteins. Generally speaking, a high identity leads to more and stronger constraints, which in turn result in a more precise three-dimensional model of the unknown protein. MODELLER has been shown previously to predict correctly about 90% of main-chain atoms of the human eosinophil neurotoxin with an RMS of approximately 1 Å, when compared to the 1.8 Å structure, based on a sequence identity of 40% to the template (Sali et al., 1995). The final model derived for the human methylmalonyl-CoA mutase was evaluated using the program PROCHECK (Laskowski et al., 1993) and the QUANTA Protein Health Module (MSI, Burlington, Massachusetts). The quality of the model was sufficiently good that the coordinates for enzyme-bound B_{12} and coenzyme-A could be fitted directly from the template structure to the model.

A model for the homodimeric human enzyme was generated by rigid body superposition of the human subunit onto the interface helices of the *P. shermanii* β subunit. Twelve conserved amino acids between human MCM and the β subunit of the *P. shermanii* enzyme, positioned on the 2 interface helices and the N-terminal extension, were used to fit the 2 molecules with an RMSD of 1.4 Å for 99 atoms.

Acknowledgments: N.H.T. thanks the Studienstiftung des deutschen Volkes for financial support.

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