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New Crystallographic Snapshots of Large Domain Movements in Bacterial 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase

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Abstract

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGR) catalyzes the first committed step of the mevalonate pathway, which is used across biology in the biosynthesis of countless metabolites. HMGR consumes 2 equiv of the cofactor NAD(P)H to perform the four-electron reduction of HMG-CoA to mevalonate toward the production of steroids and isoprenoids, the largest class of natural products. Recent structural data have shown that HMGR contains a highly mobile C-terminal domain (CTD) that is believed to adopt many different conformations to permit binding and dissociation of the substrate, cofactors, and products at specific points during the reaction cycle. Here, we have characterized the HMGR from *Delftia acidovorans* as an NADH-specific enzyme and determined crystal structures of the enzyme in unbound, mevalonate-bound, and NADH- and citrate-bound states. Together, these structures depict ligand binding in both the active site and the cofactor-binding site while illustrating how a conserved helical motif confers NAD(P)H cofactor specificity. Unexpectedly, the NADH-bound structure also reveals a new conformation of the CTD, in which the domain has “flipped” upside-down, while directly binding the cofactor. By capturing these structural snapshots, this work not only expands the known range of HMGR domain movement, but also provides valuable insight into the catalytic mechanism of this biologically important enzyme.

Keywords

HMG-CoA reductase; mevalonate pathway; cholesterol; steroid; isoprenoid; X-ray crystallography; protein structure; cofactors

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AUTHOR CONTRIBUTIONS

Y.K. conceived of the project and designed the experiments together with E.R.R. E.A. cloned the DaHMGR construct. E.R.R. expressed and purified DaHMGR, performed the kinetic work, crystallized the protein, collected X-ray diffraction data, and determined and refined the structures. Y.K. and E.R.R. analyzed the data and wrote the paper.

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

Supporting Information is available free of charge on the ACS Publications website. Figures that depict steady-state kinetics and inhibition data, the lack of electron density in the cofactor-binding site of the mevalonate-bound DaHMGR structure, crystal packing in the CTD region of the NADH-bound DaHMGR structure, and a steric clash between the CTD in the “flipped” conformation and CoA (PDF).

INTRODUCTION

The mevalonate pathway is found in all kingdoms of life and is responsible for the biosynthesis of an immense range of molecules, from steroids such as cholesterol to isoprenoids, also known as terpenes or terpenoids, the largest and most structurally diverse class of natural products. Catalyzing the reduction of HMG-CoA to mevalonate using 2 equiv of NAD(P)H, HMGR performs the first committed step of the mevalonate pathway, and thus its key role in steroid biosynthesis makes HMGR the target of cholesterol-lowering statin drugs. The enzyme has evolved into two distinct classes that can be identified by sequence,^{1–4} where class I HMGRs are present in eukaryotes and in some bacteria and archaea while class II HMGRs are found only in bacteria and archaea.

Although class I and class II HMGRs exhibit similar overall folds, with substrates and cofactors binding at a homodimeric interface, they differ in key catalytic regions and in their NAD(P)H cofactor preferences. All class I enzymes including the human HMGR use NADPH only, yet class II enzymes are known to display a wide range of cofactor preferences. Some class II HMGRs use only NADH, such as HMGRs from *Pseudomonas mevalonii* (PmHMGR)⁵ and *Burkholderia cenocepacia*,⁶ while others have been found to use only NADPH, such as HMGR from *Enterococcus faecalis*.⁷ Still other class II HMGRs can use both NADH and NADPH, with preferences for one or the other, including HMGRs from *Staphylococcus aureus*,⁸ *Listeria monocytogenes*,⁹ *Archaeoglobus fulgidus*,¹⁰ and *Streptococcus pneumoniae* (SpHMGR).¹¹

Through structural studies on SpHMGR, we recently described how a highly mobile C-terminal domain (CTD) present in all class II HMGRs and often disordered in crystal structures alternately adopts multiple conformations during the reaction cycle (Figure 1A–D). Briefly, the CTD has been observed to assume an “open” conformation both in the absence of any bound ligands (Figure 1A) and when the enzyme binds its cofactor, NAD(P)H (Figure 1B).¹¹ In this conformation, the CTD is distant from both HMG-CoA- and NAD(P)H-binding sites, exposing them to permit entry and exit of the substrate and cofactor alike. However, when only HMG-CoA is bound to HMGR, the CTD undergoes a large conformational change and adopts a “partially closed” conformation (Figure 1C).¹¹ Here, the domain makes direct interactions with the CoA moiety, covering the substrate-binding site while also leaving the cofactor-binding site open. When both the substrate and the cofactor are bound simultaneously, the enzyme changes conformation to the “closed” conformation (Figure 1D), where the CTD covers the active site, making interactions with both the substrate and the cofactor.¹² Importantly, in this “closed” conformation, the domain contributes a catalytically essential histidine residue to the active site,^{12–15} which is believed to serve as a general acid in the HMGR mechanism.

HMGR and mevalonate pathway enzymes have recently been exploited in synthetic biology and metabolic engineering for the biological production of isoprenoid compounds in microbial hosts.^{16–20} In one study, HMGR homologues from many different organisms were expressed alongside the remaining mevalonate pathway enzymes from *Saccharomyces cerevisiae* within an *Escherichia coli* strain engineered to produce amorphadiene, the isoprenoid precursor to the antimalarial drug artemisinin.²¹ Interestingly, among the several

HMGR homologues tested, the final amorphadiene yield was greatest with the class II HMGR from *Delftia acidovorans* (DaHMGR), a soil-dwelling bacterium and opportunistic human pathogen that is also known for its ability to produce gold nuggets via biomineralization.²²

To gain greater insight into HMGR catalysis and to examine DaHMGR in particular, we sought to investigate the structure and kinetics of this uncharacterized bacterial class II HMGR. In this work, we first show that DaHMGR has a strong preference for NADH, with very low activity for NADPH. We then determined crystal structures of DaHMGR that depict the unbound apoenzyme, the mevalonate-bound state, and the NADH-bound state, which provide crystallographic support for the idea that a helical structural motif of class II HMGR confers cofactor specificity. In the NADH-bound structure, the active site also contains bound citrate, a molecule highly similar to the product mevalonate and the reaction intermediate mevaldehyde that was present in the crystallization solution and exhibits the same binding features. Citrate inhibition studies show a weak degree of competitive inhibition with respect to the substrate, consistent with citrate binding in the active site. Surprisingly, this crystal structure also depicts a novel conformation of the CTD that illustrates a large protein movement relative to the previously described HMGR structures that depict ordered CTDs. Even as it shares a general location with the known “closed” conformation, the CTD appears in a completely upside-down orientation, compelling us to revise the current model of HMGR domain movement during the enzyme’s reaction cycle while also providing insight into HMGR catalysis.

MATERIALS AND METHODS

Cloning, Expression, and Purification.

A codon-optimized, linear *mvaA* gene that encodes DaHMGR (Integrated DNA Technologies) was cloned using NdeI and BamHI restriction enzymes into pSKB3, a modified pET28b vector that encodes an N-terminal, TEV-protease-cleavable hexahistidine tag and a kanamycin resistance cassette. The plasmid was transformed into *E. coli* DH10B cells, and its gene sequence was confirmed (Quintara Biosciences) before transformation into BL21(DE3) cells for protein expression.

Cells were grown in lysogeny broth supplemented with 50 µg/mL kanamycin at 37°C until the OD₆₀₀ reached ~0.6. Protein expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and proceeded for 18 h at 16°C. Cells were harvested by centrifugation at 5000g for 12 min, flash-frozen in liquid nitrogen, and stored at -80°C. Cells were resuspended in lysis buffer [50 mM Tris (pH 7.8), 400 mM NaCl, 1 mM tris(2-carboxyethyl)phosphine (TCEP), 10% glycerol, and 10 mM imidazole] with 1 unit/µL Pierce Universal Nuclease (ThermoScientific) and 1.0 mM phenylmethanesulfonyl fluoride (PMSF) and lysed by sonication on ice at 40% amplitude for 6 min with 3 s bursts and 5 s rests. The lysate was clarified by centrifugation at ~37000g for 30 min at 4°C, and the supernatant was applied to a Ni-NTA column equilibrated with lysis buffer. DaHMGR was eluted from the column in fractions using lysis buffer with 250 mM imidazole. Fractions containing the highest protein concentration as assessed by the absorbance at 280 nm (*A*₂₈₀) were pooled and applied to a PD-10 desalting column (GE Healthcare) pre-equilibrated with

storage buffer [50 mM Tris (pH 7.4), 400 mM NaCl, 1 mM TCEP, and 10% glycerol] and eluted in fractions. The DaHMGR purity was assessed by SDS-PAGE, and fractions were pooled to obtain a 8.5 mg/mL final sample, as determined by the absorbance at 280 nm using a calculated extinction coefficient of $27000 \text{ M}^{-1} \text{ cm}^{-1}$. The final yield was 2.6 mg of protein/g of cell paste. Aliquots of DaHMGR were flash-frozen in liquid nitrogen and stored at -80°C .

Kinetic Characterization.

Steady-state kinetic characterization of DaHMGR was performed by monitoring the rate of NAD(P)H oxidation by the absorbance at 340 nm using a Multiskan FC Microplate Photometer (ThermoFisher Scientific), with an extinction coefficient of $6200 \text{ M}^{-1} \text{ cm}^{-1}$. To assess NAD(P)H cofactor usage, reaction mixtures contained 50 mM Tris (pH 7.4), 50 mM NaCl, 5 mM DTT, 300 μM HMG-CoA, 5–400 μM NAD(P)H, and 20 nM DaHMGR for experiments using NADH or 100 nM DaHMGR for experiments using NADPH. To assess HMG-CoA kinetics, reaction mixtures contained 50 mM Tris (pH 7.4), 50 mM NaCl, 5 mM DTT, 400 μM NADH, 20 nM DaHMGR, and 2–300 μM HMG-CoA. The effect of citrate on the DaHMGR reaction was tested using the same reaction mixtures as with the HMG-CoA reactions, plus 0–350 mM sodium citrate pH 7.4. Nonlinear regression analysis in GraphPad Prism 7 was performed by plotting the initial reaction rates, v_0 , against the substrate concentration and fitting the data to the Michaelis-Menten equation to determine the Michaelis-Menten constant, K_m , and the maximum velocity, V_{max} . Values for k_{cat} were calculated by dividing V_{max} by the molar enzyme concentration. Assays were performed in triplicate, and the values reported are the means $\pm$ the standard error of the mean (SEM).

Crystallization.

Crystallization conditions were identified via sparse-matrix screening by sitting-drop vapor diffusion using a Crystal Gryphon (Art Robbins Instruments) with 8.5 mg/mL DaHMGR in storage buffer. Protein crystals that ultimately gave the NADH-bound structure were identified first and optimized by hanging-drop vapor diffusion at room temperature using 1 μL of the DaHMGR sample with 1 mM NADH and 1 μL of the crystallization solution over 0.5 mL of the crystallization solution. The optimized crystallization solution contained 100 mM sodium citrate (pH 5.0), 500 mM ammonium sulfate, and 600 mM lithium sulfate. Crystals were soaked in a cryoprotectant solution containing the crystallization solution with 1 mM NADH and 26% ethylene glycol before being flash-cooled in liquid nitrogen. Subsequently, protein crystals were identified that ultimately yielded the apo and mevalonate-bound structures and were optimized in the same fashion. For the apo structure, the optimized crystallization solution contained 100 mM Bis-Tris (pH 5.5), 600 mM lithium sulfate, and 18% PEG 3350. Crystals were soaked in a cryoprotectant solution that contained a 1:1 volume ratio of the crystallization solution and a solution containing 16% ethylene glycol, 16% glycerol, 18% sucrose, and 4% glucose before being flash-cooled in liquid nitrogen. For the mevalonate-bound structure, the protein was crystallized in the presence of 1 mM mevalonate and 1 mM NAD^+ , and the optimized crystallization solution contained 100 mM Bis-Tris (pH 6.5), 600 mM lithium sulfate, and 19% PEG 3350. Crystals were soaked in a cryoprotectant solution composed of the crystallization solution with 1 mM mevalonate, 1 mM NAD^+ , and 21% sucrose before being flash-cooled in liquid nitrogen.

X-ray Data Collection, Structure Determination, and Refinement.

X-ray diffraction data were collected at the Advanced Photon Source (APS) beamlines 24-ID-C and 24-ID-E and were indexed, merged, and scaled using iMOSFLM.²³ NADH-bound DaHMGR crystals belonged to space group $P6_522$ with three molecules in the asymmetric unit and diffracted to 2.14 Å resolution. The structure was determined by molecular replacement using Phaser²⁴ in the Phenix suite²⁵ using one monomer from the structure of PmHMGR (PDB entry 1QAX, sequence 72% identical with that of DaHMGR) with the substrate, cofactor, and CTD removed as the search model. Iterative rounds of model building in Coot²⁶ and reciprocal space refinement in phenix.refine²⁷ were performed. Initial electron density maps indicated that CTDs were located in novel locations; therefore, these regions were built manually in Coot with an initial polyalanine model, and side-chains were added in subsequent rounds of refinement.

Crystals that ultimately yielded the apo and mevalonate-bound DaHMGR structures were identified after the NADH-bound DaHMGR structure was refined. These crystals belonged to space group $P321$ with one molecule in the asymmetric unit and diffracted to 1.93 Å and 1.49 Å resolution for the apo and mevalonate-bound DaHMGR structures, respectively. As these crystals had a different unit cell and symmetry, their structures were determined by molecular replacement in Phaser²⁴ using one protein chain from our previously refined NADH-bound DaHMGR with the CTD and ligand removed as a search model. The same test set of reflections was preserved for both apo and mevalonate-bound DaHMGR data sets. Iterative rounds of model building in Coot²⁶ and reciprocal space refinement in phenix.refine²⁷ were performed to yield the final models for both structures. Electron density was not resolved for the CTD in these structures. Final data collection and refinement statistics are listed in Table 1.

RESULTS

Kinetic Characterization of DaHMGR.

To characterize and quantify the cofactor preferences of purified DaHMGR, we performed steady-state kinetic experiments with varying concentrations of NADH, NADPH, and substrate HMG-CoA (Table 2 and Figure S1). With respect to the cofactor NADH, DaHMGR has a K_m of 28.5 ± 1.4 μM and a k_{cat} of 16.6 ± 0.2 s⁻¹. Although DaHMGR was also active when utilizing NADPH instead of NADH, the activity was extremely low (Figure S1A), and the reaction did not reach a maximal velocity, even at cofactor concentrations that exceeded the upper limit of detection using the absorbance at 340 nm. Therefore, kinetic parameters could not be determined for DaHMGR using NADPH as a cofactor, due to low activity. With respect to HMG-CoA (Figure S1B), the enzyme has a K_m of 7.34 ± 0.71 μM and a k_{cat} of 22.3 ± 0.5 s⁻¹ when using its preferred NADH cofactor. These kinetic data show that although DaHMGR can use either cofactor for HMG-CoA reduction, NADH is strongly preferred over NADPH. Sodium citrate, present in the crystallization condition of the NADH-bound structure at a final concentration of 50 mM, was found to inhibit the reaction competitively with respect to HMG-CoA (Figure S1C), with a K_i of 170 mM.

Overall Structure.

Both apo and mevalonate-bound DaHMGR crystallized with one monomer in the asymmetric unit, in which the opposite monomer that completes the HMGR homodimer is generated by crystallographic symmetry. For both structures, electron density was observed for the entire protein chain except for the CTD (residues 378–429), which was disordered. Therefore, the final models for both structures contain residues 3–376 (of 429). On the other hand, DaHMGR bound to its preferred NADH cofactor crystallized with three molecules in the asymmetric unit, in which chains A and B form one HMGR homodimer, while chain C forms a homodimer with a second chain C monomer (C') through crystallographic symmetry. In the monomers of this structure, continuous electron density was observed for all three chains in the asymmetric unit, except for the CTD of chain A, which was disordered. Therefore, in the final model chain A contains residues 2–377, chain B contains residues 2–428, and chain C contains residues 2–429. If the CTDs are excluded, described below, the structures of all protein monomers across the three structures align well with each other and with those of other class II HMGRs, with a root-mean-square deviation for C α atoms (rmsd) of 0.75–0.84 when compared to the structure of cofactor-bound SpHMGR (PDB entry 5WPJ).

Cofactor- and Substrate-Binding sites.

In all HMGRs, two active sites are present per homodimer, where each monomer contains its own substrate- and cofactor-binding sites. Although the reactive groups of the substrate and cofactor are positioned together in the active site, buried within the homodimeric interface, the long HMG-CoA substrate and NAD(P)H cofactor molecules extend out from the active site in opposite directions, mostly interacting with opposite monomers. In the apo DaHMGR structure, both the active site and the cofactor-binding site are unoccupied. However, in the mevalonate-bound DaHMGR structure, positive difference maps showed density in the active site, representing mevalonate binding (Figure 2A). Here, the carboxylate group of mevalonate forms a salt bridge with Arg261, and the 5-hydroxyl group interacts with Glu83, Lys267, and Asn271; these mevalonate-binding interactions are all identical to those previously identified in structures of mevalonate-bound PmHMGR (Figure 2B).^{12, 28} Although the protein was co-crystallized with 1 mM mevalonate and 1 mM NAD⁺, no electron density for the cofactor was observed (Figure S2).

In the NADH-bound DaHMGR structure, positive difference maps showed electron density in the cofactor-binding sites of all three chains of the asymmetric unit, representing NADH binding (Figure 3A). In this structure, the amide group of the cofactor's nicotinamide ring forms hydrogen bonds with Asn216, while the 2'-hydroxyl group of the nicotinamide's ribose forms a hydrogen bond with Asp283 (Figure 3B). From this buried location, the cofactor extends out toward the protein surface and reaches the solvent at its diphosphate moiety, which interacts with the backbone NH groups of Met185, Gly186, Ala187, Asn188, and Thr189 as well as with the side chain of Thr189. More notably, the NADH diphosphate also interacts with the side chains of Arg428' and, through a water molecule, Arg405', both of which are from the CTD of the opposite monomer. The NADH adenosine group is solvent-exposed, with the ribose 2'-and 3'-hydroxyl groups forming hydrogen bonds with

Asp146. The adenine ring of NADH is sandwiched between Val328 and Leu152 side chains while also making hydrogen bonds with both the backbone amide and side chain of Asp183.

In addition, in the NADH-bound DaHMGR structure electron density consistent with a molecule of citrate was found in the active sites of all three monomers of the asymmetric unit (Figure 4A). Citrate, present in the crystallization solution at a final concentration of 50 mM, binds the active site in an analogous position as observed with the product mevalonate described above as well as the HMG-moiety of the substrate, HMG-CoA, which are both highly similar in structure to citrate (Figure 4B). To illustrate, a superposition of this NADH- and citrate-bound DaHMGR structure, the mevalonate-bound DaHMGR structure, and the previously determined HMG-CoA-bound SpHMGR structure¹¹ shows a strikingly similar binding mode (Figure 4C), involving the same salt bridge between a carboxylate of the ligand and conserved Arg261 (DaHMGR numbering), as well as the other interactions observed with mevalonate binding that involve conserved Glu83, Lys267, and Asn271, as described above. Citrate binding in the same position and exhibiting the same interactions has also been previously observed in a structure of SpHMGR (PDB entry 3QAE).

C-Terminal Domain.

As in most structures of HMGR, the CTD is disordered in the structures of apo and mevalonate-bound DaHMGR. However, the CTD is ordered and visualized in chains B and C of the NADH-bound DaHMGR structure. Relative to the rest of the protein, these two CTDs are located in the same position as each other (Figure 5A), representing a single, previously unseen CTD conformation (Figure 5B). Although the location of the CTD is near the previously described “closed” and “partially closed” positions, the domain is in a new orientation, turned completely upside-down relative to the “closed” structure observed previously in PmHMGR (Figure 6A-B). This ~180° in-place rotation of the CTD is facilitated by a linker region that connects the domain with the rest of the protein (residues 375–380 in DaHMGR). In the prior structures of “closed” and “partially closed” HMGR, these linker residues are primarily helical, located at the beginning of the first of three helices in the CTD. However, in our NADH-bound DaHMGR structure, this linker region is an unstructured loop in which the helix appears to have partially unwound and lifted away from the protein surface, enabling sufficient flexibility for the CTD to turn upside-down in a new position that we term the “flipped” conformation.

Upon adoption of this new conformation, key residues of the CTD are observed in new locations. In the “closed” PmHMGR structure observed when both the substrate and the cofactor are bound simultaneously, a catalytically essential His381 (PmHMGR numbering) points into the active site (Figure 6C), and His385 binds the diphosphate group of bound NADH. However, in our “flipped” conformation, the rotation of the CTD causes both histidine side chains (also His381 and His385 in DaHMGR numbering) to be flipped out of the active site and point to the solvent (Figure 6D). In addition, in such an overturned position the orientation of the three helices of the CTD is altered. In the “closed” PmHMGR structure, the first helix of the CTD (blue-cyan in Figure 6) packs closely against the active site, with His381 and His385 pointing toward the bound ligands as described above, as it lays down on the surface of the opposite monomer. The two subsequent helices (green-

yellow and orange-red in Figure 6) are then positioned away from the active site on the outer surface of the protein, toward the solvent. However, in the “flipped” conformation the first helix is now on the outer surface of the protein, distant from the active site. Instead, it is the two subsequent helices that lay on the surface of the protein, with Arg405 and Arg428 making interactions with bound NADH, as mentioned above.

DISCUSSION

HMGR is a pivotal enzyme that catalyzes the first-committed step in the mevalonate pathway, which is used in the biosynthesis of a diverse array of molecules, from steroids such as cholesterol to the precursors to isoprenoid natural products, many of which possess bioactivity as anticancer, antimicrobial, or antimalarial drugs or may be used as other commodity chemicals. Given the crucial role of HMGR in cholesterol biosynthesis, its inhibition by statins in human class I HMGR is well understood. However, the structural and mechanistic attributes of class II HMGR are less clear. Greater structural insight into HMGR mechanism, catalysis, and ligand-binding may also lead to the development of novel antibiotics, as class II HMGRs are present only in microorganisms, including human pathogens.

Here, we characterized the class II HMGR from *D. acidovorans* as an NADH-preferring HMGR and determined crystal structures of the apo, mevalonate-bound, and NADH- and citrate-bound forms of the enzyme both to examine the structural features that lead to its cofactor specificity and to shed light on the structural mechanisms underpinning reactivity. These DaHMGR structures are consistent with prior HMGR structures that depict ligands bound, in which the product mevalonate and a molecule of citrate bind to DaHMGR in the same way as previously observed in PmHMGR and SpHMGR, respectively. Additionally, NADH binds to the enzyme in a similar fashion as previously observed with NAD⁺-bound PmHMGR and NADPH-bound SpHMGR, illustrating that the mode of cofactor binding is also highly conserved.

We previously hypothesized that the composition of a short helix unique to class II HMGR termed the “cofactor helix” plays a crucial role in conferring cofactor specificity.¹¹ In particular, the cofactor helix is positioned against the adenosine moiety of the cofactor such that the three residues in helical positions 1, 3, and 7 point toward the adenosine of NAD(P)H, making cofactor-specific interactions. In the structure of PmHMGR (Figure 7A), an aspartate in the first position of the cofactor helix that is conserved among NADH-preferring HMGRs makes hydrogen bonds with the key 2'-OH of the NADH ribose.¹² This aspartate was believed to prevent the key 2'-phosphate group of NADPH from binding, though its mutation to alanine or glycine to make room for the phosphate group did not switch the cofactor specificity of PmHMGR.⁵ Meanwhile, hydrophobic leucine residues in positions 3 and 7 of the PmHMGR cofactor helix face the aromatic adenine ring of NADH. However, in NADPH-preferring SpHMGR (Figure 7B), a bulky tyrosine residue in the first position of the cofactor helix replaces the aspartate of PmHMGR, sterically shifting the cofactor's adenosine moiety toward helical positions 3 and 7.¹¹ Instead of hydrophobic residues, in SpHMGR positions 3 and 7 are now serine and arginine, respectively, conserved among NADPH-preferring HMGRs, which both interact with the key 2'-phosphate of

NADPH. These observations together with a sequence analysis of this region led to the identification of a possible motif in the cofactor helix that gives rise to NAD(P)H specificity: ¹¹ DX(V/L)XXXL for NADH and YSXXXXR for NADPH, where “X” is a variable amino acid.

Our studies of DaHMGR here offer the first new test of this hypothesis. In DaHMGR, the cofactor helix contains the sequence DKVLIGL (Figure 7C), which would classify it as an NADH-specific enzyme. Although an *in vivo* study in which DaHMGR was expressed in *E. coli* found a greater isoprenoid product yield when using NADH,²¹ we have now demonstrated NADH specificity through kinetic characterization of purified DaHMGR, lending biochemical support to the notion that the cofactor helix functions as an NAD(P)H-specifying structural motif. We may now anticipate that this principle could be generalized for all class II HMGRs.

Our NADH-bound DaHMGR structure also reveals a surprising new orientation of the CTD of class II HMGR. Of the many HMGR crystal structures that have been determined to date, the fact that only a small handful of structures depict an ordered CTD has long hinted at an inherent flexibility of this domain of the protein. Indeed, the CTD is also disordered in the apo and mevalonate-bound DaHMGR structures presented here. The CTD was first visualized in the structure of a ternary complex of PmHMGR bound simultaneously with substrate HMG-CoA and oxidized cofactor NAD⁺.¹² Here, the CTD covers the active site in a “closed” conformation, contributing a catalytically essential histidine to the reaction (His381 in both PmHMGR and DaHMGR). Mutation of this histidine residue virtually abolishes activity,^{12–15} and its position within the active site of this “closed” structure suggests that its role is to protonate the thiolate sulfur of CoA following HMG-CoA reduction.^{12, 15}

Until now, the remaining HMGR structures that depict an ordered CTD have been of SpHMGR. In contrast to the “closed” conformation, in an apo-SpHMGR structure (PDB entry 3QAU) the CTD was located in a position far from the ligand-binding sites, illustrating an “open” conformation that would permit both substrate and cofactor entry. The recent structure of NADPH-bound SpHMGR showed similar “open” conformations of the CTD, suggesting that cofactor binding alone does not trigger “closing” of the CTD.¹¹ Finally, an HMG-CoA-bound SpHMGR structure showed a third unique CTD position termed “partially closed”, in which the CTD directly interacts with the CoA moiety of the substrate while also leaving the cofactor-binding site exposed.¹¹ Taken together, these crystal structures portray the CTD as being key to controlling substrate and cofactor entry and exit, where large CTD movements alternately expose or cover both the cofactor- and substrate-binding sites at various points of the reaction.

However, the upside-down orientation of the “flipped” CTD conformation in our NADH-bound DaHMGR structure complicates our understanding and interpretation of CTD movements during the reaction cycle. With the enzyme bound with NADH but not HMG-CoA, we had expected that the CTD might adopt an “open” conformation as previously observed in the analogous NADPH-bound SpHMGR structure. However, the “flipped” CTD in DaHMGR covers the cofactor-binding site, with Arg405 and Arg428 of the CTD

interacting with NADH. The discovery of both “open” and “flipped” conformations in cofactor-bound HMGR suggests that CTD flexibility is maintained regardless of cofactor binding, as the CTD has now been observed in completely different positions when the cofactor is present but the substrate is absent. Therefore, it is likely that a single “true” cofactor-bound state is fully represented by neither the “open” nor the “flipped” conformation. Indeed, differing “open” conformations were observed in the NADPH-bound SpHMGR structure.¹¹ Instead, these data indicate that crystal packing interactions have allowed us to capture multiple placements of a CTD that would otherwise remain mobile when the cofactor is bound. Our apo and mevalonate-bound structures of DaHMGR from a different space group do not contain an ordered CTD, further suggesting that crystal packing interactions have trapped a mobile CTD in the “flipped” conformation of NADH-bound DaHMGR. In this way, visualization of a “flipped” orientation expands the known range of CTD motion in class II HMGR.

The fact that citrate is also bound to the enzyme in this structure may suggest that the “flipped” CTD conformation is simply an artifact of citrate binding. However, citrate binding in the active site is highly unlikely to have itself caused “flipping” of the CTD into an otherwise unattainable position, as citrate does not interact with the CTD. In addition, the interactions that active site residues make with citrate are strikingly similar to the interactions with both the product mevalonate, a molecule that is highly similar to citrate, as well as the HMG-moiety of the substrate HMG-CoA (Figure 4B-C), indicating that citrate does not uniquely induce a significant structural change. Additionally, a prior structure that depicts citrate bound to the active site of SpHMGR in the same fashion has been determined (PDB entry 1QAE), in which the CTD was not “flipped” but was disordered, further indicating that citrate does not itself cause CTD “flipping”, but that an otherwise mobile CTD was crystallographically trapped in a “flipped” orientation in our DaHMGR structure due to crystal packing. Indeed, the “flipped” CTDs are observed to pack closely against neighboring monomers in the crystal lattice (Figure S3).

To provide a closer look at the “flipped” conformation, a superposition of this NADH-bound DaHMGR structure with the structures of HMG-CoA-bound SpHMGR and NAD⁺- and HMG-CoA-bound PmHMGR shows that the “flipped” CTD position clashes with the CoA portion of the substrate (Figure S4). This may appear counterintuitive: in the absence of the substrate, the “flipped” CTD appears to obstruct substrate binding via the CoA moiety. However, the CTD is likely still mobile when the substrate is absent, as described above, and we have trapped only one possible conformation in the crystal. In addition, although the substrate is not bound in our structure, the active site is occupied by citrate, a small molecule that is highly similar to the HMGR intermediate mevaldehyde and the product mevalonate (Figure 4B). We also found that citrate, present during crystallization at a final concentration of 50 mM, inhibits the DaHMGR reaction with a K_i of 170 mM, a high value indicating a weak level of inhibition that is unlikely to be physiologically relevant. Citrate inhibition is competitive with respect to the substrate HMG-CoA (Figure S1C), consistent with our structure depicting citrate as occupying the HMG-moiety’s binding site, where the same interactions are observed between citrate and DaHMGR, HMG-CoA and SpHMGR,¹¹ and mevalonate and both DaHMGR and PmHMGR¹² (Figure 4C). Therefore, though the CoA moiety of the substrate HMG-CoA cannot be bound if the CTD is in the “flipped”

conformation, the active site may be bound with the intermediate mevaldehyde, the product mevalonate, or citrate.

Mevaldehyde and mevalonate differ only in their redox state; where mevaldehyde contains a terminal carbonyl at an sp^2 -hybridized carbon, mevalonate contains a primary alcohol at an sp^3 -hybridized carbon (Figure 4B). In this way, citrate, with a terminal sp^2 -hybridized carboxylate carbon, arguably more closely resembles mevaldehyde than mevalonate, thus mimicking an intermediate-bound state and indicating that the NADH- and citrate-bound structure may provide additional useful insight into the catalytic mechanism (Figure 8), as detailed here.

In the absence of any bound ligands, the CTD is flexible and disordered, as indicated by the apo-HMGR structures with disordered CTDs, including the apo-DaHMGR structure presented here, and may therefore adopt many conformations, including the “open” CTD conformation visualized in the apo-SpHMGR structure, which would allow both the substrate and the cofactor to bind. Although it is not known whether there is ordered binding of the substrate and cofactor, crystal structures for both HMG-CoA- and NAD(P)H-bound HMGR have been determined, indicating that either the substrate or the cofactor may bind first. If the cofactor binds first, the capture of multiple “open” CTD conformations in the NADPH-bound SpHMGR structure¹¹ and the “flipped” CTD conformation in the NADH-bound DaHMGR structure presented here suggests that the CTD remains flexible when the cofactor is bound, rather than adopting a single, static conformation. If instead the substrate binds first, the “partially closed” structure of HMG-CoA-bound SpHMGR illustrates how the CTD can bind directly to the substrate while keeping the cofactor-binding site open and available for NAD(P)H binding.¹¹

Once both HMG-CoA and NAD(P)H are bound, the mobile CTD adopts a “closed” conformation as depicted in the structure of the ternary PmHMGR complex to contribute its catalytic His381 residue and to seal the active site for chemistry to occur. Here (Figure 8), HMG-CoA is reduced by the first equivalent of NAD(P)H to form mevaldyl-CoA, where protonation of the carbonyl is proposed to be performed by either Glu83 or Lys267. Crystal structures of HMG-CoA-, mevalonate-, and citrate-bound HMGRs all show Glu83 and Lys267 located in the same positions (Figure 4C), directly interacting with the analogous oxygen atom of the ligand. Together with biochemical data in which mutation of Lys267 to alanine strongly compromises activity,¹² these structures suggest that Lys267 acts to polarize, and possibly to protonate, the substrate carbonyl during reduction by NAD(P)H. Glu83 may also protonate the carbonyl; mutation to glutamine virtually abolishes activity,³⁰ and a computational study indicated that the transfer of a hydride from the cofactor is more favorable if Glu83 is protonated.³¹ Therefore, the proposed mechanism shown in Figure 8 depicts Glu83 as the catalytic acid to form the mevaldyl-CoA intermediate, though Lys267 cannot be completely ruled out.

Once mevaldyl-CoA is formed, the order of the next two steps is uncertain. (1) Cofactor exchange must occur in which the now-oxidized $NAD(P)^+$ is released and a second equivalent of reduced NAD(P)H binds the enzyme, and (2) mevaldyl-CoA must be cleaved to form the mevaldehyde intermediate and CoA. Crystallographic trapping studies have

suggested that a stable CoA-containing intermediate can remain bound to the enzyme,^{11, 28} and indeed, the “partially closed” SpHMGR conformation illustrates how the CTD can bind the CoA moiety while opening up the cofactor-binding site for cofactor exchange.¹¹ Prior studies indicated that reaction intermediates are not released by the enzyme during the reaction;^{32–35} instead, the “partially closed” conformation demonstrates how the CTD can hold CoA-containing intermediates while simultaneously leaving the cofactor-binding site exposed.¹¹ These observations suggest that cofactor exchange is possible while the enzyme remains bound to the mevaldyl-CoA intermediate and before decomposition to mevaldehyde and CoA, as shown in Figure 8.

To facilitate mevaldyl-CoA cleavage to mevaldehyde, Glu83 may be involved in deprotonation. Indeed, our mevaldehyde-mimicking citrate-bound DaHMGR structure presented here shows Glu83 interacting with the corresponding oxygen atom of citrate (Figure 4C), suggesting that Glu83 is in fact protonated when this oxygen is deprotonated. Upon CoA cleavage, the thiolate moiety is likely protonated by His381 from the CTD of the opposite monomer,^{13–15} as discussed above. Our citrate-bound structure further suggests that the CoA product need not remain bound while mevaldehyde is in the active site and may be released, as shown in Figure 8. Indeed, in the “flipped” conformation where the enzyme is bound to both the cofactor and citrate, the CTD would sterically clash with any bound CoA moiety (Figure S4).

With both the second NAD(P)H molecule and mevaldehyde now bound, the enzyme can once again undergo a change in conformation to the “closed” CTD state for reduction of mevaldehyde to mevalonate. Both our citrate-bound DaHMGR structure that mimics mevaldehyde-binding and the structures of mevalonate-bound PmHMGR and DaHMGR show Glu83 and Lys267 continuing to interact with the analogous oxygen atom, indicating that either Glu83 or Lys267 may protonate mevaldehyde to form the mevalonate alcohol. For the reasons discussed above regarding Glu83 or Lys267 acting as a catalytic acid, we have again shown Glu83 as protonating mevaldehyde in Figure 8. It is interesting to note that whichever residue serves as the acid during this reaction, the transfer of a proton from the bulk solvent to the active site that might alter or restore the protonation states of catalytic residues does not occur and is not necessary during the reaction cycle. Despite the large CTD movements described above, neither Glu83 nor Lys267 is exposed to the solvent, as each remains blocked off by the intermediate itself, which does not leave the active site until the reaction is complete.^{32–34} Although His381 would become exposed to the solvent because it resides on the CTD, its mechanistic role is not in HMG-CoA or mevaldehyde reduction, but in protonation of the leaving group CoA. In any case, once the product is formed, the CTD may once again become mobile, including adopting an “open” conformation, which would allow for mevalonate and NAD(P)⁺ release, leaving the active site residues exposed to the solvent for reprotonation and “resetting” of the catalytic cycle.

In summary, we have biochemically characterized the class II HMGR from *D. acidovorans* and determined crystal structures in the unbound, mevalonate-bound, and NADH- and citrate-bound states. These structures support a structural model of NAD(P)H preference in which the composition of the “cofactor helix” motif confers the cofactor specificity of the enzyme. We have also observed an unexpected new orientation of the CTD, a highly mobile

domain that assumes many different conformations that appear to control substrate and cofactor binding. In particular, the CTD is crystallographically captured in a “flipped” conformation, making contacts with NADH while being oriented upside-down relative to prior HMGR structures that contained ordered CTDs. This conformation not only exemplifies the remarkable flexibility of this domain, but also provides greater insight into the catalytic mechanism of HMGR, a biologically essential enzyme.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS.

HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HMGR	HMG-CoA reductase
CoA	coenzyme A
CTD	C-terminal domain
TCEP	tris(2-carboxyethyl)phosphine
IPTG	isopropyl β-D-1-thiogalactopyranoside
PMSF	phenylmethanesulfonyl fluoride
NTA	nitrilotriacetic acid
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
APS	Advanced Photon Source
PDB	Protein Data Bank
rmsd	root-mean-square deviation for C _α atoms

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Figure 1.

Conformational movements of the class II HMGR C-terminal domain (CTD) (blue). (A) Structure of apo-SpHMGR (PDB entry 3QAU) with the CTD in an “open” conformation. (B) Structure of NADPH-bound SpHMGR (PDB entry 5WPJ) with the CTD in an “open” conformation. (C) Structure of HMG-CoA-bound SpHMGR (PDB entry 5WPK) with the CTD in a “partially closed” conformation. (D) Structure of the PmHMGR ternary complex bound with both NAD⁺ and HMG-CoA (PDB entry 1QAX) with the CTD in a “closed” conformation. One monomer of the HMGR homodimer is shown as a gray surface, and the other monomer as an orange cartoon except for the CTD (blue). Bound ligands are shown as spheres, with the C atoms of cofactors colored pink, the C atoms of HMG-CoA colored green, the N atoms colored blue, the O atoms colored red, the P atoms colored orange, and the S atoms colored yellow.

Figure 2. Mevalonate-binding site of the mevalonate-bound DaHMGR structure. (A) Polder $mF_o - DF_c$ omit density calculated in Phenix²⁹ is contoured at 3.0σ (green mesh), with mevalonate shown as sticks (C colored pink and O colored red). (B) The mevalonate-binding site, with mevalonate from DaHMGR (C colored pink), mevalonate from superimposed PmHMGR (PDB entry 1I6Y, C colored teal), and DaHMGR residues (C colored gray) shown as sticks, with N colored blue and O colored red. Dashed lines represent hydrogen bonding or salt-bridge interactions between mevalonate and DaHMGR.

Figure 3.

Cofactor-binding site of the NADH-bound DaHMGR structure. (A) Polder $mF_o - DF_c$ omit density calculated in Phenix²⁹ is contoured at 3.0σ (green mesh), with NADH shown as sticks. (B) Stereoview of the NADH binding site. NADH shown as sticks (C colored pink) and DaHMGR residues shown as sticks (C colored gray), with N colored blue, O colored red, P colored orange, and S colored yellow. A water molecule is shown as a red sphere and labeled “W”. Dashed lines represent hydrogen bonding or salt-bridge interactions.

Figure 4. Citrate-binding site of the NADH-bound DaHMGR structure. (A) Polder $mF_o - DF_c$ omit density calculated in Phenix²⁹ contoured at 3.0σ (green mesh), with citrate shown as sticks. (B) Comparison between the structures of the substrate (*S*)-HMG-CoA, intermediate (*R*)-mevaldehyde, product (*R*)-mevalonate, and citrate. Annotated colors refer to the corresponding ligand coloring in panel C. (C) Stereoview of citrate binding in the structure of NADH-bound DaHMGR superimposed with mevalonate in the structure of mevalonate-bound DaHMGR and HMG-CoA in the structure of HMG-CoA-bound SpHMGR (PDB entry 5WPK). Citrate (C colored pink), NADH (labeled, C colored pink), mevalonate (C colored cyan), HMG-CoA (C colored green), and DaHMGR residues (C colored gray) are shown as sticks, with N colored blue, O colored red, P colored orange, and S colored yellow. Dashed lines represent hydrogen bonding or salt-bridge interactions involving citrate and DaHMGR.

Figure 5.

Structure of the CTD in NADH-bound DaHMGR. (A) Superposition of the two monomers in the asymmetric unit that contain ordered CTDs in the same position as each other. Chain B is colored orange with the CTD colored blue and chain C is colored green with the CTD colored red. (B) The DaHMGR homodimer in the same orientation as the structures in Figure 1, with one monomer shown as a gray surface and the other monomer as an orange cartoon, except for the CTD (blue). NADH is shown as spheres, with C colored pink, N colored blue, O colored red, and P colored orange.

Figure 6.

Comparison between the “closed” and “flipped” CTD conformations. (A) “Closed” CTD in the structure of PmHMGR (PDB entry 1QAX) with NAD⁺ and HMG-CoA bound. (B) “Flipped” CTD in the structure of DaHMGR with NADH and citrate bound. (C) Catalytically important histidine residues in the “closed” and (D) “flipped” CTD conformations. Same coloring as in Figure 5, except the CTDs are colored in a blue-to-red gradient to clarify directionality. In panels C and D one monomer of the homodimer and all bound ligands have been omitted for the sake of clarity, and histidines are shown as sticks and semitransparent spheres, with C colored pink and N colored blue.

Figure 7.

Cofactor helix of class II HMGR that is involved in determining NAD(P)H cofactor specificity. Composition of the cofactor helix in (A) NADH-preferring PmHMGR,¹² (B) NADPH-preferring SpHMGR,¹¹ and (C) NADH-preferring DaHMGR (this work). HMGR is shown as a cartoon, and residues are shown as sticks, with C colored gray. NAD(P)H is shown as sticks, with C colored pink, N colored blue, O colored red, and P colored orange.

Figure 8.

Proposed catalytic mechanism for HMG-CoA reduction to mevalonate by class II HMGR, involving residues Glu83 and Lys267, as well as His381 from the CTD.

Table 1.

X-ray Data Collection and Refinement Statistics.

	NADH-bound	apo	mevalonate-bound
PDB ID	6DIO	6EEU	6EEV
Data Collection			
Beamline	APS, 24-ID-E	APS, 24-ID-E	APS, 24-ID-C
Wavelength (Å)	0.9792	0.9792	0.9791
Space group	<i>P</i> 6 ₅ 22	<i>P</i> 321	<i>P</i> 321
Unit cell			
<i>a</i> = <i>b</i> , <i>c</i> (Å)	113.00, 437.48	100.54, 75.86	100.37, 75.81
Resolution (Å)	97.86–2.14 (2.18–2.14)	57.20–1.93 (1.99–1.93)	57.12–1.49 (1.52–1.49)
Wilson B (Å ²)	21.39	20.59	14.85
Total reflections	675,186 (34,374)	138,912 (9,597)	267,310 (13,124)
Unique reflections	92,208 (4,476)	33,218 (2,243)	70,102 (3,542)
Multiplicity	7.3 (7.7)	4.2 (4.3)	3.8 (3.7)
Completeness (%)	100.0 (100.00)	98.9 (100.0)	97.6 (99.7)
Mean <i>I</i> / σ (<i>I</i>)	5.2 (2.4)	8.1 (2.0)	10.5 (2.0)
<i>R</i> _{merge}	0.287 (0.843)	0.117 (0.823)	0.067 (0.629)
<i>R</i> _{meas}	0.309 (0.904)	0.134 (0.941)	0.078 (0.735)
CC1/2	0.986 (0.40)	0.994 (0.602)	0.993 (0.700)
Refinement			
<i>R</i> _{work}	0.1905 (0.2945)	0.1597 (0.2325)	0.1351 (0.2131)
<i>R</i> _{free}	0.2389 (0.3378)	0.1980 (0.2845)	0.1646 (0.2632)
r.m.s.d. bonds (Å)	0.007	0.006	0.005
r.m.s.d. angles (°)	0.905	0.794	0.821
Number of atoms	9,429	3,097	3,228
Average B factors (Å ²)			
All atoms	24.23	22.95	23.73
Protein	23.27	21.70	20.14
NADH/citrate/mevalonate	25.48	N/A	41.40
Water	30.11	31.76	37.30
Ramachandran analysis			
Favored (%)	97.14	98.12	97.30
Allowed (%)	2.86	1.88	2.69
Outliers (%)	0.00	0.00	0.00
MolProbity Clashscore	5.80	1.62	1.41

Statistics for the highest-resolution shell shown in parentheses.

Table 2.

DaHMGR kinetics.

	NADH	NADPH	HMG-CoA
K_m (μM)	28.5 ± 1.4	N.D.	7.34 ± 0.71
k_{cat} (s^{-1})	16.6 ± 0.2	N.D.	22.3 ± 0.5
k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)	5.82×10^5	N.D.	3.04×10^6

N.D.: Not determined