Hydroxymethylglutaryl-CoA (HMG-CoA) reductase is the primary target in the current clinical treatment of hypercholesterolemias with specific inhibitors of the "statin" family. Statins are excellent inhibitors of the class I (human) enzyme but relatively poor inhibitors of the class II enzymes of important bacterial pathogens. To investigate the molecular basis for this difference we determined the x-ray structure of the class II Pseudomonas mevalonii HMG-CoA reductase in complex with the statin drug lovastatin. The structure shows lovastatin bound in the active site and its interactions with residues critically involved in catalysis and substrate binding. Binding of lovastatin also displaces the flap domain of the enzyme, which contains the catalytic residue His381. Comparison with the structures of statins bound to the human enzyme revealed a similar mode of binding but marked differences in specific interactions that account for the observed differences in affinity. We suggest that these differences might be exploited to develop selective class II inhibitors for use as antibacterial agents against pathogenic microorganisms.

Isoprenoids are branched-chain lipids formed by polymerization of the five-carbon monomer isopentenyl diphosphate. Present in all known organisms, isoprenoids function in processes as diverse as maintenance of membrane structure, cell wall and glycoprotein synthesis, defense mechanisms, and modification of proteins and tRNAs. Formation of isopentenyl diphosphate can occur by either of two pathways: the mevalonate pathway (1) or the glyceraldehyde 3-phosphate-pyruvate (GAP-pyruvate) pathway (2). Animals and Archaea use only the mevalonate pathway, and plants utilize both pathways (2–4). Many true bacteria, including Bacillus subtilis and Escherichia coli, employ the glyceraldehyde 3-phosphate-pyruvate pathway. Gram-positive cocci and the spirochete Borrelia burgdorferi possess only genes that encode the enzymes of the mevalonate pathway (5). The rate-limiting reaction of the mevalonate pathway is catalyzed by HMG-CoA-reductase, which converts (S)-HMG-CoA to (R)-mevalonate (Fig. 1). The reaction proceeds in three stages, the first and third of which are reductive.

In humans, the mevalonate pathway is responsible for the endogenous synthesis of cholesterol. For this reason, HMG-CoA reductase is the target of the statin drugs that severely attenuate cholesterol synthesis and lower blood cholesterol levels in human subjects (6) by competitive inhibition with respect to the substrate, HMG-CoA.

There are two distinct classes of HMG-CoA reductases, which appear to have arisen by divergent evolution from a common ancestor (7). The class I HMG-CoA reductases are present in eukaryotes and several Archaea. Characterized class II HMG-CoA reductases include those from Pseudomonas mevalonii (8), Archaeoglobus fulgidus (9), Staphyloccocus aureus (10), Enterococcus faecalis (11), and certain streptomycetes (12, 14). Crystal structures of a representative of each class of the enzyme have been determined, the class I human enzyme (13) and the class II HMG-CoA reductase of P. mevalonii (14, 15). Both enzymes share a common catalytic mechanism but exhibit significant differences in their three-dimensional structure (14, 16) and in sensitivity to inhibition by statins (17). The class II enzymes are considerably less sensitive to inhibition by statin drugs, with K<sub>i</sub> values in the millimolar range, in contrast to the nanomolar values for the class I enzymes (9, 10, 17, 18).

Apart from multiple genes for acetyl-CoA acetyltransferase, which also functions in fatty acid catabolism, the enterococci, streptococci, and staphylococci possess only single copies of the genes encoding the subsequent five enzymes of the mevalonate pathway. The mevalonate pathway is essential for the growth of Streptococcus pneumoniae. Strains carrying double allelic replacements of the genes encoding HMG-CoA synthase and HMG-CoA reductase are auxotrophic for mevalonate, and the virulence of an HMG-CoA synthase mutant of a pathogenic strain of S. pneumoniae is severely attenuated in a mouse model (5). Significantly, the low (20–80 nM) concentration of mevalonate in human plasma (19) cannot support growth of the HMG-CoA synthase-null mutant. Because the mevalonate pathway is essential for the survival of Gram-positive cocci, the class II HMG-CoA reductases, and possibly other enzymes of the mevalonate pathway, represent attractive potential targets for the development of active site-directed inhibitors for use as antibiotics against multiple drug-resistant strains of the enterococci and streptococci that constitute a major cause of nosocomial infections.

We have shown in previous crystallographic work (14, 15) that the active site of class II HMG-CoA reductase is a large open cavity located at the subunit interface of this dimeric enzyme. Three distinct subsites that bind cofactors and substrates can be described. One site, formed by the large domain of one monomer, binds the CoA portion of HMG-CoA. The small domain of the second monomer forms the NAD(H) binding site. A deep pocket at the dimer interface forms the binding site for mevalonate and the HMG portion of HMG-CoA. The crystal structures of the ternary complexes (15) revealed that binding
of the substrates and cofactors induces folding of the last 50 residues at the C-terminal region of the monomer to form a three-helix flap domain. This region is disordered in the original structure of the apoenzyme (14). The flap domain closes over the active site and positions the catalytic histidine, His-381, proximal to the thiester of HMG-CoA, in an orientation consistent with its proposed role in protonating the leaving CoAS⁻ anion (15, 20). The structures also presented evidence that a new residue, Lys-267, is critically involved in catalysis as confirmed by mutagenesis and chemical modification of Lys-267 (21).

The determination by Istvan and Deisenhofer (22) of multiple crystal structures of statins complexed to the human class I HMG-CoA reductase has revealed their molecular interactions. The low sequence homology between class I and class II HMG-CoA reductases, significant differences in their three-dimensional structures, and wide differences in their substrate sensitivity suggested that there might be differences in the way they bind statins. We therefore have determined the crystal structure of a class II HMG-CoA reductase complexed with the statin lovastatin. This reveals the specific interactions of the statin in the active site and explains the molecular basis for the wide differences in substrate sensitivity of the two classes of HMG-CoA reductase. More importantly, this structure provides new insights for the ultimate development of "class II statins," compounds selectively directed against HMG-CoA reductase of pathogenic microorganisms.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Following expression in E. coli of the mvaA gene that encodes P. mevalonii HMG-CoA reductase, the enzyme was purified to homogeneity as described previously (1).

Crystallization—Crystals of HMG-CoA reductase were grown in 1.2 M ammonium sulfate at 20 °C for few days following the method described previously (23). Large crystals were produced by microseeding and grown over several weeks up to 1.2 × 1.2 × 0.5 mm in size. Lovastatin (Merck) was dissolved in KOH at pH 9.0 to convert the enzyme to the ternary complex (Fig. 1). The reaction catalyzed by HMG-CoA reductase.

Lovastatin Contacts Four Different Regions of the Active Site—Four distinct subsites or pockets of the active site of P. mevalonii HMG-CoA reductase accommodate specific functional groups on lovastatin (Fig. 2). The LOV-1 site accommodates the C5-OH group, the LOV-2 site the C3-OH group. The LOV-3 site, at the top of the active site, accommodates the carboxylic group, and the hydrophobic LOV-4 site accommodates the decalin ring. The β-hydroxy-β-acid moiety of lovastatin sits in the central pocket of the active site in the same position as the HMG moiety of HMG-CoA in the structure of the ternary complex (15) (Fig. 3A). The decalin ring and the α-methylbutyrate ester moiety twist inward and face the side wall of the pocket formed by the hydrophobic side of the α-helix that contains Ala-368 to Leu-372 (Fig. 3B). In addition to occupying the HMG/mevalonate site, lovastatin partially occupies the position of the coenzyme A moiety in the HMG-CoA site.

Lovastatin Interacts Mimic Substrate Binding—Hydrogen bond interactions ofLovastatin with several residues of the enzyme mimic the substrate binding (Fig. 3A). As for the C5 oxygen of HMG-CoA (15), the C5-OH oflovastatin hydrogen bonds to catalytic residues Lys-267 and Glu-83 in the LOV-1 pocket. The carbohydrate group oflovastatin hydrogen bonds to Arg-261 in the LOV-3 site, an interaction that mimics that of the carbohydrate groups of HMG-CoA and mevalonate in the ternary complexes. This type of interaction, which is common in other dehydrogenases, is considered critical in compensating the negative charge of the carbohydrate group (27).

Additional Hydrogen Bonds Are Formed through Water Molecules—An additional hydrogen bond is formed to Asn-365 in the LOV-2 site through one of the water molecules identified in the initial map. This previously unobserved interaction has no parallel in the ternary complexes with substrates. A second water molecule mediates a hydrogen bond between Asn-216 in the B subunit and the C3-OH group oflovastatin. This contact was unexpected because Asn-216 formed two hydrogen bonds to the NAD(H) carboxyamide group in the structures of the ternary complexes, which facilitate binding of the nicotinamide ring (15). In the lovastatin complex, the water molecule overlaps exactly the position of the oxygen from the carboxyamide of NAD(H) and establishes an indirect interaction between the statin and Asn-216. Lovastatin thus appears to make all possible direct interactions with catalytically important residues present in the HMG/mevalonate pocket. Additional water-mediated interactions suggest potential sites of modification of the inhibitor to further enhance its binding affinity. Table I summarizes each of the above interactions.
Hydrophobic Contacts Also Contribute to Lovastatin Binding—Five residues in the LOV-4 site are involved in close hydrophobic contacts with the decalin ring. These are Ala-368 and Leu-372 (3.4–4.0 Å), on the hydrophobic face of an α-helix of the large domain responsible for HMG-CoA binding, and Ser-85, Ile-86, and Ala-89 (3.8–3.9 Å) located in an α-helix downstream from the catalytic Glu-83 on the other side of the pocket (Fig. 3B).

Binding of Lovastatin Prevents Closure of the Flap Domain—Lovastatin bound in the central or HMG/mevalonate pocket blocks access of the substrate HMG-CoA. In addition, the bulky decalin ring protrudes from the pocket and interferes directly with closure of the flap domain seen in the ternary complexes of HMG-CoA reductase (15). Consequently, the flap domain appears disordered in the electron density map, similar to the structure of the apoenzyme (14). Superposition of the lovastatin complex onto the HMG-CoA-NAD$^+$ complex shows that the decalin ring of the inhibitor overlaps the position of the catalytic histidine, His-381, in the closed flap structure (Fig. 3A). Binding of the statin thus impedes closure of the flap domain, blocks formation of the active site, and prevents correct alignment of functionalities essential for catalysis.

Comparison of the Statin Complexes of Class I and Class II HMG-CoA Reductases—Complexes of simvastatin and compactin with the catalytic domain of human HMG-CoA reductase have been reported and show the drugs bound in the substrate-binding site (22). All three statins have similar structures. In compactin there is a hydrogen rather than a CH$_3$ on C6′ of the hexahydrophthalene ring, and simvastatin has an additional methyl group on C2′ of the α-methylbutyrate ester moiety (Figs. 2 and 4). Major features in the mode of binding of the statins are common to both class I and class II HMG-CoA reductases because they mimic the natural substrate binding and position. Inspection of the specific interactions and arrangement of the active site residues reveals differences, which are discussed below in more detail. For the class II P. mevalonii enzyme, all residues that have direct interactions with lovastatin belong to the same subunit of this dimeric enzyme. Four direct hydrogen bonds mediate interactions with the HMG-like moiety of lovastatin, and two structural water molecules mediate additional interactions (Fig. 4A). Lovastatin binds in the hydrophobic pocket close to non-polar side chains with its decalin ring partially exposed to solvent.

Interactions of compactin (Fig. 4B) and simvastatin (Fig. 4C) with the class I human enzyme involve interactions with resi-
dues of two subunits of the tetrameric enzyme. In both cases, four regions or pockets can be distinguished in the active site of the enzyme, similar to the lovastatin complex presented here. In the complexes of compactin and simvastatin with the human enzyme there are seven direct hydrogen bond interactions with residues in the active site and one additional interaction mediated by a water molecule (Table I). Hydrophobic interactions occur with Leu-853h,2 of the hydrophobic side of helix Lα10, which occupies a position analogous to the long helix at the HMG-CoA binding site of the P. mevalonii enzyme. Two additional hydrophobic residues are in close proximity to the decalin ring, Leu-562h and Val-653h. From this analysis it is clear that a higher number of hydrogen bond interactions is involved in the binding of the statins to the active site of the class I human enzyme, consistent with the wide differences in the inhibition constants when compared with the P. mevalonii and other class II HMG-CoA reductases.

Sequence Conservation and Divergence at the Inhibitor Pocket Sites—The hydrophobic regions of both classes of the enzyme contain residues that are semiconserved across classes but which occupy comparable positions in the three-dimensional structure of the α-helices in the large domain (HMG-CoA binding domain). Ile-86p is either Val or Leu in class II HMG-CoA reductases and is Leu in the class I enzymes. By contrast, Ala-89p is conserved in class II but is a serine in the class I enzymes. Ala-368p, Leu-372p, and their analogous residues Leu-853h and Leu-857h are conserved within classes. The apolar character and the relative position of all these residues are retained in both classes of the enzyme. This suggests that overall the hydrophobic contacts of the inhibitors with the retained in both classes of the enzyme. This suggests that the relative character and the relative position of all these residues are conserved across classes but have no apparent analogues across classes. Interestingly, these conserved residues occupy similar positions in the three-dimensional structure of the enzyme. This suggests a conserved mode of binding for the substrate, and hence for statins, critical for both types of enzyme.

The LOV-1 Pocket—The LOV-1 pocket is formed by the coenzyme A-binding site all are close to the decalin ring and could act as potential hydrogen bond donor/acceptor groups. Ser-85p and Gln-364p in the coenzyme A-binding site are close to catalysis further confirm the proposed catalytic mechanism for this enzyme and provide an explanation at the molecular level for why inhibition of lovastatin is competitive with respect to HMG-CoA. Interactions of lovastatin at the active site occur in four different subsites or pockets. Present in each pocket are residues conserved among the class II enzymes but which lack apparent homologues in the class I enzymes. In the three-dimensional structure of the human enzyme Cys-351h and His-752h occupy these positions and do not interact with the statins. These differences therefore offer potential for the design of inhibitors specific for class II HMG-CoA reductases.

DISCUSSION

Lovastatin binds to the active site of P. mevalonii HMG-CoA reductase mimicking the binding of the substrate HMG-CoA. Specific interactions of lovastatin with residues important in catalysis further confirm the proposed catalytic mechanism for this enzyme and provide an explanation at the molecular level for why inhibition of lovastatin is competitive with respect to HMG-CoA. Interactions of lovastatin at the active site occur in four different subsites or pockets. Present in each pocket are residues conserved among the class II enzymes but which lack apparent homologues in the class I HMG-CoA reductases. However, residues with analogous functional groups occupy similar positions in the three-dimensional structure and establish similar interactions with the statins. Binding of lovastatin in the active site displaces both the flap domain (class II enzyme) and the C-terminal helix Lα11 (class I enzyme) that contains the catalytic histidine. This results in a conformational disorder that prevents the visualization of this region in

2 The subscripts p and h on numbered residues refer to P. mevalonii or human HMG-CoA reductase, respectively.

### Table I

| Subsite | Lovastatin atom | Protein residue | Distance (Å) | Compactin atom | Protein residue | Distance (Å) | Simvastatin atom | Protein residue | Distance (Å) |
|---------|----------------|----------------|--------------|----------------|----------------|--------------|----------------|----------------|--------------|
| LOV-1   | O5             | Lys-267        | 2.8          | O5             | Lys-691        | 2.7          | O5             | Lys-691        | 2.8          |
| LOV-1   | O5             | Asn-271        | 3.1          | O5             | Asn-755        | 2.8          | O5             | Asn-755        | 2.9          |
| LOV-1   | O5             | Glu-83         | 3.3          | O5             | Glu-559        | 2.7          | O5             | Glu-559        | 2.7          |
| LOV-2   | O3             | Water          | 2.5          | O3             | Arg-590        | 3.1          | O3             | Arg-590        | 3.1          |
| LOV-3   | O1B            | Arg-261        | 2.7          | O1B            | Lys-735        | 2.8          | O1A            | Lys-735        | 2.8          |
| LOV-3   | O1A            | Water          | 2.5          | O1A            | Lys-692        | 3.1          | O1B            | Lys-692        | 3.2          |
| LOV-3   | O1A            | Ser-684        | 2.6          | O1B            | Ser-684        | 2.7          | Water          | O1B            | 2.9          |
| LOV-3   | O1A            | Water          | 2.6          | O1B            | Ser-684        | 2.7          | Water          | O1B            | 2.9          |
the electron density maps of the structures of the complexes.

Comparison of the structures of lovastatin bound to Pseudo-
monas HMG reductase and those of compactin and simvastatin
bound to the human enzyme revealed an overall similar mode
of binding for the statins but significant differences in the
specific interactions. For the human enzyme complexes, exten-
sive hydrogen bond interactions involve residues in the LOV-1,
LOV-2, and LOV-3 pockets. Most of the residues involved in
specific interactions are conserved in the class I enzyme but
with no apparent homology to the class II enzyme. There are
two exceptions, the catalytic glutamate and asparagine in the
LOV-1 pocket, which are conserved throughout both classes of
the enzyme. The hydrophobic region around the decalin ring of
lovastatin also appears to be highly conserved and to retain the
non-polar character across both classes of enzyme. This sug-
uggests that the hydrophobic interactions in that region are less
specific for each class of enzyme, and they would probably not
provide significant discrimination for the development of class
II specific inhibitors. In contrast, regions such as the LOV-2
pocket, which exhibit marked differences in amino acid compo-
sition between both types of enzyme, are potential “hot spots”
for further investigation and drug development. Water-medi-
atated interactions, for example, could accommodate extra func-
tional groups positioned in the statin molecule to maximize

Fig. 4. Comparison of the statin complexes of class I and class II HMG-CoA reductase. Interactions of lovastatin with active site residues in the *P. mevalonii* HMG-CoA reductase-lovastatin complex (A) (this work) and of compactin (B) and simvastatin (C) with the catalytic domain of human HMG-CoA reductase (from Ref. 22). Hydrogen bonds are represented by *dashed lines* and hydrophobic interactions by *spiked semi-circles*. Letters A and B indicate whether a residue is from the A or the B subunit of the human enzyme. This figure was prepared using LigPlot (29).
interactions. Interactions of statins with the functional groups of residues such as Ser-85p and Gln-364p might be also exploited to provide alternative binding capabilities and enhance the design of selective inhibitors for the class II forms of HMG-CoA reductases of important human pathogens.

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