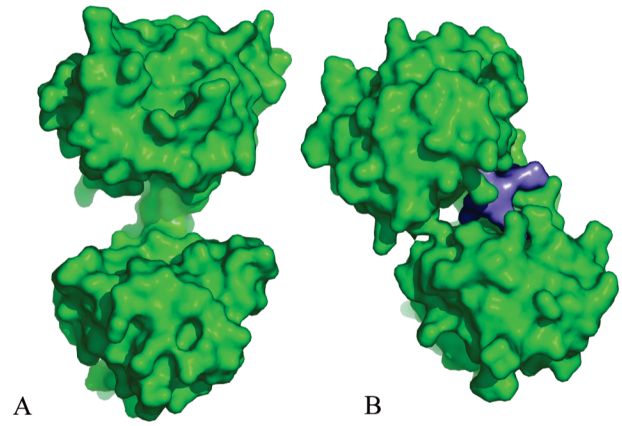

FIGURE 4: Domain closure upon ligand binding. (A) Surface representation of human apo U3S, which adopts the most open conformation observed in the various crystal structures. (B) Product bound *T. thermophilus* U3S showing how the domains close around the uro'gen ligand (purple). Both panels are in the same orientation as Figure 2.

others, Pro29 and Ala94, lie on a strand (domain 1) and helix (domain 2), respectively, and point into hydrophobic pockets of the enzyme. Thus, five of the seven invariant residues perform structural roles. Thr217 lies on a surface loop and points its hydroxyl toward the bound product at a distance of 5.1 Å. Thr228 appears to be conserved because it contributes to catalysis; mutation of the corresponding human Thr228 to Ala resulted in the greatest activity loss of residues studied, although this residue is not essential for catalysis because the mutant protein still retained 32% of wild-type activity. The one other invariant residue, Tyr155, is discussed below.

Prediction of ligand binding by the human enzyme from the known interactions of the *T. thermophilus* U3S product complex is complicated by the low sequence similarity and the large-scale domain motions that must occur upon binding. In order to build a plausible model of the human enzyme−product complex, we performed independent alignments of each domain and of the linker segment of the unliganded human U3S structure onto the TtU3S complex. In the resulting model, substitution of two residues by proline eliminates main chain amides that hydrogen bond with product in the *T. thermophilus* U3S complex. Additionally, two residues in the human model would need to be repositioned, for example by adopting an alternative rotamer conformation, in order to avoid steric clash. Overall, the human U3S product complex model seems to be reasonable if approximate.

A study of the U3S protein from *A. nidulans* reported that mutation of Tyr166 to Phe resulted in an inactive enzyme (*33*). In contrast, our earlier study concluded that the equivalent Tyr168Phe mutation in human U3S only caused 50% reduction of activity (*13*). Concern that our assays may have been performed under conditions of limiting substrate (*33*) is not valid because data were taken entirely from the range in which the reaction was linear, which is only expected to occur when substrate is saturating (*13*). Inspection of the different U3S structures reveals that this conserved tyrosine is located on the linker connecting the two domains and adopts a variety of conformations. In the TtU3S product complex, the equivalent Tyr155 residue adopts a solvent exposed conformation and is separated from the A and D rings of the uro'gen III product by Tyr131, with the closest interatomic distance between Tyr155 and the product B ring being 7 Å (Figure 5A). Our modeling suggests that, in the context of the human U3S structure, Tyr168 might approach the uro'gen III bridge carbon, C20, and be well positioned to hydrogen bond with the tyrosine to the HMB hydroxyl (Figure 5B). In this speculative model, the putative catalytic tyrosine would contribute to loss of the substrate hydroxyl and formation of the azafulvene intermediate, as suggested for the *A. nidulans* enzyme (*33*), with the different tyrosine contexts possibly contributing to differences in enzymatic activity for U3S enzymes from the different species.

Implications for Catalytic Mechanism. The HMB substrate autocyclizes in the absence of U3S to form the symmetrical uro'gen I isomer with a half-life of only 4 min (Figure 1) (*35*). U3S therefore likely functions in part by restricting HMB to conformations that avoid attack of carbon 19 on carbon 20 and formation of the I isomer, but allow attack of carbon 16 on carbon 20 and formation of the III isomer product. Unfortunately, the product complex does not provide a clear indication of how this is achieved in detail. To further investigate the mechanism we have modeled a plausible transition state based on both the uro'gen III-bound crystal structure and a crystal structure of a methylated dinitrilepyrrolenine (*6*) (Figure 1). This molecule is a synthetic precursor to the known competitive inhibitor spirolactam, and its crystallization demonstrated that the contracted ring structure is energetically viable (*6*) (Figures 1, 5B). To generate the transition state model, the dinitrilepyrrolenine was positioned in the active site by overlap of the A and B pyrroles on the product complex structure, which as discussed above are the most tightly coordinated and constrained components of the product complex. Our model depicts the methoxy side chains of the dinitrile derivative as carboxylates, and rotates the flexible side chains into a position most matching the bound uro'gen III structure (Figure 5B). The nitrile groups were replaced with a full pyrrole ring, complete with side chains, and the macro-ring pucker and orientation of the D ring carboxylates were positioned to minimize conflicts with the enzyme.

The transition state model is more compact than the product, with the distance between A and C pyrrole nitrogens reduced from 4.8 Å to 3.4 Å, and the diameter of the ring contracted from 7.0 Å to 6.2 Å when measured diagonally between bridge carbons. Despite these differences, it is trivial to accommodate all of the hydrogen bonds seen in the product complex crystal structure in the transition state complex model and the transition state model is structurally quite similar to the observed product complex structure (Figure 5B). Progression from the transition state to the second azofulvene intermediate and on to the product is expected to require easily accessible conformational shifts in the relatively unconstrained C and D pyrroles and only minor changes in the enzyme conformation.

Remaining questions of U3S mechanism include, how does the linear tetrapyrrole substrate bind, to what extent is the loss of hydroxyl facilitated by the conserved tyrosine residue in the enzyme from different species, and what conformational changes are required upon progression from substrate to the first azafulvene intermediate and on to the spiro-pyrrolenine transition state? Based upon the interactions seen in the product complex crystal structure and the known