ABSTRACT: In type II polyketide synthases (PKSs), which typically biosynthesize several antibiotic and antitumor compounds, the substrate is a growing polypeptide chain, shuttled between individual PKS enzymes, while covalently tethered to an acyl carrier protein (ACP): this requires the ACP interacting with a series of different enzymes in succession. During biosynthesis of the antibiotic actinorhodin, produced by Streptomyces coelicolor, one such key binding event is between an ACP carrying a 16-carbon octaketide chain (actACP) and a ketoreductase (actKR). Once the octaketide is bound inside actKR, it is likely cyclized between C7 and C12 and regioselective reduction of the ketone at C9 occurs: how these elegant chemical and conformational changes are controlled is not yet known. Here, we perform protein–protein docking, protein NMR, and extensive molecular dynamics simulations to reveal a probable mode of association between actACP and actKR; we obtain and analyze a detailed model of the C7–C12-cyclized octaketide within the actKR active site; and we confirm this model through multiscale (QM/MM) reaction simulations of the key ketoreduction step. Molecular dynamics simulations show that the most thermodynamically stable cyclized octaketide isomer (7R,12R) also gives rise to the most repeatable equilibrium conformations for ketoreduction. Subsequent reaction simulations show that ketoreduction is stereoselective as well as regioselective, resulting in an S-alcohol. Our simulations further indicate several conserved residues that may be involved in selectivity of C7-12 cyclization and C9 ketoreduction. Detailed insights obtained on ACP-based substrate presentation in type II PKSs can help design ACP-ketoreductase systems with altered regio- or stereoselectivity.

KEYWORDS: polyketide synthesis, protein–protein docking, computational enzymology, 2D-NMR, QM/MM
Chart 1. ActPKS octaketide and its products.

Scheme 1. Formation of Cyclized Octaketide 2 (A) and Subsequent Reactions (B)

"Atoms in blue denote the portion of PPant-octaketide (1) that forms the six-membered ring upon cyclization.

"The first transformation that likely takes place in actKR is the cyclization of actACP-1 (once spontaneously enolized) to yield actACP-2 (Scheme 1). The combination of actACP-1 binding to actKR monomers via the arginine patch and octaketide docking inside the actKR’s long but narrow active site probably allows the enzyme to exert strong regiocontrol that favors a C7 and C12 ring closure. C7−C12 cyclization is evident in the final product actinorhodin and the shunt product mutactin formed by the action of only the minimal PKS and actKR (Chart 1). In the absence of the actKR, C10−C15 cyclization of 1 competes with the natural C7−C12 ring closure. Structure−activity relationships and sequence conservation led to Thr145 and Ser158 being proposed to play a role in this regiocontrol. Thr145 has been suggested to..."
play a role in stabilizing the O11 enolate, while Ser118 may assist in the proton transfer to O7 from the solvent.

The main (second) transformation in actKR—its ketoreduction of actACP—2 is both regio- and stereoselective, producing an alcohol group on C9 (Scheme 1). The first and rate-determining step in this reaction involves hydride transfer from the actKR-bound NADPH to C9, and (asynchronous concerted) proton abstraction by O9 from actKR/Tyr157. Stabilization is provided throughout the reaction by a hydrogen bond between O9 and actKR/Ser118 (Scheme 1). The chirality set by actKR at C9 remains unresolved; both 2 and 3 are too labile to be isolated. 3 is shuttled as actACP-3 to actinorhodin aromatase (actARO) and aromatized to actACP-4, leading to a loss of the stereochemical information. In the absence of actARO, mutacin is generated, but its chirality has not been unambiguously confirmed; its designation as “9S” in Chart 1 (see chirality assignment in Supporting Information) is based on previous supposition. It has therefore not yet been possible to infer which stereoisomer and conformer of actACP-2, if any, is preferentially formed and reduced within actKR, nor how. Therefore, to fully understand the factors that control the regio- and stereoselectivity, both the protein–protein and protein-substrate interactions between actKR and actACP-I should be considered in detail. Although binding models have been suggested, no crystal structure of the complex exists and detail on the interactions between actACP and actKR is lacking. Most solved enzyme-ACP complexes feature FASs, and only two feature a PKS component (namely, KS). Moreover, to study protein–substrate interactions, a complex with actACP-I is required, but this is unfeasible. In this work, we combine protein–protein docking, molecular dynamics (MD) simulations, 2D protein-NMR spectroscopy, and hybrid quantum mechanics/molecular mechanics (QM/MM) simulations to obtain detailed information on actKR–actACP binding and actKR–octaketide interactions: our aim is to provide a unified picture of actKR structure and function and address the lack of fundamental knowledge on type II PKSs.

**MATERIALS AND METHODS**

**Protein–Protein Docking**

Rigid docking calculations of actKR–NADPH and apo actACP were performed using the Bristol University Docking Engine (BUDE), with GPU acceleration. The structure for actKR–NADPH was obtained from previous simulations starting from PDB ID 2RH4, and the structure of actACP was taken from model 13 of the NMR ensemble PDB ID 2MVU, wherein the octaketide mimic is most unsheathed into the solvent (further details in Supporting Information). To maximize docking efficiency, the search space was restricted to areas of each protein’s accessible surface interfaces and excluded areas too far away from the arginine patch. For docking, a “generation zero” of 4600 poses was randomly generated for each of the 43625 possible pairs of chosen actKR–NADPH and actACP surface points. The 50 highest-scoring poses were evolved into 2500 “generation-one children” using a Monte Carlo algorithm, and the...
process (50 new parents, 2500 new children) was repeated to generation five, resulting in ~43000 fifth-generation binding modes. Seventeen binding modes (labeled M4–M20) were selected (based on BUDE score and the distance of actACP/Ser42 to the arginine patch), and for comparison, three actKR–actACP models obtained or derived from previous works24,31 (M1–M3) were included. Detailed procedures and coordinates for all models are provided as Supporting Information.

MD Simulations of actKR–actACP Complexes

Tetrmeric (actKR–NADPH)2–(actACP)4 structures for molecular mechanical (MM) MD simulations were assembled from the docking results, initially using different docking models (M1–M20) at each actKR chain in the tetramer, without the PPant-octaketide (see Figure 2, series Ia). For three docking models that gave the most promising results in series Ia (M10, M14, and M17), further simulations were run with four actACPs from the same model bound to one actKR tetramer (see Figure 2, series Ib). For the model selected after NMR assessment (M14), further MD simulations of the tetrmeric complex were performed after introducing the PPant-octaketide moiety (2), with all combinations of the possible cyclization conformers (stage II, Figure 2; Scheme 2; Table S3). 2 was modeled from different starting positions in the active sites of each system by finding a balance between: (1) conformational agreement with the PPant of octaketide mimics crystallized with KR53–55 and (2) maintaining catalytic interactions with residues Ser144 and Tyr157. The latter was not possible with C9 positioned for pro-R hydride attack (i.e., attack from the Si-face, which would yield an R-alcohol at C9); starting structures therefore were modeled for pro-S attack in all cases (i.e., attack from the Re-face, resulting in an S-alcohol, as depicted in Scheme 1B). Moreover, while all ketone groups on 2 are potentially prone to keto-enol tautomerization, C=O groups 1, 3, 5, 9, 11, 13, and 15 on all isomer conformers of 2 were always modeled as carbonyls, to keep our work computationally tractable.

Compared to the MD simulations without PPant-octaketide, the α6-α7 loops of actKR and adjacent residues (188–229) were positioned in a more “closed” form, as suggested previously,39 with the Tyr202 side chain projecting inside the active site (and a water molecule bridging Tyr202 and the octaketide), as indicated by the recently obtained actKR-octaketide mimic structures;40 see details in the Supporting Information.

For both stage I and II MD, all residues were in their standard protonation states (consistent with pKₐ predictions from PROPKA 3.1)46 with actKR His162 protonated on Nδ1 and His153 and His201 on Nε2 (according to the surrounding H-bond network). All systems were solvated in a rectangular box extending at least 11 Å from any protein atom and neutralized by the addition of Na⁺ ions. The ff14SB force field47 and the TIP3P model48 were used, alongside NADPH parameters from Holmberg and co-workers.49 GAFF50 parameters with HF/6–31(d) RESP point charges were used for the PPant-Ser42 fragment (details and libraries in SI; calculations in ioChem-BD).51,52 Multiple independent 32 ns periodic-boundary MD runs were performed in the NpT ensemble (after an equilibration procedure), using 2 fs timestep (with SHAKE for bonds containing hydrogen). The temperature was maintained at 303 K, in line with kinetic assays45 and recommended assessment of protein–protein docking stability,53 and pressure at 1 atm. All simulations are conducted using AMBER 1654,55 with GPU acceleration where applicable. CPPTRAJ56 is used for trajectory analysis and post-processing. Further details on generation of starting structures and MD procedures are provided in Supporting Information.

QM/MM Reaction Simulation of Ketoreduction

QM/MM MD Umbrella Sampling (US) reaction simulations were run with sander from AMBER 16.57,58 Simulation conditions were identical to the MM production runs, except for a shorter time-step (1 vs 2 fs) and no SHAKE restraints59 on the QM region. This region was limited to one active site and comprised the cyclo-octaketide moiety of 2 from C4 to C16; Ser144 and Tyr157 side chains from Cβ; and the nicotinamide moiety of NADPH up to the first ribose (Figure S2). The QM region was treated with the semiempirical method PM660 as used and benchmarked in our previous study on actKR (PM6 overestimates the barrier, but the mechanism is correct).27 QM/MM MD US simulations of reductive hydride transfer from NADPH to 2’s C9 were run as previously reported,27 using the difference (x – y) as the reaction coordinate, where y is the distance NADPH: H−2/C9 and x is the distance NADPH: H−NADPH/Cα. (Figure S2). Simulations were started from 11 or 12 different “reactive” or “reaction competent” conformations selected from stage II MD runs for each of the three isomers of 2 for which reaction competent conformations were
regularly sampled (vide infra). The reaction coordinate was followed using US windows 0.1 Å apart until reaching 1.8 Å, and free energy profiles were obtained by combining all sampling (~1 ns per isomer) using the weighted histogram analysis method.61,62 Further details are reported in the Supporting Information.

2D-NMR Titration of actKR into actACP

actKR5 and uniformly 15N-labeled actACP24 were expressed and purified as described previously. All NMR data were acquired with a Varian INOVA 600 MHz spectrometer at 25 °C. Titrations of actKR into 15N-labeled holo-actACP were monitored by 1H–15N HSQC-TROSY experiments. The molar ratios of actKR/actACP at each titration point were 0.08, 0.47, 0.33, 0.67, 1.00, 1.34, 1.67, and 2.34, respectively. Stock solution of actKR was 1.66 mM KR in 100 mM potassium phosphate pH 6.5, 10 mM EDTA, and 1 mM DTT. This was added to 500 μl of 0.5 mM 15N-labeled actACP in the same buffer.

— RESULTS AND DISCUSSION

 Approach for actKR–actACP Model Generation and Validation

To obtain and validate a reliable, detailed structural model for actACP–2 binding to the tetrameric actKR, a stepwise computational procedure was followed (Figure 2), integrated with NMR spectroscopy. This general approach is in line with recent recommendations1,63 on integrative structural biology studies of protein–protein and –substrate interactions, whereby evidence from spectroscopic techniques is typically pieced together with computational techniques (in this case, molecular dynamics and docking). The approach is also in line with a previous work on related systems.64,65 First, protein–protein docking was used to explore potential actKR–actACP binding modes. Selected modes were then refined63 through extensive classical molecular dynamics simulations, in the absence of the PPant-substrate (Figure 2; Stage I). Structural analysis based on chemical shift perturbations (CSPs) of actACP obtained from 2D 1H–15N HSQC actKR titration data helped select the most likely binding mode. Then, all four stereoisomers of 2 were modeled into this binding mode, using all eight possible cyclized species, referred to as “isomer-conformers” (see Scheme 2 below). Thereafter, detailed molecular mechanical and hybrid QM/MM molecular dynamics simulations test the enzyme–substrate interactions and expected reactivity (final two stages in Figure 2). In the following subsections, we describe results from each stage in more detail and discuss how the validated model informs on the origins of stereo- and regioselectivity of actKR–actACP.

actKR–actACP Binding Poses from Docking and MD Simulation

Previous work5,8 has indicated that the actACP–actKR interaction is guided by a patch of three arginines on actKR (Figure 1), which recognize and bind the PPant phosphate attached to Ser42 in actACP-1.24 Extensive protein–protein docking of an actKR monomer (with NADPH bound) and actACP (in the absence of substrate) was assessed (protein–protein docking in Figure 2) alongside two previously suggested binding modes (M124 and M226) and one (M3) derived from the crystal structure of Escherichia coli enoyl reductase FabI complexed to its ACP (PDB 2FHS).31 By considering a combination of the BUDE docking score (i.e., an approximate assessment of the actACP–actKR interaction energy in each model, from here on referred to as BUDE interaction energy) and a cutoff for the distance [d(PPant–actKR)] between actACP/Ser42/Cγ (bound to the Oγ, which carries the PPant) and actKR/Arg38/Cζ (representing the arginine patch), henceforth referred to as Ser42-patch distance, we selected 17 further docking models (M4–M20) that are structurally distinct (see Supporting Information).

The full set of models M1–M20 were then further assessed using classical molecular dynamics (MD, MD Stage I in Figure 2) simulations to compensate for the rigidity in docking and to help eliminate false positives.53 For each model, 8 MD-refined binding poses were obtained through 8 independent MD simulations of 32 ns and subsequent clustering (series I8 in Figure 2; details in Supporting Information). For each MD refined binding pose (8 for each of the 20 docking models, i.e., 160 in total), BUDE scores and d(PPant–actKR) were measured (Figure S4). We then use (arbitrary) BUDE interaction energy and d(PPant–actKR) thresholds of ~90 kJ mol−1 and <9 Å, respectively, to select for poses that (1) are...
likely to occur with reasonable frequency and (2) are in line with PPant-octaketide insertion into the KR channel. Based on this, binding modes M1−M3, M5−8, M11, M12, and M19 were deemed unlikely to be representative after MD refinement: all poses from M2 and M3 both had large Ser42-patch distances and unfavorable BUDE interaction energies; all poses from M1 and M5−8 had Ser42-patch distances >9 Å; all poses from M11, M12, and M19 had consistently poor BUDE interaction energies (−90 kJ mol−1). MD refinement of the remaining docking models (M4, M9, M10, M13−M18, and M20) yielded several examples of binding poses with greater thermodynamic likelihood and compatibility with PPant insertion [i.e., low BUDE interaction energy and Ser42-patch distance, compatibility with PPant insertion [i.e., low BUDE interaction energy and Ser42-patch distance, d(PPant−actKR) < 9 Å, 13 additional binding poses were found for M10, 9 for M14, and just two for M17 (Figures 3 and S5)]. (All but one of the additional poses again improved their BUDE interaction energy from docking.) Notably, even for these three poses that frequently exhibit favorable BUDE interaction energies, much less favorable interaction energies (−90 kJ mol−1; Figures 3 and S4 and S5) also occur within 32 ns of MD simulation. This likely reflects a transient actKR−actACP binding interaction.

To further narrow down the selection of binding poses to those that are consistent with PPant-octaketide insertion, we monitored whether the Ser42-patch distance remained within 15% of its original value during the last 4 ns of the MD simulation from which each pose was selected (Figure 3; filled-in symbols). Combining this criterion with the most negative BUDE score and the shortest Ser42-patch distance resulted in the selection of refined poses M1014IB, M1416IB, and M171IA (see framed symbols in Figure 3 and structures in Figure 4). The subscripts denote the MD replica (number 14, 16, or 1) and series (I or II). This selection should ensure that the three selected models are thermodynamically likely (favorable BUDE interaction energy; stability until the end of their MD runs) representations of possible (transient) actACP−actKR interaction modes, which are in agreement with PPant phosphate recognition by the arginine patch.5,24

NMR and Structural Analyses of the actACP−actKR Interaction

All three thermodynamically plausible actACP−actKR binding modes selected after docking and MD simulation (Figure 4) feature actACP/Ser42 (at the N-terminus of actACP’s α2 helix) relatively close to Arg38. Only in M1416IB and M171IA, however, are all three arginines in the patch5,6 positioned to capture the phosphate in the PPant moiety of 2 (Figure 4): actACP/Ser42:Oγ is 3.4 and 5.2 Å away from the center of mass of the arginine guanidinium moieties, respectively (vs 12.9 Å away in M1014IB). All three binding modes exhibit several electrostatic interactions between actACP and actKR (Table S5). In M1014IB, however, none of these interactions are formed with the arginine patch5,24 or NADPH (with most contacts between the actACP α2 and actKR α6 helices). In contrast, in M1416IB and M171IA, charge−charge interactions are formed with the arginine patch by both actACP/Asp41 and actACP/Glu36 and with the phosphate moieties of NADPH by Arg67 (M1416IB) or Arg34 (M171IA). In M1416IB, actACP α3 is in the center of the actACP−actKR interface, whereas the overall binding interaction in M171IA is dominated by the α1−α2 loop and does not involve α3.

To compare the plausibility of the binding modes, we conducted 1H−15N HSQC titration experiments using 15N-labeled actACP and unlabelled actKR (Figure 4, bottom panel; Figure S6). Titration to an excess of actKR/actACP showed small, but distinct CSPs particularly across the α2−α3 loop and α3, consistent with relatively weak binding. The largest magnitude CSPs are observed in this region (I60, D62, and V68) and may also report on conformational changes in α3 as reported previously,23 again pointing to the involvement of α3 in the actACP−actKR interaction, as observed in M1416IB. Furthermore, residues of the flexible α1−α2 loop from T21-

Figure 3. Refinement and ranking of docking models by MD simulation. ActKR−actACP binding modes originating from MD simulations of M10 (black); M14 (red); and M17 (blue) are shown with their interaction energy (BUDE score, y-axis) and Ser42-patch distance [d(PPant−actKR), x-axis]. Asterisks denote original docking modes; squares, those originating from series IA (MX2,Y1A: X = 10, 14, 17; Y = 1−8); and triangles, those originating from IB (MX2,Y1B: Y = 1−16). The area bound by magenta lines indicates the region with BUDE score < −90 kJ mol−1 and d(PPant−actKR) < 9 Å thresholds. Open triangles and squares refer to binding modes whose Ser42-patch distance deviates by more than 15% from its average value in the last 4 ns of the MD simulations from which they originate. The three binding modes selected for structural analysis and validation with NMR are highlighted by framed symbols.

from docking models M4, M9, M15, M16, and M20 (Figure S4); two originating from M13 (Figure S4); three from M18 (Figure S4); and as many as four from M10 and M14 and five from M17 (Figure S4; triangles in Figure 3). All but two of these 23 refined poses improved their BUDE scores from docking, indicating that the flexibility introduced by MD simulation led to a more plausible actKR−actACP binding interface.

The only models with >50% of MD-refined snapshots within the thresholds, and therefore more likely to occur than others, were docking models M10, M14, and M17. These were selected for further MD simulation (series II in Figure 2; note that the next-best model M18 is structurally similar to M14, with a RMSD between actACP/Cα atoms of only 1.59 Å, and was therefore not selected). Simulations were performed using only one of each binding mode at each actKR−actACP interface (using 4 × 32 ns simulations for each tetramer, Figure 2). Clustering then gave 16 additional representative snapshots for each binding mode. Using the same thresholds as before [BUDE score < −90 kJ mol−1; d(PPant−actKR) < 9 Å], 13 additional binding poses were found for M10, 9 for M14, and just two for M17 (Figures 3 and S5). (All but one of the additional poses again improved their BUDE interaction energy from docking.) Notably, even for these three poses that frequently exhibit favorable BUDE interaction energies, much less favorable interaction energies (−90 kJ mol−1; Figures 3 and S4 and S5) also occur within 32 ns of MD simulation. This likely reflects a transient actKR−actACP binding interaction.

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D29 exhibited exchange broadening; this loop is fully solvent-exposed only in M1416IB. Although the interface predominantly characterized by charge−charge interactions (see above) suggests a highly specific molecular recognition, the broadly distributed CSPs overall indicate that the actKR/actACP likely forms a weak transient complex in solution. It is therefore likely that many transient actKR/actACP binding modes will occur, as opposed to one well-defined protein−protein interface. We note that for the E. coli FAS ACP-acyltransferase interface, such structural plasticity has been suggested to be a key contributor to catalytic efficiency.41

In summary, structural analysis and NMR titration suggests that binding mode M1416IB is a good representation of a thermodynamically feasible, transient actACP−actKR complex, with the following features: (1) the actACP “gatekeeper” α3 helix is central to the interface, occupying a cleft above the central NADPH phosphates and adjacent to the arginine patch;5,24 (2) the α4 helix interacts with the (mobile) α6 helix of actKR; (3) part of the α2−α3 loop, indicated by Hadfield et al. as being important for protein−protein interactions,24 is also in contact with actKR; and (4) the α1−α2 loop is solvent exposed.

**Reactivity of ACP-Bound Cyclized Octaketides in actKR**

To assess the possible binding interactions of cyclized octaketides in actKR, we performed multiple independent MD simulations of actKR−actACP with all possible cyclized conformers of the all-ketone tautomeric form of 2 (MD stage II in Figure 2): C7−C12 cyclization of 1 can, in principle, lead

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**Figure 4.** Comparison of putative actACP−actKR binding modes M1014IB, M1416IB, and M1711A and NMR titration data 15N-labeled actACP with actKR. Top: overview of actACP−actKR binding modes, with actKR (off-red cartoon) in the same orientation; actACP as gray cartoon. In M1416IB, actACP’s “gatekeeper” α3 helix is marked by a red circle; it is central to the actACP−actKR interface and has lost some of its structure. NADPH, actACP/Ser42, and the actKR arginine patch and actACP residues implicated in salt bridges (Table S5) are labeled and rendered as sticks: NADPH with C atoms in green; Arginine patch with C atoms in magenta; actACP/Ser42 in ball-and-stick with bright green C atoms; and H atoms omitted for clarity. Middle: magnification of the actACP−actKR interfaces with the ACP backbone colored according to the magnitude of the measured CSPs [as change (Δ) in weighted averages δAV] upon addition of actKR (from KR/ACP ratio of 0.08 to 2.34): 0.02 < ΔδAV < 0.04 ppm in yellow, 0.04 < ΔδAV < 0.06 ppm in orange, and ΔδAV ≥ 0.06 ppm in red. Bottom: ΔδAV values for every actACP residue. δAV is given by (δAV = (0.5[Δδ(1H)]2 + (0.2Δδ(15N))]2)1/2);66 where ΔδAV values are missing, this indicates either no significant shift, residues without −NH (Pro61, Pro71) or that assignments for these residues were tenuous. Full NMR data (1H−15N HSQC) are shown in Figure S6.
to four different stereoisomers of 2 (color-coded in Scheme 2; see chirality assignment for one isomer in the Supporting Information): (7R,12R)-2 (henceforth RR-2; black), (7R,12S)-2 (RS-2; gray), (7R,12R)-2 (SR-2; red), and (7R,12S)-2 (SS-2; orange). In turn, each of these four isomers can access two low-energy chair conformers (Scheme 2), with the C7–OH substituent oriented either axially (2OHax) or equatorially (2OHeq). To include a plausible actACP–actKR binding interaction (which will constrain the mobility of the PPant-octaketide), consistent with our NMR titration study, actACP binding mode M14was used. We note that other binding modes that similarly constrain the PPant-octaketide mobility (such as M10 or M17) would likely lead to similar results. The actKR a6–a7 loop was remodeled prior to MD simulation, based on an actKR-octaketide mimic complex structure,45 in line with the suggested role of this loop in substrate recognition.5 By using previous structural information63−45 and satisfying contacts between 2 and catalytic residues, initial placements for the PPant and the octaketide moieties were generated (two alternative starting positions were used in order to explore a greater portion of conformational space; modeling details and coordinates are included in Supporting Information).

In the resulting MD simulations, the frequency of reaction competent poses (%reac, defined by satisfying key distances; see Supporting Information)65 of 2 toward C9 ketoreduction was monitored, and the combined %reac values (from 4 active sites × 8 replicas × 32 ns × four initial systems, see Table S3) were compared for each of the eight possible cyclization isomer conformers of 2 (Figure 5 and Table S6). RR-2OHax had the highest frequency of reaction competent poses (9.1%), followed by SR-2OHax and SS-2OHax (with 2.9% and 2.2%, respectively). The remaining five isomer conformers had less than 2% such poses. Essentially all reaction competent poses of the diacenylated substrate are not ready for reaction (Figure 5, y-axis; QM calculation details in Supporting Information, optimized structures in ioChem-BD).42,52 we find that there is some degree of correlation between thermodynamic stability (after cyclization) and the propensity to form reaction competent poses in the actKR active site for the ensuing ketoreduction step (e.g., Figure 6b): RR-2OHax is most stable, followed by SS-2OHax and SR-2OHax (0.9 kcal mol−1 and 1.4 kcal mol−1 higher in energy, respectively). The remaining isomers are significantly higher in energy (2.5 to 5.6 kcal mol−1). The correlation between these chemically distinct quantities was unexpected. Similarly, there is also a correlation between the frequency of reaction competent poses for reduction and thermodynamic stability of cyclization products for the axial versus equatorial C7–OH arrangement (especially for 12R isomers): in 7R isomers, axial conformers are more stable and attain more reaction competent poses; in 7S isomers, the opposite is true. A priori, there is no reason why thermodynamic stability of the isomer conformers should correlate with their proneness to react in the successive ketoreduction step.

To simulate the chemical reaction itself, we selected three isomer conformers of 2 (RR-2OHax SS-2OHax and SR-2OHax). As indicated by the relatively infrequent occurrence of reaction competent poses (at most 9.1% for RR-2OHax), the cyclized octaketide spends the majority of the time “in standby”, that is, bound in the active site with C9 close to the catalytic residues, but not quite ready for reaction (Figure 6a). Moving to a reaction competent conformation (e.g., Figure 6b) will thus come at a slight free energy cost (1.4, 2.1, or 2.3 kcal mol−1 at room temperature for RR-2OHax SS-2OHax and SR-2OHax respectively, based on ΔG = RT ln[% reaction competent poses]). For each, we performed combined quantum mechanical/molecular mechanical (QM/MM) MD simulations of ketoreduction at C9 (see QM/MM reaction simulations in Figure 2), using the same approach as our previous work on the reduction of trans-1-decalone by actKR.27 The transition states and reaction barriers obtained here are similar (Figures 6 and S3), which demonstrates that the selected complexes modeled based on M14 can indeed represent reaction competent actACP–actKR poses, further validating this MD-refined model. As expected, the transition state corresponds to the hydride transfer between NADPH and C9, concerted with proton transfer from Tyr157 to O9 (Figure 6c). Subsequently, Tyr157 moves to coordinate a ribose hydroxyl of NADP⁺ (Figure 6d), ready for reprotonation through a proton shuttle likely involving the ribose and Lys161.16,27 Notably, our simulations show energetically feasible reactions, while the cyclized octaketide is bound to actACP, confirming that the ACP-PPant tether does not need to be broken prior to ketoreduction by actKR (in contrast to what is expected for hedamycin KR).8,9 The barriers to reaction are not significantly different between the three isomer conformers, suggesting that actKR can facilitate ketoreduction to a similar extent in all three, via axial hydride attack at C9 (Scheme 1 and Figure 6b-d). While a preference for axial attack is in line with previous findings on the reduction of small alicyclic ketones by agents such as [AlH₄]⁺ and [BH₄]⁻,8,9 it
is in contrast with findings by Østergaard et al. on reduction of the small alicyclic trans-1-decalone by another ketoreductase \(^7\) and our own findings for its reduction by actKR itself.\(^2\) It appears that the tendency of actKR to catalyze equatorial H\(^-\) attack in small, nonendogenous substrates can be overridden by factors such as binding site architecture, spatial constraints arising from actKR–actACP binding, and the presence of oxygen substituents on C7 and C11.

**Determinants of actKR Stereo- and Regioselectivity**

The overwhelming prevalence of pro-S reaction competent poses in our MD simulations (stage II; H\(^-\) attack from the Re-face) indicates that S-selectivity for ketoreduction at C9 in actKR is defined by its active site structure in combination with the position of the incoming PPant chain, which is determined by the actKR–actACP interaction, as suggested previously.\(^3\) (Figures 1d; 6; S2). The side chains of the adjacent residues actKR/Thr145 (possibly stabilizing O11 during cyclization of 1 to 2; Scheme 2)\(^3\) and actKR/Ser144 (stabilizing O9 during ketoreduction; Scheme 1) form a relatively rigid template close to the nicotinamide ring of NADPH. When O11 and O9 bind to these residues upon arrival of 1 into the active site, C7–C12 cyclization to any isomer conformer creates spatial constraints that strongly favor reductive hydride attack in a pro-S pose (i.e., from the Re-face or “from below” in Figure 6b-d) to yield an S-alcohol at C9.

We noted above that the link between C7–C12 ring conformer stability and greater propensity for (pro-S) C9 ketoreduction is unexpected, indicating that the actKR active site might have evolved to preferentially perform reduction on the most stable cyclization isomer conformers RR-2OHax, SS-2OHep, and SR-2OHep that is, those that are more likely to form upon cyclization of 1. In addition, actARO—likely having evolved in tandem with actKR—might prefer the combination

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**Figure 6.** Key steps in the ketoreduction of actACP-RR-2OHax by actKR. The sequence depicts the pro-S hydride attack on isomer-conformer RR-2OHax (i.e., “below”, from the Re-face of C9), with salient octaketide carbons labeled where possible (a) representative “nonreactive” snapshot of actACP-RR-2OHax (C atoms in cyan) inside the active site of actKR, highlighting residues (sticks; C atoms light blue) that could be important for regioselectivity per our hydrogen bond analysis (see text). Catalytic residues Ser144 and Tyr157 (C atoms in orange) and the NADPH cofactor (C atoms in green) are shown. Gly95/NH part of the XGG motif\(^1\) interacts frequently with 2/O1; other residues are discussed in the text. (b) Reaction competent pose of actACP-RR-2OHax poised for hydride transfer from NADPH. Hydride is shown as the blue sphere and actKR/Tyr157/H\(_\eta\) (i.e., −(O)H) as the white sphere. (c) Transition state of the ketoreduction reaction, with hydride being transferred from NADPH to 2/C9. (d) Product of ketoreduction, with Tyr157’s phenolic proton transferred to 2/O9. Every panel comprises a 2D representation of the C7–C12 ring mimicking its 3D rendering as closely as possible; panels (a,d) also contain representations of the C7–C12 ring with the Si-face of C9 facing the reader. Groups R and R’ are defined in Scheme 1A.
of S chirality at C9 alongside the three isomer conformers to perform its conversion of 3 to 4 (although confirming this hypothesis would require detailed mechanistic studies of actARO, which is beyond the scope of this work).

Apart from its stereoselectivity in ketoreduction, the other remarkable characteristic of actKR is its regioselectivity, namely, why cyclization occurs between C7 and C12 (if it occurs on actKR, rather than on actKS/CLF) and why ketoreduction then occurs specifically at C9 (with the link between the two already noted). To investigate if and how the binding site architecture might drive regioselectivity, we examined the formation of hydrogen bonds (direct or water-mediated) between actKR and substrate oxygen atoms in MD trajectories from stage II (Figure 6a, full details in Tables S7 and S8). Hydrogen bonds between the cyclized octaketide moiety and actKR are rather transient during our simulations. Short-lived hydrogen bonds are consistent with I and 3 “sliding” in and out of the binding channel, respectively, as suggested by Javidpour et al., as well as the “in standby” conformation of the cyclized octaketide (with catalytically competent poses only being attained for a fraction of the simulation time, Figure 5). Hydrogen bonds directly relevant for ketoreduction at C9 are observed between O9 and Ser144 and Tyr157 on actKR (Scheme 1), but not as the most frequent (average frequencies, respectively, of 5.3 and 4.8% for RR-2, 4.3 and 4.3% for SR-2, and 3.0 and 1.8% for SS-2). Instead, the most frequent hydrogen bonding for O9 occurs with nearby backbone hydrogens of actKR/Phe189 (a residue whose importance was also noted experimentally) and actKR/Gly146 (Figure 6a and Table S7). Interactions with actKR/Ser144 and actKR/Tyr157’s −OH hydrogens are typically mediated by water bridges when found (Table S8). These interactions are consistent with isomers of 2 being held “in standby” in the binding site (Figure 6a), with the C9−O9 carbonyl never far from reaching a reaction competent pose (Figure 6b). Only this carbonyl interacts with the key catalytic residues, thus achieving regioselectivity at C9.

The simulated isomers of 2 can be considered as the products of C7−C12 cyclization of the all-ketone tautomeric form of 1 (Scheme 2), and their interactions may therefore reflect how such regioselective cyclization might be promoted by the actKR active site. One possible key interaction could be hydrogen bonding between O11 and actKR/Thr145’s hydroxyl group; however, our simulations only indicate sporadic and indirect hydrogen bond interactions (through water bridges, Table S8). A different hydrogen bond interaction that may be relevant for cyclization, between 2/O7/H7 and actKR/Tyr202’s −OH group, is observed occasionally in simulations for most isomer-conformer pairs (Tables S7 and S8). This (highly conserved) Tyr202 side chain, in its orientation toward the active site (Figures 6a and S2), could thus be involved in catalyzing regioselective C7−C12 cyclization, for example, as a proton donor to O7, or aiding proton transfer from the nearby His153 and His201. Other interactions that may be relevant for cyclization are the long-lived intra-molecular hydrogen bond between 2/H7 and 2/O5, and the occasional water bridges between 2/O5 and actKR/Tyr202’s −OH, both of which may contribute to C7−C12 cyclization through stabilization of proton transfer to O7. Notably, interactions of 2/O7/H7 with actKR/Ser158’s hydroxyl group, previously proposed to play a role in proton donation in cyclization, are hardly ever sampled. While these specific hydrogen bond interactions detected for O5, O7, and O11 may be structurally and/or electronically important factors for regioselective cyclization of 1 to 2, further work is required to confirm the possible roles of actKR residues in C7−C12 cyclization, such as stabilization of the enolate species and the source for O7 protonation (e.g., involving Tyr202, His153, and/or His201).

Finally, we consider contacts at the extremities of the (cyclized) octaketide species in its all-ketone form. Zhao et al. recently used extensive MD simulations of actKR and a double mutant, which affects chain-length specificity, together with octaketide and tetraketide substrate mimics. They considered two previously proposed substrate entrance sites, a “back-patch” near Q149/R220 and a “front-patch”, identical to the “arginine patch”. In our work, only binding at the latter is considered, as this is enforced by the location of ACP, with the PPant phosphate group binding to the arginine patch. This is consistent with the preference of the PPant octaketide mimic found by Zhao et al. For the octaketide, we find frequent and fairly persistent hydrogen bonds (direct or through bridging waters) between the start of the chain (O1) and the backbone actKR/Gly95/NH (Figure 6a). This glycin is part of the highly conserved XGG motif characterizing type II PKS, which has been suggested to be an anchor point for the PPant-octaketide to be presented to the actKR active site. Our simulations further support this. At the other end, O15 forms frequent hydrogen bonds (direct or through bridging waters) with actKR/Arg220, located toward the C-terminus of the α7 loop and previously considered by mutagenesis (Figure 6a). Arg220 can “seal” the binding pocket at its far end (including through hydrogen bonding with actKR/Gln149, forming the “back-patch” that can support binding of short polyketides) and could thus be a key factor for the regioselectivity of cyclization by helping the linear octaketide buckle upon itself near O15, folding the C12−C16 fragment back onto C7−C11.

### CONCLUSIONS

In the type II actinorhodin polyketide synthase, association between actinorhodin ketoreductase (actKR) and an actinorhodin ACP (actACP) carrying a phosphopantetheinylated octaketide results in the latter being inserted into the actKR active site (as 1 or cyclized as 2). Subsequently, 2 is stereoselectively reduced at C9=O9 to yield alicyclic chiral alcohol 3. In this work, we study the actACP−actKR binding interaction in atomic detail and suggest a plausible representative binding mode, using a combination of protein−protein docking, molecular dynamics simulations, and NMR CSPs. Then, further molecular dynamics simulations (including QM/MM reaction simulations) based on this binding mode are used to investigate the mechanism and the sources of regio- and stereoselectivity of actKR toward its natural substrate.

After initial selection of simulation-refined docking models based on estimated binding affinity and proximity of actACP/Ser42 to a “patch” of three arginines on actKR (Arg38, Arg65, and Arg93), one binding mode was found to be most consistent with our 2D NMR data and previous reports. In this mode, actACP docks onto actKR with its α3 helix and the N-termini of α-helices 2 and 4. Subsequent simulations based on this binding mode of complexes with all possible C7−C12 cyclization isomers of 2 revealed an overwhelming preference for pro-S ketoreduction at C9=O9, particularly for the most thermodynamically stable cyclization isomer (i.e., (7R,12R)-2 with C7−OH oriented axially). In addition to establishing a
link between chirality at C7/C12 and chirality at C9, this finding unequivocally confirms previous experimental data on mutactin, inferring that C9 should be enantiopure; it also strongly suggests that chirality at 3;C9 should be S rather than R (i.e., with hydride attack occurring from the C9’s Re-face rather than Si), and that actKR preferentially catalyzes this attack axially rather than equatorially. The (transient) binding mode of actACP in conjunction with spatial features of the actKR active site are sufficient to cause the indicated S-selectivity. QM/MM MD reaction simulations of C9 ketoreduction were performed for the three isomers of 2 that most frequently formed reaction competent binding poses. This indicated that S-selective ketoreduction is equally efficient for these isomers (i.e., no specific C7/C12 chirality is preferred in the chemical step) and our model yields energy barriers similar to those obtained with efficiently converted small molecules (further validating our proposed actACP—actKR binding mode). Further analysis of our MD simulations of 2 inside actKR identified residues (such as Gly95 and Arg220) that are important for steering the binding of the substrate and holding it “in standby” in the actKR active site, as well as those that may aid regioselective cyclization between C7 and C12.

In summary, we have combined protein–protein docking, extensive MD simulation, NMR, and QM/MM reaction simulations to produce and validate a detailed model of the actKR—actACP interaction that is consistent with all currently available experimental data for cyclization and ketoreduction of the natural octaketide substrate. The model obtained provides important mechanistic insights, demonstrating the use of multiscale atomistic simulations to improve our understanding of biocatalytic protein–protein complexes. We have shown that the specificity of the actKR—actACP interaction, together with the architecture of the actKR active site, has direct implications for the elegant regio- and stereoselectivity of actKR toward its natural substrate. The information obtained can aid in future engineering of type II PKS ketoreductase/acyl carrier systems, for example, to make them process alternative substrates or change cyclization, regio-, and stereoselectivity; an important step toward building biocatalytic systems that can yield new polyketide derivatives with different chain lengths, stereochemistry, and/or cyclization patterns.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacsau.2c00086.

Chirality assignment, additional details on docking calculations, construction of starting structures, parametrization of isomer conformers of 2, MD simulation and analysis, NMR data, details of QM/MM simulations, and analysis of hydrogen bonds in MD runs in II (PDF)

Input files for protein–protein docking; structures of M1–M20, M1014BB, M14e6BB, and M1771A in PDB format; AMBER input (topologies and coordinates) for all runs in LFF, Ile, and II; and modified force field parameters for the PPant moiety and octaketide portions of 2 (ZIP)

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