

## Structures of riboswitch RNA reaction states by mix-and-inject XFEL serial crystallography

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Riboswitches is RNA structural elements generally located in the 5' untranslated region (3'UTR) of mRNA. In the genetic regulation, ligand binding to the aptamer domain of a riboswitch triggers a signal to the downstream expression platform. A complete understanding of the structural basis for this mechanism requires the ability to study structural changes over time. We apply femtosecond X-ray free electron laser (XFEL) pulses to obtain structural measurements from crystals so small that diffusion of a ligand can be timed to initiate a reaction prior to diffraction. We demonstrate this approach by determining four structures of the adenine riboswitch aptamer domain during the course of a reaction involving two apo, one ligand-bound intermediate, and the final bound states. These structures support a reaction mechanism model with at least four states and illustrate the structural basis for signal transmission. The two apo conformers differ significantly in the three-way junction and the P1 switch helix relative to the ligand-bound conformation. Our time-resolved crystallographic measurements with a 10-second delay captured the structure of an intermediate with changes in the binding pocket that accommodate the ligand. With a >10-minute delay, the RNA molecules were fully converted to the bound state, in which the substantial conformational changes resulted in conversion of the space group. Such drastic changes *in crystallo* highlight the important opportunities that micro/nanocrystals may offer in these and similar time-resolved diffraction studies. These results all together demonstrate the potential of "mix-and-inject" time-resolved serial crystallography to study biochemically important interactions between biomacromolecules and ligands, including those involving large conformational changes.