

**Structural Basis for Molecular Mechanism of Dihydroorotate:Fumarate
Reductase Activity Revealed by *Trypanosoma cruzi* Dihydroorotate
Dehydrogenase**

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Dihydroorotate dehydrogenases (DHODs) are flavoenzymes catalyzing the oxidation of (*S*)-dihydroorotate to orotate, the fourth step and only redox reaction in the *de novo* pyrimidine biosynthesis pathway. DHOD from *Trypanosoma cruzi* (TcDHOD) couples the oxidation of dihydroorotate (first-half reaction) with the reduction of fumarate (second-half reaction). Here we report the crystal structures of native TcDHOD determined at 1.58 Å resolution and complexes with dihydroorotate (1.36 Å), orotate (1.80 Å), fumarate (1.94 Å), succinate (1.38 Å) and oxonate (1.26 Å). The structures of TcDHOD complexed with dihydroorotate and fumarate, substrates of the first and second-half reactions, respectively, reveal that the reactions proceed by the one site ping-pong Bi-Bi mechanism, and that three amino acid residues, Lys43, Cys130 and Lys164, and two bound water molecules play essential roles in the hydride and proton transfer from dihydroorotate to FMN and FMN to fumarate. The mechanism of the dihydroorotate oxidation proposed here is unlike the conventionally proposed one, but it not only can be applied to DHODs of other families but explain the decrease in the enzymatic activities of mutant DHODs.