STRUCTURAL STUDIES OF RHIZOBIUM ETLI INDUCIBLE ASPARAGINASE MUTANTS

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L-Asparaginases are a large family of enzymes, grouped into three structural Classes. Some Class 1 asparaginases from bacteria are used to treat acute lymphoblastic leukemia (ALL) and lymphosarcoma. Unfortunately, these therapeutic regimens are often associated with a number of serious side effects. Alternative sources of therapeutic asparaginases have thus been sought, and the inducible Rhizobium etli enzyme (ReAV) emerges as an interesting candidate.

ReAV differs significantly in sequence from other microbial asparaginases, indicating a different catalytic mechanism of asparagine hydrolysis. The crystal structure of ReAV [1] shows a protein folded as some β-lactamases, but forming a unique dimeric assembly. The active site of ReAV contains two Ser-Lys tandems, centered around the hydrated Ser48 residue and located in the close vicinity of a Zn^{2+} cation, which has an unusual coordination sphere created by two cysteines, a lysine and a water molecule. The presence of a Zn^{2+} cation in the active site area is unique to ReAV; however the metal ion is not necessary for catalysis. Another characteristic residue of ReAV is an oxidized Cys249, which is involved in a network of H-bonds comprising the active site area.

To decipher the catalytic mechanism of ReAV, the most conspicuous residues implicated by the crystal structure, i.e. the two Ser-Lys tandems (Ser48-Lys51 and Ser80-Lys263), the residues involved in zinc coordination (Cys135, Lys138, Cys189), and the relatively distant Cys249, were subjected to site-directed mutagenesis and substituted with Ala. All eight alanine mutants were studied using biophysical and structural methods. With the exception of the K138A mutant, all the created ReAV muteins lost the ability to hydrolyze L-asparagine, as clearly demonstrated by the Nessler method. This confirms the significance of the implicated residues in catalysis. The replacement of Ser48 and Ser80 by Ala affected the protein stability and folding, as indicated by CD spectra and low expression yields. We were able to crystallize mutants: S48A, K51A, S80A, C135A, K138A, C189A and K263A, and solve their X-ray crystal structures. The structures reveal some intriguing variations in the active site area. With alanine substitutions of Cys135, Lys138 and Cys189, the zinc coordination site fell apart and the mutants are unable to bind Zn. Moreover, the absence of the Zn2+ cation affected the oxidation state of Cys249, which no longer carried a chemical modification. The K51A and K263A mutations disrupted the network of H-bonds in the active site region and modified the hydration pattern of Ser48. Work supported by National Science Centre (NCN, Poland) grant 2020/37/B/NZ1/03250.

1. J.I.Loch et al. *Nature Commun*., **12,** (2021), 6717.