Crystal structures of the elusive *Rhizobium etli* L-asparaginase reveal a peculiar active site

Joanna I. Loch¹, Barbara Imiolczyk², Joanna Sliwiak², Anna Wantuch¹, Magdalena Bejger ², Miroslaw Gilski ²,³ & Mariusz Jaskolski ²,³

*Rhizobium etli*, a nitrogen-fixing bacterial symbiont of legume plants, encodes an essential L-asparaginase (ReAV) with no sequence homology to known enzymes with this activity. High-resolution crystal structures of ReAV show indeed a structurally distinct, dimeric enzyme, with some resemblance to glutaminases and β-lactamases. However, ReAV has no glutaminase or lactamase activity, and at pH 9 its allosteric asparaginase activity is relatively high, with $K_m$ for L-Asn at 4.2 mM and $k_{cat}$ of 438 s⁻¹. The active site of ReAV, deduced from structural comparisons and confirmed by mutagenesis experiments, contains a highly specific Zn²⁺ binding site without a catalytic role. The extensive active site includes residues with unusual chemical properties. There are two Ser-Lys tandems, all connected through a network of H-bonds to the Zn center, and three tightly bound water molecules near Ser48, which clearly indicate the catalytic nucleophile.

¹Department of Crystal Chemistry and Crystal Physics, Faculty of Chemistry, Jagiellonian University, Krakow, Poland. ²Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznan, Poland. ³Department of Crystallography, Faculty of Chemistry, A. Mickiewicz University, Poznan, Poland.

✉email: mariuszj@amu.edu.pl
Asparaginases, which split L-Asn to L-Asp and ammonia, are a diversified group of enzymes\(^1\). The serendipitous detection of strong antileukemic properties of guinea pig serum\(^2\) was attributed by Broom et al.\(^3\) to L-asparaginase activity, and indeed low (\(\mu\)M) \(K_m\) bacterial asparaginases (Class 1 in revised classification\(^4\)) of type \(\Pi\), notably \(E. coli\) EcAII, but not type I (e.g. EcAI with mM \(K_m\)) became potent drugs in the treatment of acute lymphoblastic leukemia (ALL)\(^5\). Later, it was realized that plants possess different, Ntn-type, asparaginases (some of which, e.g. in legumes, are \(K^+\) dependent\(^6\)) but close homologs of plant-type (or Class 2) asparaginases were also discovered in bacteria, e.g. EcAII\(^7,8\).

Experiments with \(Rhizobium\) \(etli\), a nitrogen fixing bacterial symbiont of legumes, revealed that it possesses two asparaginases, a constitutive and an inducible one\(^9\). A comprehensive phylogenetic analysis of I-asparaginase sequences\(^10\) predicted early on that the inducible \(R.\) \(etli\) enzyme is not related to any known Class 1 or Class 2 asparaginases. According to a recently proposed classification\(^11\) the \(R.\) \(etli\) enzymes are termed ReAV (constitutive) and ReAV (inducible) and belong to Class 3 asparaginases. An in silico model of ReAV predicted a protein fold with structural similarity to \(\beta\)-lactamases or transpeptidases\(^12\).

Because of serious side effects of bacterial asparaginase administration, other candidate antileukemics have been sought, so far without success, e.g. by mutagenetic optimization of natural enzymes\(^13\) or by considering plant-type mammalian proteins in this role\(^16-18\). Also, interest has been directed towards \(R.\) \(etli\) asparaginases, which could offer at least a possibility for optimization. The structure of these enzymes has been, however, recalcitrant to experimental elucidation; for example, in our laboratory futile crystallographic studies have taken almost 20 years. In this work we finally describe high resolution crystal structure of ReAV in several crystal forms and show that the enzyme belongs to a folding class that is more closely related to glutaminases or penicillin-binding proteins than to any of the known \(\alpha\)-asparaginase families. We report that ReAV is an allosteric asparaginase with high pH optimum and with \(K_m\) of 4.2 mM at pH 9, and that it is a metalloprotein with a puzzling active site.

**Results**

**Expression and biophysical characterization of WT ReAV and its mutants.** WT ReAV was expressed in \(E. coli\) in large amounts in soluble form (~100 mg/L of bacterial culture). The correctness of the WT ReAV sequence was confirmed by mass spectrometry. Based on analysis of the crystal structures of the WT protein, five ReAV mutants with substitutions in the putative active site area were generated (Supplementary Table 1). A similar expression yield was observed for mutant K51A, and a slightly lower yield for K263A. On the other hand, mutants S48A and S80A were produced mostly in insoluble form as inclusion bodies (Supplementary Fig. 1). Nevertheless, it was possible to recover from the cell lysate 1–5 mg of each variant for routine activity and stability tests.

Poor signal in the far-UV CD spectra indicated that variants S48A and S80A are incorrectly folded or carry a strong structural distortion (Supplementary Fig. 2). These observations were confirmed by nanoDSF experiments, as it was impossible to determine a reliable \(T_m\) for the S48A and S80A mutants due to poor fluorescence. \(T_m\) determined for WT ReAV is 51 °C (nanoDSF), in agreement with previous results\(^12,19\). The \(T_m\) was similar for variant K51A (50 °C), and slightly higher for K263A (52 °C) or lower for C135A (48.5 °C) (Supplementary Fig. 2). A series of DSC experiments performed for WT ReAV in buffers of different pH and NaCl content showed a two-step melting process at pH 6.5 and 7.5 and only one transition at pH 8.5. At pH 7.5 in the presence of 10 mM NaCl, the second peak was significantly reduced (Supplementary Fig. 3).

**Crystal forms of ReAV.** In the course of the crystallization experiments, a number of different crystal forms of WT ReAV were obtained, four of which are presented herein as follows (Supplementary Table 2): orthorhombic form OP (the highest resolution, 1.29 Å, of X-ray diffraction) with one ReAV dimer in the asymmetric unit (ASU) and tight packing of the molecules; monoclinic form MC with one dimer in ASU; and another monoclinic form with four protein chains (two dimers) in ASU, reported here as two variants, MP1 and MP2, differing in the hydration pattern of Ser48.

The orthorhombic (OP) and monoclinic MP1/MP2 forms show similar packing pattern with solvent content of 49.4–52.6%, whereas the monoclinic form MC, with a significantly different arrangement of the dimers in the unit cell, has 53.2% solvent content.

In all cases the electron density maps have excellent definition for almost the entire protein molecules, except short disordered regions at the N- and C-termini. Even a preliminary analysis of the crystal structures clearly suggested that the active site is located in the area with an accumulation of residues showing unusual behavior, centered around the hydrated Ser48 side chain, and accentuated by a nearby Zn\(^{2+}\) ion coordinated by Cys135, Lys138 and Cys189.

**Protein fold topology.** The ReAV protomer has an \(\alpha\)/\(\beta\) fold formed by two tightly packed domains. The larger, mostly \(\alpha\)-helical catalytic domain built of helices H2–H14, harbors the active site (Fig. 1). This domain also contains a small three-stranded \(\beta\)-sheet (strands B4–B6). The second (dimer stabilization) domain consists of a six-stranded antiparallel \(\beta\)-sheet (strands B1–B3 and B7–B9) located in the core of the molecule. This \(\beta\)-sheet also includes a very long and unique \(\beta\)-strand B2 involved together with helix H7 in dimer stabilization.

The oligomeric state and location of the active site. ReAV is a homodimer with approximate \(C_2\) symmetry (Fig. 2). This symmetry is preserved quite well, as illustrated by the ~180° rotation and Ca rmsd of ~0.5 Å between the subunits. The shape of the whole dimer resembles a basket, with the handle formed by the pair of helices H7 from both subunits. The bottom of the basket is stabilized by an extended network of H-bonds and hydrophobic interactions formed between the longest \(\beta\)-strand B2, strand B1 and the extended loop connecting helix H19 and strand B10 from one subunit, and the residues located in strands B4, B5 and B6 from the other subunit (Fig. 2).

There is a characteristic tunnel with ~20 Å diameter between the subunits, suggesting that ReAV might interact with other biomolecules. The tunnel leads to the two active sites (Fig. 3), each located in a cleft on the protomer surface. Calculations of the electrostatic potential revealed strong positive charge in the cleft area that would be excellent for attracting negatively charged ligands/substrates (Fig. 3d). This analysis clearly confirmed that the putative active site is located in the middle of the \(\alpha\)-helical domain, in the cleft surrounded by helices H2, H4, H9 and the loop connecting strands B5–B6, in agreement with the conclusion drawn from the chemical character of the residues in this region. This finding has been subsequently confirmed by site-directed mutagenesis in this area, coupled with enzymatic tests. In addition to the unusual arrangement of the putative catalytic residues, the active site cleft is occupied in its distal area by a zinc ion with a singular coordination sphere.
Metal binding site. The zinc ion was found near helix H9 and the loop connecting strands B5–B6, with a tetrahedral coordination sphere formed by Cys135, Lys138, Cys189 and a water molecule (w1) (Fig. 3a). The position of Lys138 is stabilized by two water-mediated H-bonds to the side chain of Gln54 (Fig. 3b). These water molecules (w7, w8) and the position of the Lys138 side chain are conserved in all analyzed ReAV structures. The position of Cys189 is nearly identical in all the structures and is additionally held in place by an H-bond to the side chain of His139. Cys135 interacts not only with the Zn ion but also with Lys51, and weakly with Asn134. The two Zn-S distances are almost identical (~2.30–2.31 Å). The distance between the water molecule w1 and Zn$^{2+}$ is in the range ~1.89–2.05 Å and the position of this water is the same in all structures. The coordination geometry together with an X-ray fluorescence scan and anomalous map, plus ITC titration results (vide infra), all unambiguously identify the metal cation as Zn$^{2+}$.

The catalytic center. Close to the Zn$^{2+}$ site, a system of four conspicuous residues, Ser48, Ser80, Lys51 and Lys263, is located. In the close vicinity of the Ser48 hydroxyl, there is a characteristic, almost tetrahedral pattern of three closely-spaced water molecules w2, w3, w4 (Fig. 3e), visible in all crystal structures, except MP2.
These waters must have extremely strong H-bonds with the O\text{y} atom of Ser48, as indicated by the O–O distances, which in the highest-resolution structure OP refined to 2.2–2.5 Å. The water molecules are also tightly H-bonded to each other. Water w\text{3} is also H-bonded to w1 coordinated by the Zn\textsuperscript{2+} cation (Fig. 3f).

In all structures, regardless of the hydration state of Ser48, the side chain of Ser48 is also H-bonded to the N\text{c} atom of Lys51 and O\text{y} of Ser80. On the other hand, Ser48 is not always hydrated (structure MP2) or the hydration may be incomplete and even different in the same crystal structure (triclinic crystals, not included in this study). In structure MP2, the side chain of Ser48 has two conformations: one as in the hydrated state but with H-bonds to w1, Lys51, Ser80 and another water molecule (different from w2/w3/w4) (Fig. 3g, Supplementary Fig. 4). The second conformer of Ser48 is rotated ~110° and H-bonded to the N atom of Ala266 and carbonyl oxygen of Leu264. Except for the absence of the w2-w3-w4 water triad, no other structural rearrangements are observed in the vicinity of Ser48. The different hydration state might be the effect of differences in crystal vitrification prior to X-ray data collection. Nevertheless, the structural data indicate that Ser48 is not always hydrated.

Ser48 is H-bonded to Ser80, which is also important for catalysis but even more important for protein stability as it is the kingpin of a network of H-bonds that lead to Lys51 (N\text{c}), Lys263 (N\text{c}), Ans134 (O\text{b}1) and w4. This network stabilizes the position of Ser48 and the water triad w2-w3-w4. (Fig. 3g, Supplementary Fig. 4). The potential origin/role of the Cys249 modification is unclear, but without it the Cys249-w5-Lys263-Ser80-Lys51-Cys135-Zn network cannot be built. In the MS experiments Cys249 was very reactive and was mapped in the MS spectrum as carbamido-methyl cysteine as a result of reaction with iodoacetamide, which is commonly used to prepare MS samples.

Enzymatic activity of WT ReAV and its active-site mutants. In qualitative assays of L-asparaginase activity, WT ReAV was most active at alkaline pH 7.5 but it was also active at alkaline pH 10–12 (Supplementary Fig. 5), in agreement with the pH optimum of 9–11 reported previously\textsuperscript{20}. Our quantitative ITC measurements yielded the following kinetic parameters $K_m$, $k_{cat}$ and $k_{cat}/K_m$: 15.8 ± 1.5 mM, 278 ± 23 s\textsuperscript{-1} and 18 ± 3 mM\textsuperscript{-1} s\textsuperscript{-1} at pH 7.5; 6.9 ± 0.5 mM, 441 ± 32 s\textsuperscript{-1} and 44 ± 9 mM\textsuperscript{-1} s\textsuperscript{-1} at pH 8.0; and 4.2 ± 0.3 mM, 438 ± 32 s\textsuperscript{-1} and 104 ± 15 mM\textsuperscript{-1} s\textsuperscript{-1} at pH 9.0 (Fig. 4). Fitting the titration data with the Hill equation showed that the Hill coefficient is different than 1 and equal to 1.4 ± 0.1 (at pH 7.5 and 8.0, both with and without Zn\textsuperscript{2+} cations) or to 1.6 ± 0.1 (at pH 9.0).
The same experiments performed with L-Glu or ampicillin showed no heat effect, indicating that these compounds are not substrates of ReAV. Likewise, spectroscopic measurements did not detect any reaction with the CENTA (a chromogenic cephalosporin) β-lactam antibiotic.

Since the crystal structures revealed that ReAV is a zinc metalloprotein, we determined the kinetic parameters (in 30 mM phosphate buffer pH 8.0) of the enzyme deprived of Zn²⁺ ions (by TPEN treatment) in order to probe the role of Zn²⁺ ions in the l-asparaginase activity. The results are: $K_m = 6.5 \pm 0.6 \text{mM}$, $k_{cat} = 432 \pm 7 \text{s}^{-1}$ and $k_{cat}/K_m = 66 \pm 7 \text{mM}^{-1} \text{s}^{-1}$. We also determined the influence of high concentration of zinc ions by comparing the rate of asparagine conversion without Zn²⁺ and with controlled Zn²⁺ presence at 100 µM, which is about the maximum Zn²⁺ concentration achievable in the bacterial cell. In the case of ReAV deprived of zinc this rate was determined as 1.1 ± 0.1 µM s⁻¹ of substrate depletion under the conditions of our experiment. This rate was lowered by 29% in the presence of 100 µM Zn²⁺ ions.

For the five residues implicated by the crystal structures as belonging in the active site area, alanine site directed mutants (S48A, K51A, S80A, C135A and K263A) were generated. Although at room temperature all variants retained the dimeric structure of the WT protein, as indicated by gel filtration (Supplementary Fig. 2), the Nessler test clearly showed that all the mutants were unable to hydrolyze l-Asn (Fig. 5).

Microcalorimetric measurements of divalent cation binding by ReAV. Titration of ReAV pre-treated with a strong chelating agent (TPEN), using different divalent metal cations (Zn²⁺, Mg²⁺, Ni²⁺, Mn²⁺, Cd²⁺) revealed that only interaction with Zn²⁺ produces a heat effect (Fig. 6), with the following binding parameters: $N = 1.0 \pm 0.1$, $K_d = 2.7 \pm 0.9 \text{µM}$, $\Delta H = -4562 \pm 200 \text{cal/mol}$, $\Delta S = 10.2 \pm 0.6 \text{cal/mol/deg}$. This indicates a strong and specific binding of the zinc ion by ReAV.

Structural homologs of ReAV. The crystal structures of ReAV reveal a unique architecture, with only distant similarity to known protein folds. A Dali22 search of the PDB found the closest structural homologs among bacterial enzymes (rmsd 1.5–2.2 Å), mostly glutaminases (Fig. 7), e.g., from Bacillus subtilis (1mki), E. coli (1u60), or Micrococcus luteus (3i55) (Supplementary Fig. 6), but also among human or mouse mitochondrial liver-type (4bqm) or kidney-type (4jkt) glutaminases 23. A more distant similarity (rmsd 2.8–3.1 Å) was detected to various proteins involved in bacterial cell wall biosynthesis or with the ability to bind antibiotics, e.g., penicillin binding protein (PBP) from E. coli (1nzo), Acinetobacter baumannii (3u1e), Staphylococcus aureus (6c39), Listeria monocytogenes (5sxz) or Neisseria gonorrhoeae (6hjz, 6p54) (Supplementary Figure 6). Additional homologs (rmsd 2.4–3.2 Å) were found among serine β-lactamases, e.g., from E. coli (3qnc, 6skp, 1tem)31,32, Klebsiella pneumoniae (6vlo)33, Pseudomonas aeruginosa (5eua)34 or Streptomyces clavuligerus (2xgn)35, and peptidoglycan glycosyltransferase from Atopobium parvulum (4r1g, 4jbf)36 (Supplementary Fig. 6). Sequence similarity between ReAV and the above homologs is low and varies from random to 29% (for E.coli glutaminase, 1u60).

Discussion

Legumes have developed symbiotic relationship with soil bacteria, such as R. etli, which fix atmospheric nitrogen in their roots. In this system, the plant supplies the bacteria with carbon sources, i.e., R. etli supplies ammonia, l-Asp and l-Ala37. In Rhizobiales, genes essential for the symbiotic lifestyle are located not only on the chromosome but also on plasmids38. In particular, plasmid p42e contains the asnRPAB operon19 encoding proteins involved in the degradation of l-asparagine: l-asparaginase (AnsB) and thermolabile (inducible) l-asparaginase ReAV (AnsA)39. The gene encoding ReAV might have arisen by horizontal gene transfer from Eukarya, especially Ascomycetes.
fungi\textsuperscript{20}. As another curiosity, \textit{R. etli} does not encode any proteins that could be identified with the well-established Classes (1 and 2) of asparaginases found in other organisms.

Our kinetic experiments showed that ReAV hydrolyzes \textit{l}-Asn with a Michaelis constant of $15.8 \pm 1.5$ mM and $k_{\text{cat}} = 278 \pm 25$ s$^{-1}$ even at pH 7.5, i.e. away from its high pH optimum of 10–12. These results are in agreement within the order of magnitude with the values reported previously for the same assay conditions but using a different method\textsuperscript{20} ($K_m = 8.9$ mM, $k_{\text{cat}} = 106$ s$^{-1}$). At pH 9.0 the enzyme is much more active, with $K_m = 4.2 \pm 0.3$ mM and $k_{\text{cat}} = 438 \pm 32$ s$^{-1}$. Apart from the quite different Berthelot assay, which differs from the calorimetric method not only in the measured signal (absorbance vs heat) but also in its mechanics (premixed reaction solutions vs fast titration with constant stirring), the previous study used a different protein construct, with a long (7.5 kDa, 20\% of protein mass) artificial sequence (His-tag + antibody epitope + protease recognition sequence) attached at the N terminus. However, in terms of enzyme efficiency, the two measurements are very similar ($\sim 18$ mM$^{-1}$ s$^{-1}$ (this study) vs $\sim 12$ mM$^{-1}$ s$^{-1}$ (Moreno et al.)\textsuperscript{20}. To make the two studies comparable, our kinetic curves, with clearly hyperbolic shape (Fig. 4), were fitted to the Michaelis-Menten equation. An additional fitting to the Hill equation yielded a Hill coefficient $n_h > 1$, indicating that ReAV exhibits positive allosterism, and this effect was stronger at higher pH ($n_h \sim 1.4 \pm 0.1$ at pH 7.5 and 8.0 pH vs $\sim 1.6$ at pH 9.0). Moreover, zinc-free protein had the same Hill coefficient, indicating that zinc is not the allosteric modulator of ReAV. Examples of allosteric asparaginases belonging to Class 1 were reported before, e.g. \textit{l}-asparaginase 1 from \textit{Saccharomyces cerevisiae}\textsuperscript{40}. EcAI and human \textit{l}-asparaginase \textsuperscript{41}. However, those enzymes exhibited much stronger positive cooperativity and a sigmoidal substrate dependence curve, with Hill coefficients 2.2, 3.5 and 3.9\textsuperscript{11}, respectively. Moreover, they did not follow the Michaelis-Menten kinetics at all. It was established that the substrate itself is the allosteric effector of those enzymes\textsuperscript{41}.

Our ITC experiments also established that there is no heat effect on interaction with \textit{l}-Gln, indicating that ReAV is most likely free of \textit{l}-glutaminase activity. This result is in agreement with earlier observations\textsuperscript{20,42}. We couldn’t use the simple Nessler test for this reaction because even the highest grade commercial \textit{l}-Gln (supplied by Sigma-Aldrich/Merck or BioShop) contains impurities that give positive Nessler effect even without any enzyme\textsuperscript{43}. L-DON, a typical \textit{l}-glutaminase inhibitor, did not inhibit the ReAV activity, which indicates that it is not able to bind in the ReAV active site.

All the crystal structures reveal the presence of a zinc ion in the active site area. ReAV affinity for Zn was measured by ITC titration with Zn$^{2+}$ after removal of all metals with a strong chelating agent (TPEN). The results showed that Zn$^{2+}$ binding is
strong ($K_d \approx 2.7 \mu M$) and highly selective, as similar divalent cations (Mg$^{2+}$, Ni$^{2+}$, Mn$^{2+}$, Cd$^{2+}$) were not bound by the protein. The kinetic parameters of ReAV treated with TREN (Km = $6.5 \pm 0.6 \mu M$; $k_{cat} = 432 \pm 7 \, s^{-1}$) are very similar to those of the native protein, suggesting that zinc does not play a catalytic role in l-Asn hydrolysis. Comparison of l-Asn conversion rates by protein devoid of zinc and supplied with maximum physiological concentration of Zn$^{2+}$ (100 \mu M) in Tris buffer revealed 29% lower activity in the latter case, in general agreement with the results of Moreno et al.\textsuperscript{30}. However, we note that Moreno et al. used 1 mM Zn$^{2+}$ in alkaline phosphate buffer, where Zn$_2$(PO$_4$)$_2$·4H$_2$O precipitation occurs\textsuperscript{34}, and protein construct with a metal-chelating His-tag. Thus, the exact cause of the reported effect was not obvious.

Our enzymatic assays included several ReAV mutants carrying substitutions in the active site area, designed on the basis of our structural analyses. The results clearly show (Fig. 5) that all variants were unable to hydrolyze l-Asn, suggesting that the two serine residues, Ser48 and Ser80, as well as the accompanying Lys51 and Lys263, are crucial for catalysis. Similar lack of activity was observed for the C135A substitution, which would affect the zinc coordination (Fig. 3). As the ITC experiments showed that Zn$^{2+}$ is not necessary for catalysis, it is reasonable to assume that Cys135 and the Zn binding site may be important for protein folding and stability.

The recently proposed classification\textsuperscript{1,4} divides l-asparaginases into three classes: bacterial-type (Class 1), plant-type (Class 2) and R. etli-type (Class 3)\textsuperscript{1,13}. The homology of structure and sequence within the first two classes is high but there is no similarity across the classes. The sequence of ReAV that has been available so far, clearly indicated that R. etli defines yet another, completely different class of l-asparaginases, with sequence identity to Class 1 and 2 enzymes at noise level (~15%). In this completely different class of L-asparaginases, with sequence available so far, clearly indicated that αβ belong to different protein families, viz. glutaminases, PBPs and -lactamases. This conjecture is supported by the observation that the structural homologs of ReAV found in the PDB by DALI have no structural homology to any known l-asparaginase (or glutaminase-asparaginase) or lactamases. Nevertheless, a UniProt search revealed that a lot of sequence homologs of ReAV (with 35-89% sequence identity) appear in proteobacteria, terrabacteria, and in filamentous ascomycetes fungi.

The structural homologs of ReAV found in the PDB by DALI belong to different protein families, viz. glutaminases, PBPs and -lactamases. Superposition of ReAV on these PDB models revealed relatively high structural similarity in the α-helical domain for helices H2, H4, H9, H10, H12, H13 and H17, with most significant differences seen in the region of helix H1 (and the N terminus), H7, H18 and H19 (Fig. 7). A degree of similarity is also visible at the β-strands B2, B3, B8, B7 and B9; however, in the superposed homologs the β-strand corresponding to B2 is shorter and strands B1 and B10 are absent altogether (Fig. 7). Some structural homologs of ReAV have evolved additional domains, thus a degree of structure similarity is visible only for the catalytic domain but not for the peripheral motifs that are absent in ReAV (Supplementary Fig. 6).

Interestingly, the ReAV dimerization motif formed by the β-strands B2, B1, B10, is replaced in many of the analyzed glutaminases, -lactamases and PBPs by a long α-helix (Supplementary Fig. 7). However, this α-helix is not involved in dimer formation of these proteins. Not all proteins listed above are dimeric. The -lactamases from E. coli\textsuperscript{31} and K. pneumoniae\textsuperscript{42} are dimeric, but they dimerize utilizing short antiparallel β-strands originating from the protein core (Supplementary Fig. 7). The elements corresponding to helix H7 and strands B1, B2 and B10, that are responsible for ReAV dimerization, are absent in those -lactamases.

These observations suggests that ReAV might have common evolutionary ancestors with bacterial glutaminases, lactamases or PBPs. This conjecture is supported by the observation that the putative active site of ReAV corresponds quite well to the active sites of these structural homologs, albeit only in the part encompassing Ser48, Ser80, Lys51 and Lys263 (Fig. 8). In most of the structural homologs, the active site is located in a region of the protein core corresponding to the ends of helices H2, H4, H9, H13 and β-strand B7 (Fig. 3c). In these structures, the catalytic Ser maps to Ser48 of ReAV and the counterparts of Ser80, Lys51 and Lys263 are also preserved. However, in some of the homologs the hydroxyl group corresponding to Ser80 is provided by a Tyr residue (Fig. 8).

These observations strongly suggest that the catalytic nucleophile of ReAV is Ser48. In most of the structures presented in this study, Ser48 is tightly surrounded by three water molecules, w2-w3-w4, in a nearly tetrahedral arrangement and with excellent definition in the electron density maps (Fig. 3e). A similar triad was also found in the crystal structure of B. subtilis glutaminase (1mki). However, it was interpreted in that structure as a phosphate group covalently linked to the putative nucleophilic serine, despite the fact that MS experiments performed by the authors did not confirm phosphorylation\textsuperscript{23}. Likewise, MS experiments carried out for ReAV did not reveal any phosphorylation, or any covalent modification of Ser48 for that matter. Consequently, the three electron density peaks surrounding the Oy atom of Ser48 in ReAV are interpreted as water molecules.

The presence in ReAV of two serine-lysine dyads, Ser48-Lys51 and Ser80-Lys263, resembles the arrangement of the catalytic residues in the model -lactamases and other ReAV homologs (Fig. 8). Their catalytic mechanism was studied in detail\textsuperscript{46}, revealing that the counterpart of Ser48 acts as the nucleophile, while the assisting lysine (Lys51 in ReAV) polarizes the hydroxyl...
group of the nucleophile, and also activates a water molecule in the second step of the hydrolytic reaction. The second Ser-Lys pair, corresponding to Ser80-Lys263 of ReAV, is involved in substrate binding. It was concluded that for its role in catalysis, the counterpart of Lys51 must be unprotonated, i.e., in free-base form. It was also found that both lysines contribute to the high pH optimum (9.5–10.5) for PBPs catalysis. This observation is in excellent agreement with our findings that ReAV is most active at alkaline pH.

We can conclude that in ReAV all four residues, Ser48, Lys51, Ser80 and Lys263, are also important for L-Asn hydrolysis, as their mutations rendered the enzyme inactive. In this view of the reaction, Ser48 launches a nucleophilic attack on the amide group of the substrate, leading to an acyl-enzyme intermediate and NH₃ release. In the second step of the reaction, the ester intermediate is hydrolyzed by an activated water molecule. For the first step of the reaction, the Ser48 nucleophile is strongly polarized by Lys51. The attraction of the three water molecules w2-w3-w4 by the Ser48 side chain is a clear evidence of this polarization. In the absence of a substrate, the arrangement of w2-w3-w4 might mimic the tetrahedral transition state of the catalytic event (Supplementary Fig. 8). However, in our numerous collection of ReAV structures, we also see cases with incomplete or absent (MP2) water triad. The corresponding crystals were grown at different pH, detergent and salt conditions, which would certainly affect the protonation state of the key lysine residues and, therefore, polarization of Ser48. It can be hypothesized that in the crystal structure with no w2-w3-w4 triad and with double conformation of Ser48 (MP2), the hydroxyl group of Ser48 is not polarized and this structure shows the enzyme in its resting state, between catalytic cycles.

ReAV has yet another characteristic residue, namely Cys249 with an oxidative modification at the ε atom, located ~5 Å from the putative nucleophilic Ser48. The physiological function of this cysteine is unclear; however, symbiotic Rhizobium species have a very intensive metabolism, generating high amounts of reactive oxygen species (ROS). Oxidative modifications of cysteine side chains play an important protective role against ROS. In ReAV, Cys249 may be a “suicide” quencher, protecting the sensitive zinc binding site from oxidative damage. Cys249 is not only very reactive, but is also involved in a network of H-bonds that extends to the Ser-Lys dyads and to Cys135 in the Zn coordination sphere. Cys249 does not have a counterpart in the structural homologs of ReAV.

Except ReAV, there are no other L-asparaginases with a metal ion right in the catalytic center. Previously, zinc binding sites were also found in the E. coli enzyme EcAII, however, the metal ions were located distinctly away from the active site. It must be emphasized that none of the analyzed structural homologs of ReAV contains a metal ion near the active site, close to the Ser-Lys pairs. The presence of Zn²⁺ very close to the two Ser-Lys pairs, and coordinated by an additional lys residue, is a highly unusual feature, uniquely characteristic of ReAV. Interestingly, in the structurally homologous PPBs, lactamases and glutaminases, the space occupied by the Zn²⁺ cation in ReAV is preserved, but is usually filled with a Tyr or Glu side chain (Fig. 8) that is often involved in catalysis.

The Zn²⁺ ion in the active site of ReAV has an unusual coordination sphere formed by two cysteines, a lysine, and a water molecule (Fig. 3). Our ITC experiments revealed that Zn binding is highly selective and specific. On the other hand, our kinetic data show that zinc is not necessary for L-Asn hydrolysis. The enzyme kinetic data contradict, therefore, the conclusion about the role of the Zn²⁺ cation that might be drawn from its own coordination sphere. We cannot exclude, of course, that ReAV might also have other catalytic properties and act as an enzyme with dual (or even multiple) activity and that for the other activity zinc might be necessary. This speculation, however, would need to be based on additional work (and even serendipity) to be verified. To date, we have only checked the potential β-lactamase and glutaminase activities and achieved negative results.

Finally, R. etli is also interesting as an evolutionary curiosity. How did it lose the “standard” asparaginase found in other bacteria? And how did it acquire asparaginase activity in an entirely unrelated protein? Is it possible that a serine β-lactamase (not to be confused with zinc metallo-β-lactamase) was hijacked as a scaffold for an asparaginase in R. etli (or its ancestor) by blocking the original activity by Zn coordination? In this speculative scenario Zn acts as an inhibitor by sterically blocking the original active site. Is ReAV a freak of nature or can similar enzymes be found in other groups of organisms? The questions can go on as this is only the beginning of a fascinating research project.

Methods

Cloning, mutagenesis, expression, and purification. The gene encoding inducible asparaginase (strain CFN 42) wild type, WT) was obtained from Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH, and cloned to pET151-D-TOPO expression vector (Invitrogen) carrying a TEV-cleavable N-terminal His-tag. Protein production in the E. coli strain BL21 STAR (DE3) (Invitrogen) was induced with 0.5 mM IPTG. Cells were grown overnight at 16 °C and the expression level was checked by SDS-PAGE. Protein was re-suspended in 100 mM Tris buffer pH 8.0, containing 500 mM NaCl and 10 mM imidazole. Cells were disrupted by sonication and centrifuged cell lysate was loaded on a chromatography column filled with HisTrap Ni resin (GE Healthcare). ReAV was eluted from the column using imidazole gradient (to 500 mM). Fractions containing ReAV were pooled and dialyzed overnight in the presence of TEV protease at 4 °C against 100 mM Tris buffer pH 8.0, containing 100 mM NaCl and 5 mM TCEP. His-tag debris (including His-tagged TEV protease) was removed by another HisTrap Ni column. Finally, the protein was loaded on HiLoad 16/600 Superdex 200 (GE Healthcare) gel filtration column and eluted using 100 mM Tris pH 8.0, with 100 mM NaCl.

Protein purity and homogeneity was checked by SDS-PAGE and DLS. L-Asparaginase activity was routinely monitored colorimetrically at 420 nm by the Nessler method using 10 mM L-Asn (in 20 mM Tris buffer pH 8.0) as substrate and commercially available K₃[Fe(CN)₆] (Chempur). The same method was used to find the optimum pH of the enzymatic reaction, but with Tris buffer replaced by a series of 25 mM Britton-Robinson buffers covering pH 2–13. The pH activity profile was additionally confirmed by Berthelot reaction.

Protein identity was confirmed by MS as follows. Protein was reduced with NHHCO₃ and DTT at 95 °C; alklylation was carried out with iodoacetamide. Protein was digested overnight at 37 °C with sequencing-grade trypsin (Promega). Peptide solutions were deposited on a MALDI AnchorChip plate (Bruker Daltonics GmbH) with saturated solution of α-cyano-4-hydroxydynamic acid in 50% ACN and 0.1% TFA (matrix). Spectra were acquired with a minimum of 2500 laser shots in a MALDI TOF/TOF UltraFlx mass spectrometer (Bruker Daltonics GmbH). The MS/MS data of combined peptide mass fingerprints were used for searching UniProt database installed on Mascot server (Matrix Science, London, UK). Site-directed mutagenesis of ReAV was carried out according to Quik-site mutagenesis protocol (New England Biolabs) using site-specific non-overlapping mutagenic primers (Supplementary Table 1) to introduce substitutions into the following positions: S48A, S80A, K51A, K263A, and C315A. Presence of mutations was confirmed by sequencing (Genomed S.A., Poland). Mutants were produced and purified as described for WT protein. Their activity was checked by the same method using the same protocol as for WT ReAV.

Folding, monitoring of oligomeric state and thermal stability. The correct folding and thermal stability were routinely checked for the WT and mutant proteins by circular dichroism (CD) and nano-scale differential scanning fluorimetry (nanoDSF). Prior to the measurements, protein samples were applied to analytical Superdex75 10/300 GL column (GE Healthcare) and were eluted using 50 mM phosphate buffer pH 7.5 with 20 mM NaCl. These experiments were also used to assess the oligomeric state of the proteins. Far-UV CD spectra were recorded using a Jasco J-815 spectropolarimeter. Protein concentration was kept in 50 mM phosphate buffer pH 7.5 with 20 mM NaCl and 0.1% TFA (matrix). Spectra were acquired with a minimum of 2500 laser shots in a MALDI TOF/TOF UltraFlx mass spectrometer (Bruker Daltonics GmbH). The MS/MS data of combined peptide mass fingerprints were used for searching UniProt database installed on Mascot server (Matrix Science, London, UK).

Site-directed mutagenesis of ReAV was carried out according to Quik-site mutagenesis protocol (New England Biolabs) using site-specific non-overlapping mutagenic primers (Supplementary Table 1) to introduce substitutions into the following positions: S48A, S80A, K51A, K263A, and C315A. Presence of mutations was confirmed by sequencing (Genomed S.A., Poland). Mutants were produced and purified as described for WT protein. Their activity was checked by the same method using the same protocol as for WT ReAV.
recorded as fluorescence emission for protein samples subjected to a temperature ramp of 60 °C/hr from 20 to 95 °C.

Detailed HT ReAV melting profile at different pH and ionic strength was monitored by differential scanning calorimetry (DSC) using a MicroCal PEAQ-DSC system (Malvern Instruments Ltd.). Each DSC experiment consisted of two measurements, both at the same conditions: (i) five reference scans with buffer-filled cells to establish instrument thermal history and to achieve near perfect baseline repeatability; (ii) one sample-buffer scan to acquire data for analysis. The best experiment conditions were: temperature range 25–70 °C, scan rate 15 °C/hr, and low feedback mode. Protein concentration varied between 100 and 500 µM. Before the DSC experiments protein was dialyzed against specified buffers: 50 mM Tris of pH 6.5, 7.5, 8.5, or 50 mM Tris pH 7.5 containing 10 mM NaCl. All buffers contained 3 mM TCEP.

Microcalorimetric assessment of divalent metal cation binding. In order to remove any bound Zn2+ prior to ITC titrations the WT protein was incubated for 2 h on ice with 2-fold excess of TPEN (N,N,N',N'-tetraakis(2-pyridylmethyl)-1,2-ethanediamine) dissolved in 96% ethanol. After incubation, TPEN was removed by dialysis and gel filtration on a HiLoad 16/60 Superdex 200 (GE Healthcare) column (50 mM Tris pH 8.0, 100 mM NaCl, 1 mM TCEP).

Titrations were performed at 25 °C using a MicroCal iTC200 calorimeter (GE Healthcare). The protein in the cell at ~100 µM concentration (determined at 280 nm) was titrated with 2 µl aliquots of 1 mM XCl2 salts (X = Mn, Mg, Ni, Mn, Cd). Both, metal and protein were in 25 mM Tris buffer pH 8.0, 300 mM NaCl and 1 mM TCEP. The raw ITC data were analyzed with the Origin 7.0 software (OriginLab) to obtain the thermodynamic parameters of the complexation reactions: stoichiometry (N), dissociation constant (Kd), as well as changes in enthalpy (ΔH) and entropy (ΔS). The measurements were performed in duplicate.

X-ray data collection, structure solution and refinement. Structural analysis. X-ray diffraction data were collected using synchrotron radiation from beamlines at Petra III EMBL/DESY in Hamburg or BESSYII in Berlin. Initially, the diffraction data were collected at EMBL beamline P13 in the Petra III storage ring. Since in the 367-residue amino acid sequence of ReAV there are as many as 21 Cys/Met residues, long-wavelength (λ = 0.664 Å, 6.0 keV) anomalous X-ray diffraction data were collected for S-SAD phasing. The data were measured at seven positions of a rod-shaped crystal with approximate dimensions 350 × 200 × 150 µm. At each position, 3600 frames of 0.1° oscillation and 40 ms exposure were collected. The anomalous data (as well as all subsequent X-ray diffraction images) were indexed and integrated with XDS and further processed with XSCE27. Out of the seven anomalous data sets, the best four were selected, scaled and merged together to serve as an experimental S-SAD data set (START). A high-resolution native data set (1.29 Å, OP) was collected at λ = 0.9762 Å for the same crystal.

The data were input to SHELEXC and SHELXD58 for sulfur-substructure determination using the HKL2MAP interface59. An iterative density modification and main-chain auto-building as implemented in SHELEXC was used to build the initial map. ARP/WARP60 was used to build the first model (START), with ~95% docked residues and with the initial refinement R value of ~35%. The model was manually rebuilt with Coot61 and refined using REFMACS62. After structure solution, a high electron density peak was found close to Ser48, suggesting coordination of a metal cation. The peak height and tetrahedral coordination were consistent with Zn2+. The metal identity was confirmed by an X-ray fluorescence scan in the range of Zn absorption edge (Supplementary Fig. 9). Since no zinc was used in the crystallization conditions, the metal must have been bound by the protein during expression/purification.

The consequent structures discussed here were solved by molecular replacement using Phaser63. Structures were refined with REFMACS using anomalous signal (OP) or TLS (START, MP1, MP2, MC) protocols. The electron density maps were inspected in Coot. The statistics of data collection and structure refinement are summarized in Supplementary Table 2.

The structures were analyzed and visualized using PyMOL64, DALI65 and PISA66. The electrostatic potential distribution was calculated using Adaptive Poisson–Boltzmann Solver (APBS) algorithm67–68, as implemented in the PyMOL Plugin. Due to low sequence similarity, superpositions of ReAV and its structural homologs identified by DALI were calculated using the Secondary Structure Matching tool69 from the cp4d package70. Sequence similarity between ReAV and selected structural homologs was assessed by pairwise sequence alignments in EMBOSS Needle71. Additional software used in this work is listed in Supplementary References.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Atomic coordinates and structure factors corresponding to the final crystallographic models of ReAV generated in this study have been deposited in the Protein Data Bank (PDB) under accession codes 7om3 (START), 7om4 (OP), 7om5 (MP1), 7om6 (MP2), and 7om7 (MC). The corresponding low-resolution electron microscopy images and the Macromolecular X-raylography Raw Data Repository (https://mxdrx.icm.edu.pl) under DOI numbers: 10.18150/74YTYQ (START), 10.18150/MUKYHI (OP), 10.18150/XBX6JE (MP1), 10.18150/VQIQI9Q (MP2), and 10.18150/6W9HGI (MC). Source data are provided with this paper.

Received: 29 June 2021; Accepted: 1 November 2021; Published online: 18 November 2021

References

1. Loch, J. I. & Jaskolski, M. Structural aspects of L-asparaginases - a growing family with amazing diversities. IUCrJ 8, 514–521 (2021).
2. Kidd, J. G. Regression of transplanted lymphomas induced in vivo by means of a transplanted lymphoma extract. J. Exp. Med. 88, 565–582 (1953).
3. Broome, J. D. Evidence that the L-asparaginase activity of guinea pig serum is responsible for its antilymphoma effects. Nature 191, 1114–1115 (1961).
4. da Silva, L. S., Doonan, L. B., Pessoa, A., de Oliveira, M. A. & Long, P. F. Structural and functional diversity of asparaginases: overview and recommendations for a revised nomenclature. Biotechnol. Appl. Biochem. 1–11 (2021).
5. Derst, C., Henselg, J. & Röhm, K.-H. Engineering the substrate specificity of Escherichia coli asparaginase II. Selective reduction of glutaminase activity by amino acid replacements at position 248. *Protein Sci.* 9, 2009–2017 (2000).

6. Yun, M. K., Nourse, A., White, S. W., Rock, C. O. & Heath, R. J. Crystal structure and allosteric regulation of the cytoplasmic Escherichia coli L-asparaginase I. *J. Mol. Biol.* 369, 794–811 (2007).

7. Moharir, M. V., Vongpitakkul, S. S. & Wang, E. S. L. The structure and function of D- and L-asparaginase from Kluyveromyces lactis. *Biochemistry* 37, 9908–9918 (1998).

8. Bejger, M. A. & Oue, M. et al. N-acetylglutamyl-tRNA synthetase from Pseudomonas aeruginosa is a novel member of the ancient class of tRNA synthetases. *J. Biol. Chem.* 278, 28126–28133 (2003).

9. Borek, D. & Jaskolski, M. Crystal structure of D-asparaginase from Vibrio cholerae reveals conservation of structure and allosteric regulation of the cytoplasmic Escherichia coli D-asparaginase. *Acta Cryst.* D70, 1854–1872 (2014).

10. Ajewole, E., Santamaria-Kisiel, L., Pajak, A., Jaskolski, M. & Marsolais, F. Structural basis of potassium activation in plant asparaginases. *FEBS J.* 285, 1528–1539 (2018).

11. Michalka, K., Hernandez-Santoyo, A. & Jaskolski, M. The mechanism of autocalcification activation of plant-type L-asparaginases. *J. Biol. Chem.* 283, 13388–13397 (2008).

12. Borek, D. & Jaskolski, M. Crystallization and preliminary crystallographic studies of a new L-asparaginase encoded by the Escherichia coli genome. *Acta Cryst.* D56, 1505–1507 (2000).

13. Huerta-Saquero, A., Evangelista-Martínez, Z., Moreno-Enríquez, A. & Perez-Rueda, E. Rhizobium etli L-asparaginase II: an alternative for acute lymphoblastic leukemia in children and adolescents: a systematic review. *Hematol. Transfus. Cell Ther.* 42, 54–61 (2020).

14. Jeong, J. H., Cha, H. J. & Kim, Y. G. Crystal structures of penicillin-binding protein D2 from listeria monocytogenes and structural basis for antibiotic specificity. *Antimicrob. Agents Chemother.* 62, e00796–18 (2018).

15. Bellini, D., Koekemoer, L., Newman, H. & Dowson, C. G. Novel and improved crystal structures reveal multiple conformational changes and a new penicillin-binding protein structure of *Neisseria gonorrhoeae* PBPs1 and P2. *J. Mol. Biol.* 431, 3501–3519 (2019).

16. De Luca, F. et al. Evolution to carbapenem-hydrolyzing activity in noncarbapenemase class D β-lactamase OXA-10 by rational protein design. *Proc. Natl Acad. Sci. USA* 115, 13824–13829 (2018).

17. Mave rash, L. et al. Crystal structure of 6a-(hydroxymethyl)penicillanic acid complexed with the TEM-1 β-lactamase from Escherichia coli: Evidence on the mechanism of action of a novel inhibitor designed by a computer-aided process. *J. Am. Chem. Soc.* 118, 7435–7440 (1996).

18. Vologod, K. et al. Structural and mechanistic studies of the orf21 gene product from the clavulanic acid biosynthesis pathway. *Acta Cryst.* D69, 1567–1579 (2013).

19. Lee, D., De Beer, T. A. P., Laskowski, R. A., Thornton, J. M. & Orengo, C. A. 1,000 structures and more from the MCGS. *BMC Struct. Biol.* 11, 2 (2011).

20. Resende-Antonio, O., Reed, J. L., Encarnacíon, S., Collado-Vides, J. & Palsson, B. Metabolic reconstruction and modeling of nitrogen fixation in Rhizobium etli. *PLoS Comput. Biol.* 3, 1887–1895 (2007).

21. González, V. et al. The mosaic structure of the symbiotic plasmid of Rhizobium etli CFE414 and its relation to other symbiotic genome compartments. *Genome Biol.* 4, R36 (2003).

22. Landeta, C. et al. Plants with a chromosome-like role in rhizobia. *J. Bacteriol.* 193, 1317–1326 (2011).

23. Costa, I. M. et al. Recombinant L-asparaginase 1 from Saccharomyces cerevisiae: an allosteric enzyme with antiangiogenic activity. *Sci. Rep.* 6, 1–11 (2016).

24. Karamitros, C. S. & Konrad, M. Human 60-kDa lysophospholipase contains an N-terminal L-asparaginase domain that is allosterically regulated by L-asparaginase. *J. Biol. Chem.* 289, 12963–12975 (2014).

25. Huerta-Zepeda, A., Durán, S., Du Pont, G. & Calderón, J. Asparagine degradation in Rhizobium etli. *Microbiology* 142, 1071–1076 (1996).

26. Wootton, J. C., Kavanagh, J. P., Baron, A. J. & Lovett, M. G. Re-investigation of the effects of L-glutamine and L-asparagine on the Neurospora crassa NADP-specific glutamate dehydrogenase. *Biochem. J.* 159, 803–806 (1976).

27. Gustafson, J. P. Visual MINTEQ 3.1. User Guide. KTH Royal Institute of Technology. 1–73 (2017).

28. D ocurier, J. D. et al. Crystal structure of the OXAs–β-lactamase reveals mechanistic diversity among class D carbapenemases. *Chem. Biol.* 16, 540–547 (2009).

29. Atanasov, P. B., Mustafi, D. & Makinen, M. W. Protonation of the β-lactam nitrogen is the trigger event in the catalytic action of class A β-lactamas. *Proc. Natl Acad. Sci. USA* 97, 3160–3165 (2000).

30. Zhang, W., Shi, Q., Meroueh, S. O., Vakulenko, S. B. & Mobashery, S. Catalytic mechanism of penicillin-binding protein 5 of Escherichia coli. *Biochemistry* 46, 10113–10121 (2007).

31. Matamoros, M. A. & Beck, R. Redox control of the legumiose-rhizobium symbiosis. *Adv. Bot. Res* 94, 67–96 (2020).

32. Roos, G. & Messens, J. Protein sulfenic acid formation: from cellular damage to redox regulation. *Free Radic. Biol. Med.* 51, 314–326 (2011).

33. Waszkczak, C. et al. Oxidative post-translational modifications of cysteine residues in plant signal transduction. *J. Exp. Bot.* 66, 2923–2934 (2015).

34. Borek, D., Kozak, M., Pei, J. & Jaskolski, M. Crystal structure of active site mutant of antileukemic L-asparaginase reveals conserved zinc-binding site. *FEBS J.* 281, 4097–4111 (2014).

35. Zhang, Y. et al. Personalized nanomedicine: a rapid, sensitive, and selective UV–vis spectrophotometry method for the quantification of nanostructured PEG-asparaginase activity in childrens plasma. *Int. J. Nanomed.* 13, 6337–6344 (2018).

36. Krom, M. D. Spectrophotometric determination of ammonia: a study of a modified Bethrel test reaction using salicylate and dichloroisocyanurate. *Analyst* 105, 305–316 (1980).

37. Wang, Y., Wang, G., Moetessier, N. & Mittermaier, A. K. Enzyme kinetics by isothermal titration calorimetry: allosteroy, inhibition, and dynamics. *Front. Mol. Biosci.* 7, 1–19 (2020).

38. Mueller, U. et al. The macromolecular crystallography beamlines at BESSY II of the Helmholtz-Zentrum Berlin: current status and perspectives. *Eur. Phys. J.* 130, 1–10 (2015).

39. Cianci, M. et al. P3, the EMBL macromolecular crystallography beamline at the low-emittance PETRA III ring for high- and low-energy phasing with variable beam focusing. *J. Synchrotron Radiat.* 24, 323–332 (2017).

40. Kabsch, W. Integration, scaling, space-group assignment and post-refinement. *Acta Cryst.* D66, 133–144 (2010).

41. Sheldon, G. M. A short history of SHELL. *Acta Cryst.* A64, 112–122 (2008).

42. Papp, L. & Schneider, T. R. HKL2MAP: a graphical user interface for macromolecular phasing with SHEXL programs. *J. Appl. Crystallogr.* 37, 843–844 (2004).
60. Perrakis, A., Harkiolaki, M., Wilson, K. S. & Lamzin, V. S. ARP/wARP and molecular replacement. Acta Cryst. D57, 1445–1450 (2001).

61. Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot. Acta Cryst. D66, 486–501 (2010).

62. Murshudov, G. N. et al. REFMAC5 for the refinement of macromolecular crystal structures. Acta Cryst. D67, 355–367 (2011).

63. McCoy, A. J. et al. Phaser crystallographic software. J. Appl. Crystallogr. 40, 658–674 (2007).

64. DeLano, W. Pymol: an open-source molecular graphics tool. CCP4 Newsletter On Protein Crystallography. vol. 40 (DeLano Scientific San Carlos, California, USA, 2002).

65. Krissinel, E. & Henrick, K. Inference of macromolecular assemblies from crystalline state. J. Mol. Biol. 372, 774–797 (2007).

66. Baker, N. A., Sept, D., Joseph, S., Holst, M. J. & McCammon, J. A. Electrostatics of nanosystems: application to microtubules and the ribosome. Proc. Natl Acad. Sci. USA 98, 10037–10041 (2001).

67. Dolinsky, T. J., Nielsen, J. E., McCammon, J. A. & Baker, N. A. PDB2PQR: an automated pipeline for the setup of Poisson-Boltzmann electrostatics calculations. Nucleic Acids Res. 32, W665–W667 (2004).

68. Holst, M. & Saied, F. Multigrid solution of the Poisson–Boltzmann equation. J. Comput. Chem. 14, 105–113 (1993).

69. Krissinel, E. & Henrick, K. Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions. Acta Cryst. D60, 2256–2268 (2004).

70. Winn, M. D. et al. Overview of the CCP4 suite and current developments. Acta Cryst. D67, 235–242 (2011).

71. Madeira, F. et al. The EMBL-EBI search and sequence analysis tools APIs in 2019. Nucleic Acids Res. 47, W636–W641 (2019).

Acknowledgements

We thank Dr. Milosz Ruszkowski for crystal vitrification and participation in synchrotron data collection at EMBL/DESY and Dr. Jakub Barciszewski for data collection at BESSY. We thank Dr. Piotr Bonarek and Dr. Jakub Nowak for help with the CD and nanoDSF experiments. We acknowledge Dr. Lukasz Marczak for carrying out the MS measurements. We thank the Helmholtz-Zentrum Berlin für Materialien und Energie for the allocation of synchrotron beamtime, and Dr. Thomas Schneider for his interest in the S-SAD experiment at EMBL/DESY. Work supported by National Science Centre (NCN, Poland) grant 2020/37/B/NZ1/03250.

Author contributions

J.I.L. designed the experiments, performed site-directed mutagenesis, grew crystals, refined the structures, analyzed the results and wrote the manuscript. B.I. produced and purified the protein, grew crystals, refined the structures, analyzed the results and participated in manuscript preparation. J.S. carried out enzymatic assays, analyzed their results and participated in manuscript preparation. A.W. carried out expression of ReAV mutants and biophysical measurements. M.B. established the preliminary crystallization conditions and conducted the DSC experiments. M.G. collected and processed X-ray diffraction data, solved the structures, analyzed the results and participated in manuscript preparation. M.J. conceived and coordinated the project, designed the experiments, analyzed the results and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-27105-x.

Correspondence and requests for materials should be addressed to Mariusz Jaskolski.

Peer review information Nature Communications thanks Jacek Łukkowski and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021