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Research

Stability of The Tyrosyl Radical in The Beta Subunit of *Arcobacter bivalviorum* Ribonucleotide Reductase

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ABSTRACT

Arcobacter spp., such as *Arcobacter bivalviorum* (*A. bivalviorum*), are free-living organisms found in diverse environments and associated with animals. They are considered emerging enteropathogens and potential zoonotic agents. Ribonucleotide reductase (RNR) is the key enzyme that is used to convert ribonucleotides into deoxyribonucleoside triphosphates (dNTPs). This process utilises radical-based chemistry and is crucial for DNA biosynthesis and repair. There are three RNR classes, with class I RNR the most studied, present in *A. bivalviorum*, eukaryotes, and many prokaryotes. Class I RNRs are further divided into three subclasses: Ia, Ib, and Ic. Class Ib RNRs use a dimanganese-oxo centre, unlike class Ia RNRs, which use a diiron-oxo centre. *A. bivalviorum* possesses a class Ia enzyme that requires a diferric tyrosyl radical cofactor located within its beta (β) subunit. Indeed, both the efficiency and fidelity of DNA synthesis are influenced by the stability of the tyrosyl radical (Y•) in the RNR, which is a critical aspect of its enzymatic function. This study investigates the stability of the Y-radical (Y•) site within the RNR β subunit of *A. bivalviorum* and the nature of the neighbouring amino acid residues. To achieve these goals, we developed a model of the RNR β subunit of *A. bivalviorum*, using the RNR β subunit of *Aquifex aeolicus* as a reference template (7aik.1. A PDB). The results provide some important details about the radical site and its surrounding residues, highlighting the influence of the protein structure on the stability of the radical. These findings may guide the development of novel inhibitors targeting this enzyme in *A. bivalviorum*.

Key words: *Arcobacter bivalviorum*, food safety, iron centre, ribonucleotide reductase, tyrosyl radical

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INTRODUCTION

The *Arcobacter* genus comprises gram-negative spiral-shaped bacteria. They were first isolated in 1977 from aborted bovine foetuses and human clinical specimens. Unlike bacteria in the phylum Campylobacterota, *Arcobacter* species can be found in both animal and environmental sources (Wesley, 1998). Some *Arcobacter* species are human or animal pathogens that cause diarrhoea, gastroenteritis, bacteraemia, and endocarditis. *Arcobacter bivalviorum* is a species of the *Arcobacter* genus that was discovered relatively recently in shellfish, primarily in bivalves. Initial identification was based on isolates from mussels and oysters (Levican *et al*., 2012). Its presence in molluscs underscores its significance in both marine and aquatic ecosystems. The emergence of this species has sparked concerns regarding food safety, particularly the consumption of shellfish. There are various classes of ribonucleotide reductases. Class III ribonucleotide reductase (RNRs) are oxygen-sensitive and function under anaerobic conditions; their radical-generating mechanisms are inactivated by oxygen. In contrast, Class I RNRs are oxygen-dependent and function under aerobic conditions, with tyrosyl radical generation requiring molecular oxygen. Genomic analysis revealed that *A. bivalviorum* possesses a combination of (RNR) class I and III enzymes.

In all living cells, RNR is a crucial enzyme that catalyses the conversion of ribonucleotides into deoxyribonucleotides

(dNTPs), which are essential for DNA synthesis and repair (Hofer *et al*., 2012). RNRs are classified into three main groups based on the type of radicals utilised, metal cofactors, and oxygen dependence. Class I RNRs are oxygen-dependent heterocomplexes comprising three subclasses. The Ia subclass is comprised of NrdA (R1) and NrdB (R2). This subclass contains a diferric centre and a tyrosyl radical (Fe^{III}-O-Fe^{III}-Y$^{\bullet}$). The Fe^{III}-Y$^{\bullet}$ cofactor in this subclass can self-assemble from apo-β2, Fe^{II}, O_2, and a reducing agent. The Ib subclass, which consists of NrdE (R1) and NrdF (R2), utilises manganese (Mn^{III}-O-Mn^{III}) instead of iron for the formation and stabilisation of tyrosyl radical Y$^{\bullet}$. The Ic subclass uses a combination of manganese and iron as metal cofactors (Mn^{IV}-O-Fe^{III}). Class II RNRs are oxygen-independent (anaerobic) homocomplexes that utilise adenosylcobalamin (AdoCbl) to form radicals (Pedraz López, 2020). Class III RNRs are strictly anaerobic homocomplexes that rely on glycyl radicals as cofactors (Torrents, 2014).

Class Ia (RNR) enzyme consists of two distinct subunits, a catalytic alpha (α) subunit and a radical-generating beta (β) subunit. In solution, the class Ia enzyme primarily exists as homodimers, forming α2 and β2 structures. These conformations are crucial for their function and regulation. Within the β subunit of class Ia RNR, a diiron-oxo cofactor which is remarkably significant is essential for generating a stable tyrosyl radical. In *Escherichia coli*, the cofactor is identified as Y122β (Rofougaran *et al*., 2008). The presence of this radical is essential for the catalytic activity of enzymes, undergoing a journey of approximately 30 angstroms, through a series of proton-coupled electron transfer (PCET) steps within the α2β2 active complex. The journey of the tyrosyl radical for 30 angstroms to form a thiyl radical in the active site of the enzyme is critical. The actual reduction of the ribonucleotides (ATP, GTP, CTP & TTP) is facilitated by this catalytic thiyl radical. One unique aspect of class Ia RNR is the role of the tyrosine residues in PCET (Reece & Seyedsayamdost, 2017). Tyrosine is particularly suitable for this process because the thermodynamics of oxidation at physiological pH necessitate proton transfer accompanied by electron transfer. This coupling of the proton and electron transfer stabilises the tyrosyl radical, making it an efficient intermediate in the reduction process. This concerted transfer is crucial because it prevents the formation of high-energy charged intermediates which could be detrimental to enzyme function (Reece & Seyedsayamdost, 2017) (Greene *et al*., 2020). RNR is an important enzyme not only in providing cells with dATP, dGTP, dCTP, and dTTP but also in maintaining the correct balance to prevent an increased mutation rate due to imbalanced dNTP levels (Hofer *et al*., 2012). Specific ATP, GTP, CTP, and TTP reductions were determined allosterically. This regulation is dependent on the identity of the deoxyribonucleotide (dATP, dGTP, or TTP) bound at the allosteric specificity site that is located at the α2 dimer interface. The overall activity of the enzyme is regulated allosterically via a third nucleotide binding site at the N-terminus of α2 (Zimanyi *et al*., 2016) (Chen *et al*., 2018). Additionally, it is influenced by the cellular ATP/dATP ratio. To date, all characterised class Ia RNRs have been subjected to this form of allosteric activity regulation (Johansson *et al*., 2016). However, the underlying molecular mechanisms may vary. For example, in *E. coli* class Ia RNR, dATP binding at this allosteric activity site leads to enzyme inactivation. This inactivation occurs as the enzyme transitions from the active α2β2 conformation to an inactive α4β4 conformation, thereby preventing the essential radical transfer for catalysis (Ando *et al*., 2011). In contrast, human class Ia RNR exhibit a different mechanism of inhibition by dATP. In humans, the binding of dATP seems to induce the formation of a stable α6 state. This state effectively prevents the α2 and β2 subunits from interacting in a manner that is catalytically competent for radical transfer (Brignole *et al*., 2018) (Aye & Stubbe, 2011).

Among the RNR enzymes, class Ia enzyme from *E. coli* is the most well-studied. The radical site (Y122β) within the β subunit of *E. coli* shows that the tyrosyl radical Y• is located within a very hydrophobic pocket (Ormö *et al*., 1995) (Gerez *et al*., 1997). The Y • radical within this pocket was surrounded by three hydrophobic residues: Phe208, Phe212, and Ile234. These hydrophobic residues contribute to radical stability; mutating these three hydrophobic residues into more hydrophilic residues leads to proteins with significantly shorter radical half-lives. Additionally, the radical's deep location, approximately 10 angstroms away from the protein's closest surface within the β subunit, is another factor contributing to its stability. This distance makes it difficult for molecules such as radical scavengers and reducing agents, to directly reach and deactivate the radical (Gerez *et al*., 1997).

In this study**,** a computational approach was employed to investigate the stability of radicals within the RNR β subunit of *A. bivalviorum*. A protein model was developed using the β -subunit structure of *Aquifex aeolicus* as a template. Examination of the radical site has focused on two key aspects: analysing its proximity to the closest surface and investigating the nature of the surrounding amino acid residues. Research has revealed important details about the structure and stability of RNR that could help in developing inhibitors targeting the RNR enzyme in *A. bivalviorum*.

MATERIALS AND METHODS

Alignment of RNR β subunit sequences

The RNR sequence was obtained from *Aquifex aeolicus* (NCBI Accession Number 7AIK_A) and *A. bivalviorum* (NCBI Accession Number AXH11072), the National Center for Biotechnology Information (NCBI) database. After obtaining the amino acid sequences of the RNR enzymes, two different alignment tools from the European Molecular Biology Open Software Suite (EMBOSS) were used. The RNR β subunit sequences from *A. bivalviorum* and *A. aeolicus* were aligned, using Clustal Omega, known for its efficacy in multiple sequence alignment and EMBOSS Needle which employs the Needleman-Wunsch algorithm for global sequence alignment.

Mapping the active site residues of *A. bivalviorum* RNR

The sequence of RNR from *A. bivalviorum* (NCBI Accession Number AXH11072) was analysed using InterPro, an integrated resource that combines various protein signature databases to determine the active site (Paysan-Lafosse *et al*., 2023). Using this tool, the potential active sites of the enzyme (Fe^{III}-O-Fe^{III}-Y•) and residues such as Y• which play critical roles in substrate binding and catalysis, can be identified. Essential amino acid residues and domains in *A. bivalviorum* RNR, which are likely critical for its enzymatic function, were identified using InterPro's comprehensive analysis. Such an analysis is important for identifying the key residues and domains necessary for enzymatic function that help predict the three-dimensional structure of the protein, understand how the protein interacts with its substrates, and elucidate the mechanisms of catalytic actions.

Homology modelling of *A. bivalviorum* RNR

Advanced computational modelling techniques were employed to analyse the structural properties of the RNR from *A. bivalviorum*. To model the structure of the enzyme, the protein sequences of *A. bivalviorum* RNR were used with PHYRE-2 and SWISS-MODEL, both highly regarded for protein homology and analogy recognition (Kelley & Sternberg, 2009) (Waterhouse *et al*., 2018). The model was generated concerning the structure of the ribonucleotide reductase β subunit from *Aquifex aeolicus* in the RCSB Protein Data Bank (PDB ref. 7AIK) with a 42% internal diameter and a 100% confidence interval.

3D structure visualisation of *A. bivalviorum* RNR

PyMOL, a powerful molecular visualisation system, was used to analyse the three-dimensional structure of the ribonucleotide reductase β subunit from *A. bivalviorum*. This advanced visualisation tool enabled us to perform detailed 3D alignments, particularly focusing on the site of the tyrosyl radical (Y•) and the surrounding amino acids (GLU-183, HIS-220, HIS-121, ASP-87, LEU-83 & GLU-118). This analysis aimed to understand the key structural similarities and differences between RNR enzymes. Measuring the distance of the tyrosyl radical is important to demonstrate how far the tyrosyl radical site (Y•) is from the closest surface. Such measurements can be performed using the Measurement Wizard, a powerful tool in PyMOL that allows the measurement of distances, angles, and dihedral angles between atoms in a molecular structure.

RESULTS

Alignment of RNR β subunit sequences

RNR is a multimeric enzyme composed of two subunits, a large subunit (alpha) and a small subunit (β) (Chakraborty *et al*., 2022). In the Class Ia enzyme of *E. coli*, these subunits exist primarily as homodimers (α2 and β2) (Nguyen *et al*., 2023). The homodimer form of the β subunit of ribonucleotide reductase (RNR) is essential for the enzyme's function. This dimerisation, which is crucial for the stability of the radical, is made up of two β subunits that associate closely to form a functional unit (Brown *et al*., 1969). Each β subunit in the dimer contributes to radical formation. The overall conformation of the β subunit allows it to interact efficiently with the α subunit of RNR. This interaction is necessary for the transfer of the radical to the α subunit, where the actual reduction of ribonucleotides occurs. In the radical-generating β subunit of *A. bivalviorum,* a diiron cofactor which cycles between Fe^{III}-Fe^{III} and Fe^{II}-Fe^{II} states to generate radical generation (shown as red spheres) is essential for the generation of a nearby stable tyrosyl radical. Additionally, the β subunit is the site of the tyrosyl radical (Y125β) which is important in initiating the radical-based reaction mechanism that converts ribonucleotides into deoxyribonucleotides. Two different alignment tools are used in Figures 1 and 2 to illustrate the similarities among the β subunits of the ribonucleotide reductase from *A. bivalviorum* and *A. aeolicus*. This involved aligning both the protein sequences and the three-dimensional structures of the

β subunits from *A. aeolicus* (NrdB A.a) and *A. bivalviorum* (NrdB A.v). The EMBOSS Needle was used for the pairwise sequence alignment of RNR β subunits from *A. aeolicus* (NrdB A.a) and *A. bivalviorum* (NrdB A.v). The percentages of sequence identity, similarity, and gaps were 38.3% (133/347), 57.1% (198/347), and 9.8% (34/347), respectively. The alignments revealed a significant degree of similarity between the two RNR enzymes (Figure 2).

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NrdB A.v          1 --MGRKTIYNPDSKETLNERRIFGGNPDGMINFTKMKYQWALNLWDTME-     47
                      :.||.|:||......::|:|..|||..:....::||.||.:|:..|.
NrdB A.a          1 NELVRKLIFNPQGDREASKRKIIKGNPTNIFELNEIKYSWAFDLYKLMGF     50

NrdB A.v         48 ANTWFPKEVQMTGDAKDYK-YLSPAEKRMYDLVLSQLIFMDSLQTNNLMD     96
                    .|.|.|:|:||..|.|.|: .||..|||.|:||||.||.:||.|. :::.
NrdB A.a         51 TNFWIPEEIQMLEDRKQYETVLSDYEKRAYELVLSFLIALDSFQV-DMLK     99

NrdB A.v         97 NINPYITVPEINACLSRQSYEEANHSKSYAVMVESISD--NTDEIYDKWK    144
                    .....||.||:...::.|.::|:.|:.||..::||:.|  ..||||:.|:
NrdB A.a        100 EFGRMITAPEVEMAITAQEFQESVHAYSYQFILESVVDPVKADEIYNYWR    149

NrdB A.v        145 TDEKLREKNNYIADVYHNLAGDITDEKIVLAMFANQILEGLYFYAGFAAM    194
                    .||:|.|:|..||::|:........:|..:.|...|.|||.||||:|||..
NrdB A.a        150 EDERLLERNKVIAELYNEFIRKPNEENFIKATIGNYILESLYFYSGFAFF    199
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Fig. 1. EMBOSS Needle used for pairwise sequence alignment of both enzymes RNR β subunit from *A. aeolicus* (NrdB A.a) and RNR β subunit from *A. bivalviorum* (NrdB A.v). The percentage sequence Identity: 133/347 (38.3%), Similarity: 198/347 (57.1%), Gaps: 34/347 (9.8%). The radical site in *A. bivalviorum* (Y125β) is highlighted in red. I = position with fully conserved residue; = position with residues sharing strongly similar properties.. = position with residues sharing weakly similar properties.

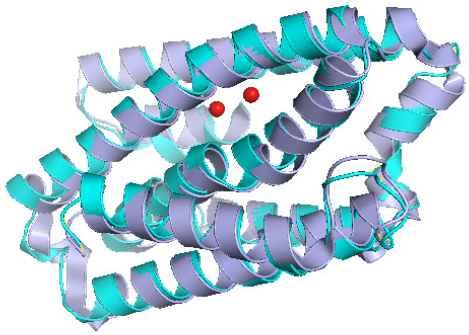

Fig. 2. 3D alignments of RNR β subunits from *A. aeolicus* and RNR β subunit from *A. bivalviorum.* 3D alignments of domains containing the radical site, using PyMOL show a high level of similarity between the two enzymes. The iron ions of RNR subunits are shown as red spheres.

Homology modelling of *A. bivalviorum* RNR

To investigate the relationship between radical stability and its topology, the 3D structure of the β subunit of RNR was developed (Figure 3)**.** Before modelling the structure of the β subunit of RNR from *A. bivalviorum*, a search was conducted in the RCSB Protein Data Bank for similar RNR structures in other bacteria. The search yielded an RNR structure similar to that of *A. aeolicus* (referred to as NrdB A.a). A model of *A. bivalviorum* RNR β subunit (referred to as NrdB A.v (was developed using SWISS-MODEL based on NrdB A.a structure with 100% confidence and visualised using PyMOL. The NrdB A.v enzyme is a homodimer that exists as a β2 structure. TYR-125 is the site of the tyrosyl radical (Y•) (Figure 4). From Figure 5, it can be seen that glutamic acid (GLU), histidine (HIS), aspartic acid (ASP), and leucine (LEU) are the amino acids surrounding the radical. These residues play a crucial role in stabilising tyrosyl radicals in RNR. The tyrosyl radical (Y•) is buried deep within the β subunit of the enzyme, far from the closest surface (≈ 9.8 Å) (Figure 4).

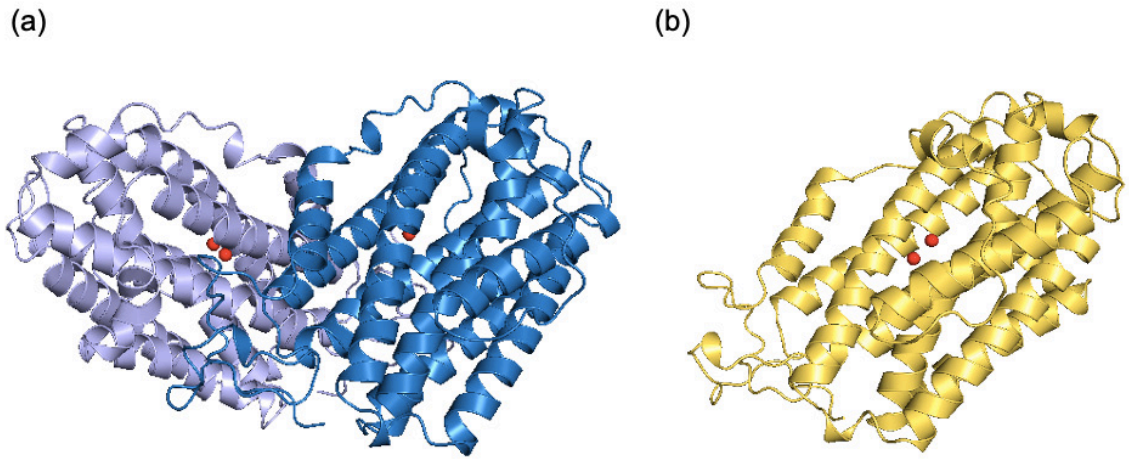

Fig. 3. Model β subunits (β2 structure) of *A. bivalviorum* ribonucleotide reductase constructed using SWISS-MODEL based on *A. aeolicus* RNR structure with 100% confidence and visualised using PyMOL. RNR is displayed as a cartoon. **(**a) Model of the dimeric β subunits (β2 structure) of *A. bivalviorum* RNR with individual monomers shown as dark blue and light blue ribbons. (b) Model of the monomeric β subunit. The iron ions of RNR subunits are shown as red spheres.

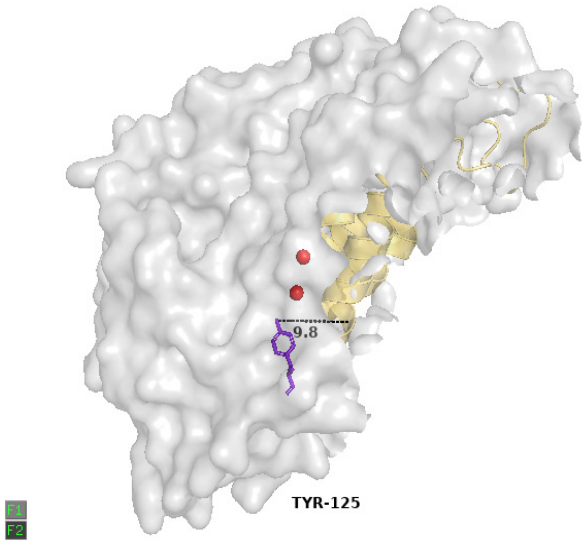

Fig. 4. Model of the monomeric β subunit of *A. bivalviorum* ribonucleotide reductase, constructed using SWISS-MODEL based on *A. aeolicus* RNR structure with 100% confidence and visualised using PyMOL. The tyrosyl radical (Y•) is located as TYR-125, which is buried far from the closest surface (≈9.8 angstroms).

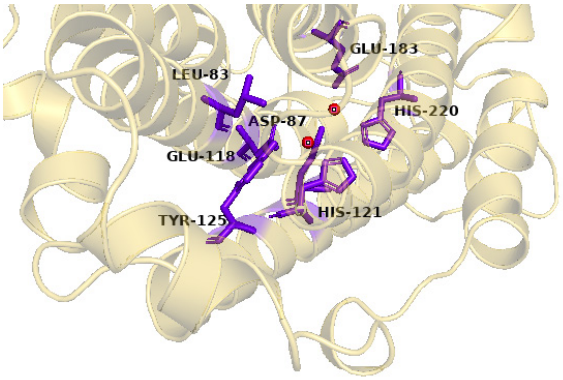

Fig. 5. Model of the monomeric β subunit of *A. bivalviorum* ribonucleotide reductase constructed using SWISS-MODEL based on *A. aeolicus* RNR structure with 100% confidence and visualised using PyMOL. Amino acids contributing to the stability of the tyrosyl radical are depicted as sticks. GLU-183, HIS-220, HIS-121, ASP-87, LEU-83 and GLU-118 are the neighbouring amino acid residues. The iron ions of RNR subunits are shown as red spheres.

DISCUSSION

The β subunit (β2) of ribonucleotide reductase may exist in the inactive metR2 form, due to the sensitivity of the radical to one-electron reductants or radical scavengers. This form (metR2) lacks the tyrosyl radical but contains a diiron centre (Fe^{III}-Fe^{III}) (Regnström *et al*., 1994). A two-step reaction forms the tyrosyl radical. In the first step, iron is enzymatically reduced by NADPH. In the second reaction, the interaction between molecular oxygen and the diiron centre generates a tyrosyl radical (Covès *et al*., 1995). The Y radical (Y•) is stabilised by surrounding amino acids that contribute to its stability through various interactions, such as hydrogen bonding and hydrophobic interactions which help maintain the precise orientation and environment necessary for the persistence and reactivity of the tyrosyl radical. Amino acids also contribute to the structural integrity of the active site, ensuring the correct positioning of the tyrosine residue. Nearly charged amino acids may influence the stability of the radical by altering the local electrostatic environment. Studies on *E. coli* RNR have shown that the tyrosyl radical (Y122β) is located in a highly hydrophobic pocket with three hydrophobic residues (phenylalanine 208, phenylalanine 212 & isoleucine 234). Replacing these hydrophobic residues with hydrophilic residues results in enzymes with shorter radical half-lives. In contrast, modifications that maintain hydrophobicity did not affect the tyrosyl radical (Y122β) stability (Gerez *et al*., 1997). Additionally, the radical is deeply buried within the enzyme (10 Å away from the closest surface). This underscores the importance of the hydrophobicity of the pocket and the tyrosyl radicals positing as stabilising factors. Such a design is crucial for protecting the tyrosyl radical from reactive species and ensuring proper electron transfer.

In the RNR β subunit in *A. bivalviorum*, the Y radical (Y125β) is surrounded by amino acid residues GLU-183, HIS-220, HIS-121, ASP-87, LEU-83, and GLU-118 which vary significantly in hydrophobicity. These amino acids play crucial roles in the stability of the Y radical within the RNR of *A. bivalviorum*. Glutamic acid (GLU) residues, represented by GLU-183 and GLU-118, are hydrophilic because of their negatively charged side chains at physiological pH. Typically, these residues are located on the enzyme's surface, where they interact with water molecules or participate in ionic interactions with other biomolecules. They are often present in enzyme-active sites, contributing to substrate specificity and stability. Histidine (HIS), found in HIS-220 and HIS-121, is generally neutral but can become positively charged depending on the pH. The imidazole ring in histidine facilitates metal ion binding and participating in enzyme catalysis, particularly in acidic or neutral environments. While histidine can be somewhat hydrophobic, but is more often found at protein-active sites or in metal binding because of its potential to switch between charged and uncharged states. Aspartic acid (ASP-87) shares some properties with glutamic acid, another negatively charged hydrophilic residue. In contrast, leucine (LEU-83) is a highly hydrophobic residue often found in the interior of enzymes. Leucine supports structural integrity through hydrophobic interactions, which help stabilise the core of the enzyme.

Its nonpolar nature helps maintain the conformation of hydrophobic pockets to which nonpolar molecules or regions of other proteins might bind. In the RNR of *A. bivalviorum*, the tyrosyl radical (Y radical) is approximately 9.8 angstroms away from the enzyme's closest surface. Because it is deeply buried within the enzyme structure, the radical is shielded from reactive oxygen species and other radical scavengers that disrupt it. Comparing the hydrophobic and hydrophilic properties of the amino acids surrounding the tyrosyl radical residues in *A. bivalviorum* and *E. coli*, it can be concluded that the tyrosyl radical in *A. bivalviorum* is unstable, in contrast to that in *E. coli*.

CONCLUSION

Research on the stability of the tyrosyl radical within the RNR β subunit of *A. bivalviorum* adds crucial insights into the enzyme's molecular function. The stability of the tyrosyl radical is essential for RNR's proper function, as it plays a fundamental role in DNA synthesis and repair by converting ribonucleotides into deoxyribonucleotides. These findings indicate that the tyrosyl radical (Y•) in *A. bivalviorum* is uniquely positioned and influenced by the surrounding amino acid environment which can either stabilise or destabilise the radical depending on its hydrophobic or hydrophilic properties. This study highlighted the significant role of the hydrophobic pocket in maintaining radical stability, which is critical for the enzyme's efficiency and fidelity in DNA synthesis (Schmidt, 2019). This stability is vital, as it protects the radical from deactivation by external molecules such as radical scavengers, thus preserving enzyme activity. Additionally, a computational modelling approach based on the β subunit structure of *A. aeolicus* provided a clearer understanding of the topology of the radical and its interactions with surrounding residues. The comparative analysis reveals that, despite their similar biochemical roles, there are distinct differences in the stability of tyrosyl radicals between species, due to variations in the amino acid environment surrounding the radical site. In *A. bivalviorum*, the Y radical (Y125β) is surrounded by a mix of hydrophobic and hydrophilic residues, which affects the radical's stability differently compared to the predominantly hydrophobic pocket in *E. coli*'s RNR.

ETHICAL STATEMENT
Not applicable

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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