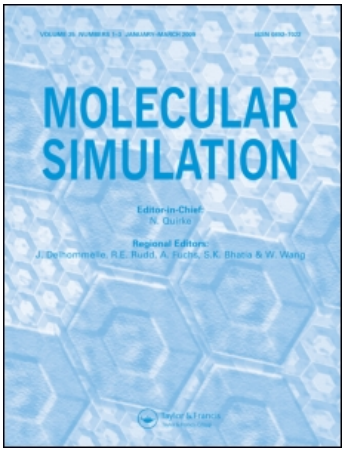

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The biogenic production of sulphide gas by sulphate-reducing bacteria (SRB) causes serious economic problems for the natural gas and oil industry. Due to their corrosive properties, biofilms produced by SRB create issues during all steps of oil and gas recovery, from early in production to storage, including increased risk of toxic oil spills and health risk to workers in the oil field and to the population living around the area of extraction. SRB are unique among micro-organisms in their ability to utilise sulphate as an electron acceptor. One of the key enzymes important in this biological process is adenosine phosphosulphate reductase (APS reductase). This article aims to discuss the structure and dynamic properties of APS reductase through computer-generated simulations using molecular dynamics.

Keywords: molecular dynamics; APS reductase; energy

1. Introduction

Sulphate-reducing bacteria (SRB) are characterised by the ability to use sulphate as an electron acceptor for the production of energy during anaerobic respiration. The presence of SRB in water creates a costly problem for industry because SRB cause corrosion of submerged metals. SRB form biofilms on metals that allow for dissimilative reduction reactions that lead to the dissolution and loss of the protective metallic surface or film coating [1]. In the natural gas and oil industry, corrosion of pipelines and gas lines by SRB is an important and costly problem. Not to mention that the production of sulphide gas (biosulphidogenesis) by SRB also adds to environmental pollution. In oil extraction from offshore reservoirs, SRB control is especially crucial due to the use of seawater in secondary extraction. Seawater is injected to help the withdrawal of the oil, but the salty water also stimulates the growth of SRB due to the high sulphate content. Several methods have been proposed and used for the control of SRB and biosulphidogenesis. The most common method used for control is the addition of biocides to the water. Unfortunately, this method is economically prohibitive and, in practice, does not produce the desired result. A second alternative, use of nitrate-reducing bacteria (NRB), establishes a competition between NRB and SRB. Also called biocompetitive exclusion technology (BET), this practice stimulates the activity of native, helpful (petrobiotic) organisms that outcompete SRB, thus reducing sulphide production [2]. However, some SRB also have the ability to reduce nitrate and nitrite, providing resistance to control by petrobiotics when nitrite and nitrate are used. So there is still need for a process to control sulphide production. Our work aims to better understand the critical pathways unique to SRB to aid in the development of novel mechanisms of control.

One of the intrinsic characteristics of SRB is the ability to reduce sulphate that is dependent on the presence of four enzymes: ATP sulphurylase, adenosine phosphosulphate reductase (APS reductase), hydrogenase and sulphite reductase. These four enzymes constitute the pathway for turning sulphur and sulphur composites into sulphide gas. APS reductase catalyses the reduction of adenosine 5′-phosphosulphate (APS) according to the following Equation (1):

$$\mathrm{APS} + 2\mathrm{e}^- \xrightarrow{\mathrm{APSR}} \mathrm{AMP} + \mathrm{HSO}_3^- . \qquad (1)$$

The process of reduction happens through the nucleophillic attack of the N5 atom of the reduced form of flavin adenine dinucleotide ($FADH_2$) on the sulphur of APS, forming a linked FAD–APS. This intermediate then breaks down into AMP and sulphite, releasing the FAD.

The structure and the function of the enzymes described above have been studied through experimental techniques, including biochemical analysis, X-ray diffraction and electron magnetic resonance [3,4]. However, new lights on the understanding of their structural, molecular and dynamic properties can be shed through computer

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simulations using molecular dynamics (MD) techniques in combination with experimental analysis in the laboratory.

The MD generates predictions of molecular conformational changes as a function of time by calculating the movement of the atoms in a molecule. The calculated coordinates then define the conformational trajectory in space.

In our work, we have used MD to analyse the aspects of the dynamic and structural behaviour of APS reductase. The MD allowed a virtual experiment that mimicked the behaviour of APS reductase in aqueous solution through experiments *in silico*, generating important information about the protein and its interactions with other molecules. Computer modelling also gave us insight into the movement of key residues during the catalytic process mediated by APS reductase.

2. Methodology

The dynamic and structural properties of APS reductase, in aqueous solution, were analysed using MD techniques taking into consideration the typical value of pressure found in oil wells in the Recôncavo Baiano region of Bahia, Brazil. The simulation was carried out using the 3D structure of APS reductase of the hyperthermophilic *Archaeoglobus fulgidus*, determined through X-ray diffraction, that is deposited in the Protein Data Bank (PDB) under the code 1JNR [3]. In accordance with the experimental data, the APS reductase is a 190 kDa metalloprotein constituted by two αβ heterodimers, forming an $\alpha_2\beta_2$ tetramer. The essential unit for catalytic function, however, is a single αβ heterodimer. Each heterodimer contains two iron–sulphur centres, $[4Fe–4S]^{+2}$, located in the β-subunit that are responsible for the transfer of two electrons from the surface of the protein to the FAD molecule contained in the active centre of the protein [4].

Despite the experimental data presenting an $\alpha_2\beta_2$ tetramer, only a single αβ heterodimer was simulated. The simulation was performed using a dual core computer with 2 Gb of RAM memory, using the GROMOS87 force field code of the Groningen Machine for Chemical Simulations (GROMACS) software package, version 3.3.2. The CPU time for the simulation was approximately 242 h. The analysis was performed for the αβ heterodimer, without the molecule of glycerol, for 10 ns at 363 K with 300 bar of pressure. The topology of the FAD cofactor was obtained from the web server PRODRG (data not shown; [13]). Partial charges of the iron–sulphur centres for configuring the field of forces were calculated with General Atomic and Molecular Electronic Structure System (GAMESS97) software (Table 1) using the Hartree–Fock theory with a set of minimum bases (RHF/STO-3G), without taking into account the cysteine atoms (SG – in the PDB file), which are covalently attached to the iron atoms. The electrostatic adjustment for the electron density was performed with the charges from electrostatic potentials using a grid (CHELPG) based method.

Table 1. Partial charges of the iron–sulphur clusters as obtained from *ab initio* calculations.

Atom	Charge
Fe1	0.7026
Fe2	0.6714
Fe3	0.6919
Fe4	0.7299
S1	−0.2051
S2	−0.1844
S3	−0.2099
S4	−0.1965

The αβ heterodimer, solvated with 18,123 water molecules of the simple point charge type, was minimised with the steepest descent and conjugated gradient algorithms for a triclinic box with an approximate volume of 665.20 nm^3 and with a minimum distance of 0.8 nm between the protein and the walls of the box. Eighteen Na^+ ions were added to neutralise the charge in the box. The whole system was balanced maintaining restricted position for 500 ps with 2 fs time steps. The SHAKE algorithm [5] was used for the bonds within the heterodimer and the SETTLE algorithm [6] was used for the bonds of the water molecules. The unligated interactions were cut at 1.2 nm and the temperature was maintained through the weak coupling of Berendsen with a constant time of $T_T = 0.1$ ps. The initial velocity used in the equilibration of the system was generated using the Maxwell distribution. The pressure was kept constant with Berendsen's weak coupling barostat with a constant time of $T_P = 1$ ps.

3. Results and discussion

Analysis of the APS reductase αβ heterodimer was carried out to explore the stability and the catalytic properties of the protein, with a special emphasis on the conformational changes of the active site region and the FAD molecule which allow for substrate accessibility. The steady state of APS reductase and the FAD molecule was analysed based on the energy and geometric properties obtained from the crystal structure. The active site region is constituted by residues HIS-A-398, TRP-A-234, ARG-A-265, VAL-A-273, GLY-A-274, GLU-A-141, GLN-A-145, ASN-A-74 and PHE-A-448. The channel that connects the active site to the surface of the molecule is surrounded by residues ARG-A-85, LYS-A-281, LYS-A-283 and ARG-A-317. The temporal change in the radius of gyration (R_g) of the cluster of residues that surrounds the channel is shown in Figure 1. The several conformations acquired by this cluster of residues suggest a movement of widening and narrowing of the channel during the period of simulation.

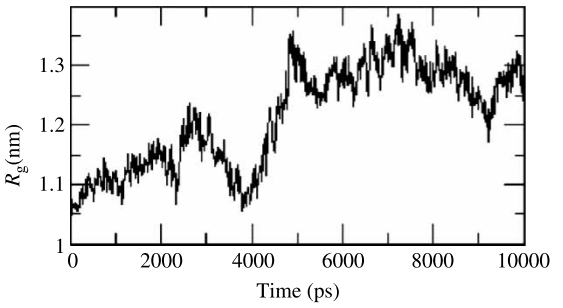

Figure 1. Time evolution of the radius of gyration of the cluster of residues (ARG-A-85, LYS-A-281, LYS-A-283 and ARG-A-317) that surrounds the active site channel.

In the more open state, the radius of gyration of the cluster of residues (ARG-A-85, LYS-A-281, LYS-A-283 and ARG-A-317) is almost 0.4 nm bigger than the R_g determined from the optimised crystal structure when it reaches a value near to 1.4 nm. It seems that the fluctuation of the channel diameter is a function of the component residues of the active site movement. Beyond such geometric aspects, other physico-chemical parameters, e.g. the charge, will influence the selectivity of the active site for the possible ligands.

To determine which residues had the largest displacement during the conformational change, we analysed the fluctuations in the mean square root (RMS) in relation to atoms of the component residues of the active site. The entrance of the substrate-binding channel is surrounded by five positively charged residues (Arg-A-83, Lys-A-281, Lys-A-283, Arg-A-294 and Arg-A-317). As can be seen in Figure 2, the α-carbon atoms 55 and 75 of residues LYS-A-281 and LYS-A-283, respectively, underwent the greatest displacements.

The presence of the FAD molecule at the active site of APS reductase strongly influences the interaction of the protein with the substrate. The FAD molecule is not covalently attached to the protein, but remains linked by electrostatic interactions and Lennard-Jones potentials (shown in Table 2), and by hydrogen bonds (shown in Figure 3).

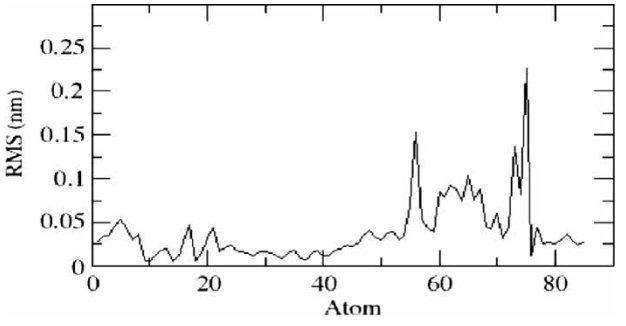

Figure 2. RMS fluctuations in the displacement of the atoms of the residues that surround the active site channel.

Table 2. Energy of ligation of the FAD molecule to the enzyme due to non-bonded forces.

Energy	Mean	RMSD
Coulomb	− 853.18	238.49
Lennard-Jones	− 375.65	44.86
Sum	− 1228.83	–

The number of hydrogen bonds formed is insufficient to maintain the FAD molecule attached to the protein. On average, 0.81 bridges were formed in the first 5 ns of simulation, declining to 0.04 being formed in the final 5 ns. The Coulomb interactions, together with London–van der Waals forces, are the key factors contributing to the attachment of the FAD molecule.

Water molecules associated with the FAD molecule also help in this stabilisation by a small network of hydrogen bonds. Moreover, the water molecules allow larger fluctuations of the side chains and facilitate the entrance of the substrate to the active site. The radial distribution function $G(r)$ was calculated over the simulation trajectory. Figure 4 shows the development of the shells of solvation surrounding the FAD molecule, with the first water molecule located about 0.012 nm away.

To study the conformational changes in the FAD molecule, the angle between the isoalloxazine and adenine rings was analysed. Free FAD in solution may exist either in a stacked conformation, in which the isoalloxazine and adenine rings couple with each other, or in an extended, unstacked conformation [7]. It is known that the stacked form of FAD is non-fluorescent due to reductive photoinduced electron transfer from the adenine moiety to the isoalloxazine moiety [8] and that this conformation is maintained mainly through London–van der Waals interactions [9]. As obtained from the simulation, the protein-bound FAD molecule has conformations that are different from those that occur in free solution. In the initial conformation, the angle between the rings is close to 41.40°, but during the course of the simulation, the rings adopt a more parallel conformation with an angle of 17.06°. As shown in Figure 5, during the first 5 ns of

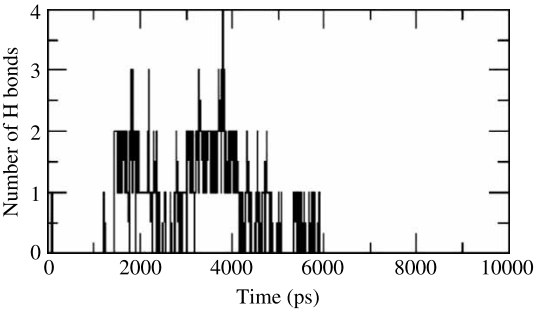

Figure 3. Number of hydrogen bonds between the FAD molecule and the enzyme as a function of time.

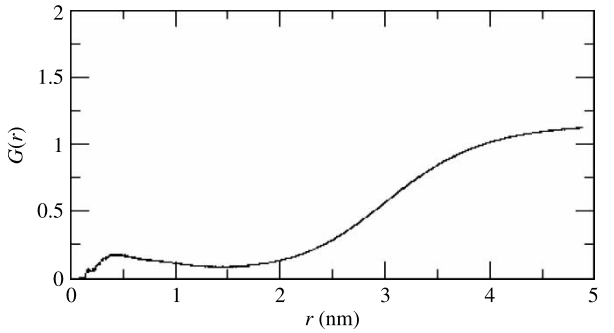

Figure 4. The radial distribution function $G(r)$ at a radius r from the FAD surface.

simulation, the angle oscillated between approximately 180° and 0°, and during the final 5 ns between approximately 0° and − 180°.

Structural analyses of different states of the enzyme indicated that substrate binding induces a shift of the isoalloxazine ring towards the channel bottom, thereby producing a compressed enzyme–substrate [10].

Each β-subunit of APS reductase contains two [4Fe–4S] centres coordinated by the cysteine residues, mediating electron transfer through the enzyme to the active site. Fe–S centres in other enzymes are known to regulate the catalytic activity through oxidation state changes or coupling redox reactions to the translocation of ions [11].

The $[4Fe–4S]^{+2}$ clusters of APS reductase differ significantly in their reduction potential (approximately − 500 mV for cluster II and − 60 mV for cluster I) and the distance edge-to-edge is 9.7 Å. The distance between the S3 of cluster I and the methyl group is 12.4 Å [3,12]. The large difference in reduction potential is caused by the surrounding protein matrix. In our simulation, the motions in the protein reduce the distance between the clusters from both β-subunits gradually during 1 ns simulation from 0.3 to 0.21 nm (Figure 6), increasing magnetic couple and electrostatic potential energy at short distances. Consequently, the electron transfer rate increases because of the proximity of the iron clusters (Figure 7).

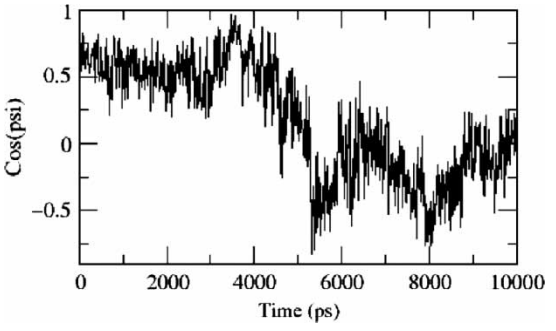

Figure 5. Time fluctuation of the angle between the isoalloxazine and adenine rings of FAD.

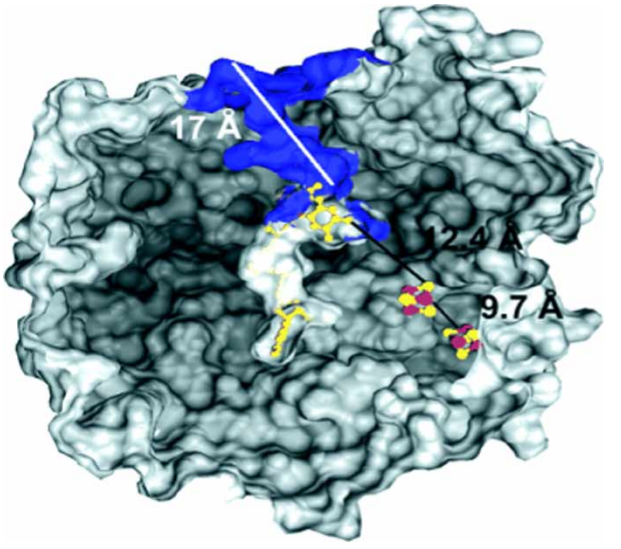

Figure 6. The active site channel of APS reductase. Shown is a cut through the molecular surface of APS reductase to indicate the active site channel (blue) and the position of the cofactors [3].

Figure 8 illustrates that the pressure of the box fluctuates with small amplitude around the reference value, whereas slightly larger fluctuations occur for the temperature. However, there is no significant influence on the total energy. This is corroborated by the comparison of the total energy with temperature–pressure. During the time course of the simulation, the total energy did not exceed $-9.0 \times 10^5\,\mathrm{kJ\,mol^{-1}}$, indicating that APS reductase is an energetically rather stable.

4. Conclusion

The present model accounts for the behaviour of APS reductase under pressure conditions found in oil wells in the Recôncavo Baiano region of Bahia. Also, it was able to account for the behaviour of specific protein regions, such as the active site and its channel as well as for the protein-bound FAD molecule.

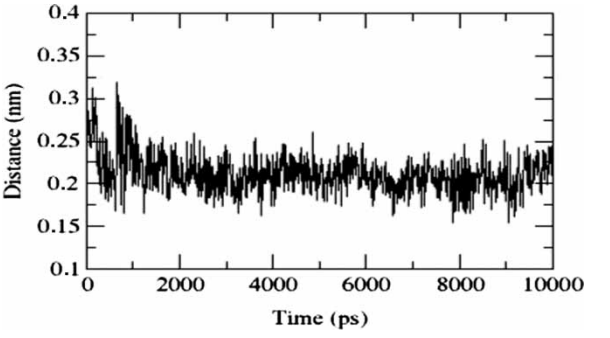

Figure 7. Fluctuation of the distance between clusters I and II during the 10 ns of simulation.

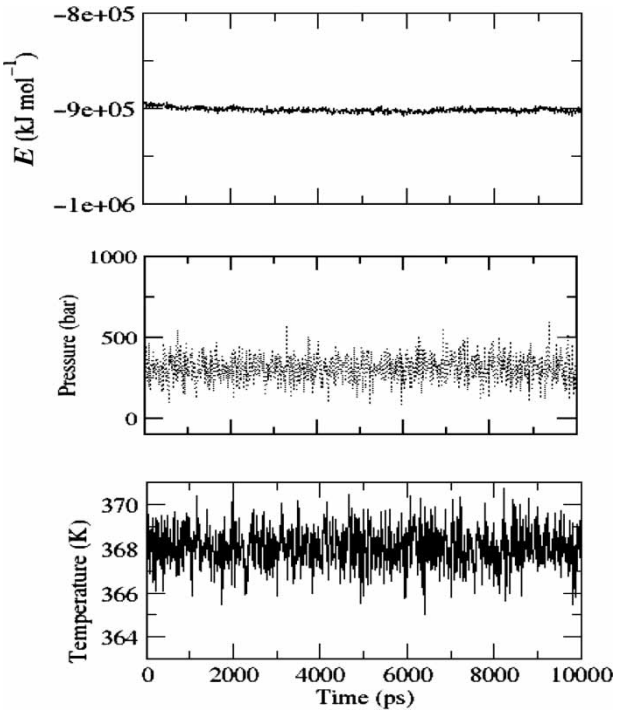

Figure 8. Significant influence of pressure and temperature fluctuations on total energy.

The parameters for the FAD molecule, obtained through a semi-empirical method, corroborated the data present in the current literature [9]. Partial electric charges of the atoms that constitute the iron–sulphur centres were obtained through *ab initio* calculations, in which only the atoms of the metallic centres had been considered. However, to obtain a highly accurate force field, it would be necessary to take into account the covalently bonded cysteine atoms.

In this study, we have used MD simulation to analyse the dynamic changes in the enzyme active site and in its channel. The results show that the protein is stable energetically, with no significant dependence on the temperature–pressure fluctuations. The temporal oscillation of the radius of gyration of the cluster of the component residues that surround the active site channel does suggest that the size and the shape of the channel change in time.

The FAD molecule remains stable in the active site region through hydrogen bonds and van der Waals interactions, and through electrostatic forces due to negatively charged protein residues. At the temperature of simulation, the FAD molecule acquires several conformations. The presence of a few water molecules near FAD should facilitate its stabilisation, and allows for substrate diffusion due to increased fluctuations of the enzyme side chains.

The motion of iron–sulphur clusters reduces the distance gradually during 1 ns simulation, whereas the distance stabilised after 2 ns simulation.

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