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RESEARCH ARTICLE

Identification of Selected Kinetoplastids 18S rRNA Residues Required for Efficient Recruitment of Initiator tRNA Met and AUG Selection *in silico*

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Abstract

High Resolution 18S rRNA structures of kinetoplastids ribosomes from theoretical methods have provided atomic level details about the process of translation. This process entails detailed information on the mRNA and tRNA binding and decoding centers within the 18S rRNA that was previously not very well understood. We identified residues in selected kinetoplastids 18S rRNA critical in recruiting the first methionyl tRNA to the small ribosome subunit during initiation and comparing them to see the differences. The Kozak sequence presence on eukaryotic mRNAs tethers it to the AUG start codon. Kinetoplastids are a closely related group, and the three chosen exhibited differences in the A-site in terms of position and nucleotides found there. Interactions are found at the A-site (543-UUU-546 for *T. cruzi*, 560-CCUA-563 for *T. brucei*, and 540-UUUG-543 for *Leishmania major*), where the different mRNA get complementary sequences at the 16th helix. The current findings show that each messenger RNA has a sequence that is complementary to the appropriate 18S rRNA sequence, tethering the mRNA to the small ribosomal subunit, which then recruits the bigger subunit. When compared to the Kozak region that flanks the AUG start codon, this method effectively promotes start codon placement.

Key Words: *18S rRNA; Ribosome; Scanning; Translation initiation; A site; Kinetoplastids*

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1. Introduction

1.1. Introduction to the ribosome

The ribosome is a ubiquitous part of the cell that is responsible for protein production. Ribosomes, which perform this translation process in all kinetoplastids, are evolutionarily similar due to operational aspect. Ribosomes are made up of a ribonucleic acid (RNA) as well as many proteins that have been coupled with one another to form a basic structure. These elements are either indirectly or actively engaged in the peptide synthesis process. Nucleotide oligomers merge to produce the polymer RNA, whereas amino acid oligomers merge to form the polymer protein. Because of the numerous functions that RNA molecules perform, these complexes are essential to molecular genetics. Those same operations may include a variety of catalytic duties as well as intricacies of transcriptomic engagement. While interacting with other molecules such as proteins, cofactors, or other RNAs, RNA can assume various three-dimensional structures to correspond with their functional diversity. It has been discovered that many relevant functional motifs have high sequence conservation, indicating the important roles that these RNAs have played throughout history. The desire to understand the underlying mechanisms for the various roles that RNA(s) play, and the RNA functions has sparked a tremendous amount of interest in structural biology. This is due, in part, to an increase in the availability of complete genome sequences. X-Ray crystallography and Nuclear Magnetic Resonance Spectroscopy are the main processes for revealing the three-dimensional configuration of biological molecules and macromolecules, though there are some concerted efforts to use other such as cryo-electron microscopy. NMR for isolated RNA domains is indeed the preferred technique for determining suitable configuration. Moreover, due to the size constraint of NMR in solving larger RNA, emerging methods must be developed [1]. Emerging conceptual techniques have been established to help reduce the time required to acquire the three-dimensional configuration of more complex structures [2]. Both homology and de novo modeling are examples of these. Considering the aforementioned techniques, the work reported in this research takes into account the highly conserved and biologically relevant ribosomal motifs from the small subunit of the selected kinetoplastids [3].

1.2. The eukaryotic 40S small subunit

From the various experimental methods that have been used to obtain the crystal structure of the ribosome, much atomic detail to the functional states has been revealed from the whole ribosome. Examples are the consorted efforts that obtained the structure of the thermos thermophillus 30S one from MRC, Cambridge [4] and that from Max Plank/Weizmann Institutes group [5]. Both groups gave evidence that the three-dimensional structures could be described clearly in personalized forms which have been further illustrated by other groups that have pursued to entangle the eukaryotic ribosome. The eukaryotic small ribosomal subunit could be subdivided into three regions the head, neck and body which incorporates a spur, shoulder and platform [2,6,7]. The subunit contains the 18S rRNA which has approximately 1700 nucleotides for Plasmodium falciparum and can be divided into four domains; the 3′ minor domain, the 3′major, the central domain and 5′domain [2,8]. The 40S ribosomal subunit also contains 34 proteins which are associated with the 18S rRNA [2,8] The main purpose of the eukaryotic 40S ribosomal subunit is to decode the genetic information from mRNA transcript [8,9]. The 40S subunit interacts with factors of initiation, initiator tRNA

and mRNA playing a key role in the assembly of the initiation complex before the commencement of the synthesis of the proteins [10,11]. The subunit also plays a key role of ensuring translational fidelity in protein synthesis by ensuring that the right anticodon-codon interaction are maintained [12-14] In the 18S rRNA, the helix 44 has been shown to be involved in the protein synthesis [8,15,16] which forms part of the A-site discussed in detail in the next section.

1.3. Inter subunit association

The small ribosomal subunit interacts with the large subunit to achieve the functionality in the course of protein synthesis using a magnesium dependent fashion [17,18]. Approximately 12 intersubunit bridges have been identified for the prokaryotic 70S ribosome [19,20]. In the process of elongation, these bridges have been proposed to undergo breakage and rearrangement during protein synthesis [19,21,22] resulting to a ratchet motion [23,24]. The bridges include interaction of RNA-RNA, RNA-protein both RNA-RNA to RNA-protein and finally protein to protein interactions [22,25,26]. The small subunit rRNA has been shown to have residues at a specific position that is kingdom specific as shown for position 702 residue [27].This position residue forms a helical region (helix 23) which adopts an interesting V-shape motif known as a kink turn which is considered to be dynamic [28,29]. In the course of translocation of both the mRNA and tRNA both the two ribosomal subunits have been shown to undergo a ratchet-like motion [21,30]. This process of contact breaks has been shown to continue during the process of translocation where bridges at both heads of the subunits are in contact [27,31]. One of the bridge contacts of the small subunit includes the A-site and the P-site [25,32] which is discussed in the following section.

1.4. Interactions of the tRNA and the ribosome

From the section above tRNA molecules interact with the ribosome during protein synthesis at three functionally distinct sites namely, the P-site, A-site, and the E-site. These sites that bind to the tRNA [25,32] are explained in detail in the following sections.

1.5. A-site

The A-site because of its role in ensuring that bases correctly pair between the mRNA codon and the tRNA having the cognate anticodon is sometimes known as the decoding site (see Table 1). Studies show a portion of the A-site is located in the small subunit 16S rRNA at helix 44 for the E.coli stretching from position 1404-1412 and 1488-1497 [33,34]. In this location, it is ensured that the addition of the new amino acid takes the correct tRNA to grow the polypeptide chain [35]. The new amino acid at 3'end of the tRNA of the A-site is made available for the formation of the peptide bond at the peptidyl transferase center (PTC) of the larger subunit [18,36]. At the small ribosomal subunit decoding center, there is a clearly visible anticodon stem loop (ASL) comprising nucleotides 26-44 of the tRNA [18,36]. The translation initiation has been suggested to happen when a stable cognate pair between mRNA/tRNA complex five hydrogen bonds binds to the three universally 16S rRNA conserved bases G530, A1492, and A1493 [34,35,37]. Translation occurs only when a stable pairing happens meaning a non-similar anticodon and codon pairing is not stable enough to propagate this process.

1.6. P-site

The site at which the initiator tRNA binds before translocation begins on the small ribosome is known as the P-site. The AUG codon recognition site on the mRNA to be translated by the small subunit signals the first amino acylated binding to the initiator tRNA [35,38]. The P-site that ensures codon fidelity is achieved during initiation and translation [34,39]. This site spans in the larger subunit becoming the second site binding the tRNA in the ribosome during translation [39].

The P-site maintains the reading frame as it has evolved to hold the tRNA tightly in a position which is important for peptidyl transfer [18]. The P-site bound to the tRNA in the small 30S subunit is stabilized by A-minor type I and type II interactions between the 16S rRNA G1338 and A1339 and GC base pair 30-40 and 29-49 of the P-site tRNA (Abdi & Fredrick, 2005; Khatter et al., 2015). Residues A1339 and G1338 in the 16S rRNA Interact with the ASL (Anti-codon Stem-Loop) of the P-site tRNA on one side and nucleotide 790 found at helix 24 of the 30S platform on the other side [34,41,42]. This ensures the tRNA does not move into the E-site. For the translocation of the tRNA from the P-site to the E-site, the two elements act as a translocation switch and as an initiator by moving apart [43-45]. Ribosomal proteins S13 and S9 interact with the P-site bound tRNA via phosphate oxygen's (For S13 position 29 and position 33 and 34 for the S9 protein) [18,43]. The P-site acceptor end interacts with the larger 50S subunit peptidyl transferase center (PTC) [46-48]. The 23S rRNA D-site at H69 makes direct minor groove interaction with the helix at nucleotide 11 and 12 and also the adjacent nucleotide 24 and 25 in the anti-codon stem, apart from just ASL and PTC interactions [22,26,34].

1.7. Peptidyl transferase center

The PTC center is the point at which the peptide bond formation takes place growing the peptide to the incoming tRNA from the A-site. This site is located at the larger ribosomal subunit. The PTC comprises 23S rRNA domain V primarily which facilitates the formation of the peptide bond by positioning first the 3' CCA ends of the aminoacyl-tRNA and peptidyl via the P- and A- loops binding [34,47]. The formation of the peptide bond in the PTC occurs via the primary amine of the A-site nucleophilic attack on the carbonyl carbon of the P-site bound tRNA's amino acid [18,49]. Studies done previously show that the larger ribosomal subunits (the 50S) are active in peptide bond formation, but the rate of catalysis is lower by a factor of about 1000 than that of intact (the 70S) ribosome [50-52]. It is a bit unclear how ribosomes accelerate the process of peptide formation, but current hypothesis suggests mostly if not entirely due to the substrate positioning within the active site [53-56] coupled to substrate-assisted catalysis [51,57-59].

1.8. E-site

The area occupied by the translocated deacylated tRNA from the P-site is known as the E-site. Both the small (30S) and larger (50S) ribosomal subunits have been proposed to bind to the E-site bound tRNA making interactions with the mRNA codon [60,61]. Recent studies from *Thermus thermophilus* 2.8 Å resolution crystalline structure [18] however, show no E-site codon-anticodon interaction. The middle anticodon base A35 was found to be closer to 16S RNA G693 than to the E-site codon [18,62]. This information is thus consistent with previous

studies [32] where the small subunit (the 30S) E-site was claimed to consist of S11 and S7 ribosomal proteins primarily [32,63]. The interactions of the E-site tRNA acceptor end in the large (50S) subunit with residues at the base of H82, and the D and T loop bases with the L1 stalk are thought to stabilize the stalk in a closed conformation [18].

2. Results and Discussion

Firstly, the three selected kinetoplastids (shown in Table 1) primary sequences and structures were analyzed to compare the 16 Helix. This showed that although the secondary structures are highly conserved there are fundamental variations when it comes to the primary sequences as shown in Figure 1. This conservation and variation may define the differences in the disease variation caused by this kinetoplastid class. Kinetoplastid parasites, unlike most eukaryotes studied thus far, use a bipartite form of RNA polymerase transcription to produce mature messenger RNAs. Pre-mRNAs are synthesized for all protein-coding genes from long head-to-tail arrangements known as polycistronic gene clumps, whereas RNAs encoding the capped 5′ ends of mature transcripts are transcribed separately from the spliced leader RNA array [64,65]. This would further suggest that the variation of the end cap regions of specific kinetoplastids mRNAs could be important to their translation by the specific ribosomes.

Table 1: *Three selected kinetoplastids T. cruzi, T. Brucei, and leishmania major structures analyzed in the study showing the rRNA class, the number of nucleotides each has, the accession number and the A-site nucleotides involved in the role of translation.*

Kinetoplastid	RNA Class	Nucleotide base number	Accession Number	A-site
T. cruzi	18S	2315	AF245382	543-UUUU-546
T. brucei	18S	2251	M12676	560-CCUA-563
Leishmania major	18S	2203	AC005806	540-UUUG-543

The structure of helix 16 of the 18S rRNA of the selected kinetoplastids gives us required information to uncover the process of transcription. This section where the A site lies unveils the base pairing concept between the sequence of the ribosomal RNA and the kinetoplastid mRNAs. This facilitates the arrangement on the start codon which complements the stabilizing role of the consensus Kozak sequence that franks the AUG start codon [66,67].

The tip of eukaryotic helix h16, which can base pair with the kinetoplastids mRNA sequence preceding the three-way junction, is the key regulatory site, according to functional and structural data. This increased interplay can sometimes recompense for weak or deficient Kozak consensus sequences at mRNAs. This tethering mechanism explains why slippage on a second start codon does not occur and provides specificity for the formation of translation initiation complexes on the first start codon of mRNAs. It increases the general affinity for the small subunit and correctly localizes the ribosome on the mRNAs start codon by directly forming base-pair interactions with the tip of the ribosomal h16, preventing scanning. Residue U can base pair with A and G residues, according to the wobble rules. As a result, the complexity of base pairing with the UUUC sequence increases, implying that the interaction with h16 may similarly help many other mRNAs. Furthermore, the presence of the three way junction folded domain stabilizes the base-pairing interactions by locking the ribosome in a pre-translocation conformation [66,67].

The mRNA binding at the tip of helix h16 (18S rRNA) shows that mRNA and the rRNA have a direct connection. This region of the 18S rRNA of the three selected kinetoplasts having an apical (543-UUUU-546 for *T. cruzi*, 560-CCUA-563 for *T. brucei* and 540-UUUG-543 for *Leishmania major*) tetra loop in which the four nucleotides are frequently flipped out in diverse kinetoplastid ribosomal configurations (Figure 2-4).

To find the nucleotides that interact with the 18S rRNA, we used nucleotide substitution to probe the mRNA structure and binding and docking experiments to monitor binding to the 18S ribosomal RNA. A-site binding was found in the nucleotides upstream of the three way junction domain [66,68] (numbering commencing on the A of the AUG codon, or h4 nucleotides.

Interestingly, mutations within positions 26 to 30 (26AAGGG30) could establish a putative interaction site at the mRNA channel's entry by base-pairing with nucleotides of the h16 tetraloop (540UUUC543). The distance between the mRNA on the AUG on the P-site and the TWJ on helix h16, as revealed by mRNA modeling, would support such a contact (Figure 1). This motivated us to alter these h4 nucleotides to see if ribosome placement was altered. Single nucleotides 26 to 30 mutants were created and tested ensuring no loss of functionalities [67]as shown in the alignments and Figure 1 below.

>*Leishmania major* (AC005806) HELIX 16 526-555 GAGUUGUCAGUCCAUUUGGAUUGUCAUUUC
>*Trypanosoma brucei* (M12676) HEKIX 16 548-580 GAGCCGACCGUGCCCUAGUGCAUGGUUGUUUUC
>*Trypanosoma cruzi* (AF245382) HELIX 16 530-560 GAGCCGACAGUGCUUUUGCAUUGUCGUUUUC

```
                1       10        20 23
                |-------+---------+--|
T.cruzi         GAGCCGACAGGC----GCAGCGC
T.brucei        GAGCCGACCGGCCCAGGCAGGGC
Leishmania      GAGGCAGCCAGG------AGCAC
Consensus       GAGcCgaCcgGc....gcAGcgC
```

Figure 1: *Sequence alignment of the three selected kinetoplastics helix 16 which contains the A site.*

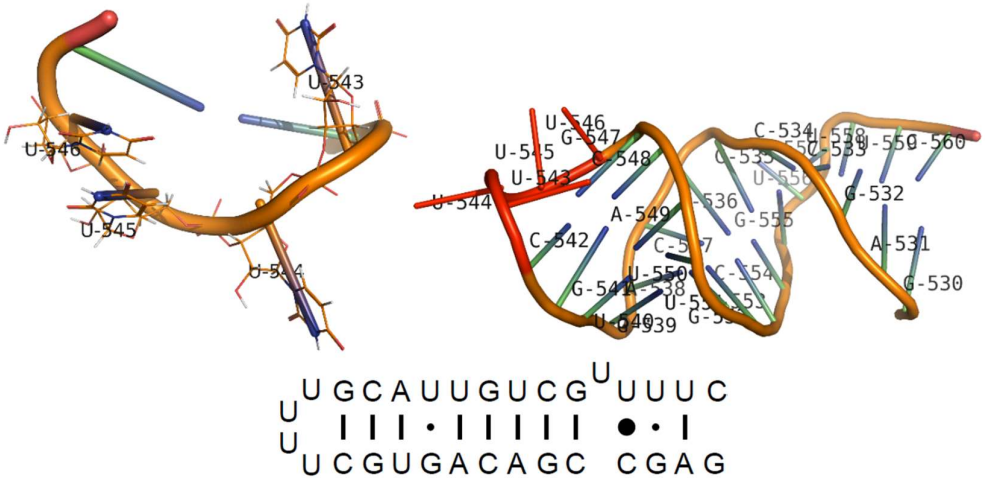

Figure 2: *Trypanosoma cruzi (AF245382) A site showing the specific residues that form the helix and the resulting site.*

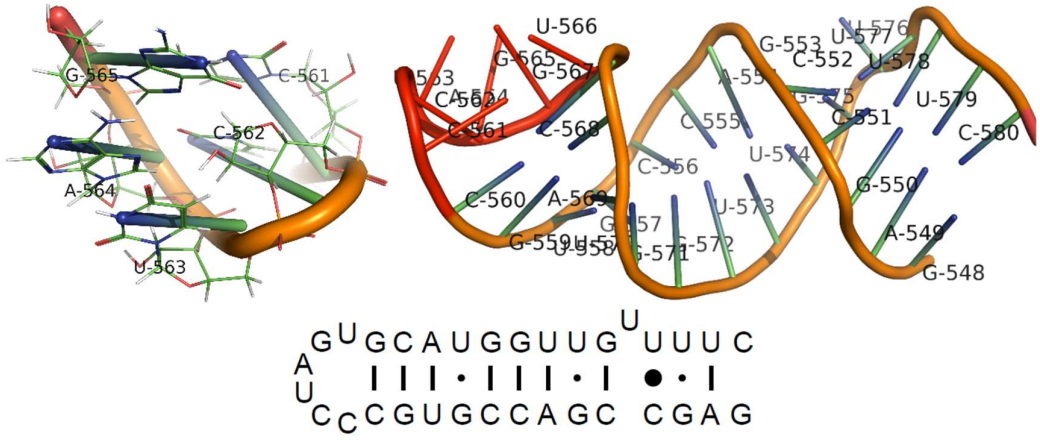

Figure 3: *Trypanosoma brucei (M12676) A site showing the specific residues that form the helix and the resulting site.*

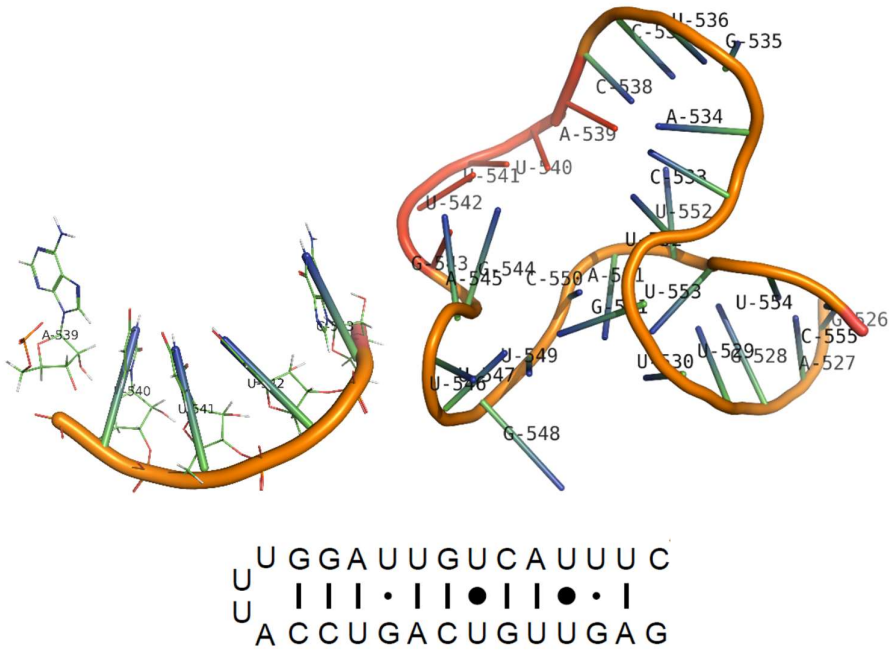

Figure 4: *Leishmania major (AC005806) A site showing the specific residues that form the helix and the resulting site.*

3. Material and Methods

3.1. Selection and three-dimensional modeling of kinetioplastids 18S rRNA

The sequences of the three kinetoplastids; *Leishmania major*, *Trypanosoma brucei*, and *Trypanosoma cruzi* 18S rRNA were obtained through blasting in the gene bank (NCBI) [36]. These are shown in the supplementary section with their details. It is important to note that most of the sequences in the gene bank are not complete, so a further process of verification was required. We checked the completeness of the sequences using information available at specialized groups that do verification of 18S rRNA sequences. Such a group is The

Comparative RNA Web (CRW) Site, which has a database that shows the completeness of sequences among other analyzed and verified annotations [26]. The sequences picked from this site were further analyzed to ascertain the sequences and minimize the errors. Back to the gene bank, the FASTA format of these refined sequences was picked and saved as text files using a notepad++ text editor (https://notepad-plus-plus.org/) (accessed on 15 December 2020). Since there is a possibility of having more than one complete sequence, the final query sequence to be modeled was obtained by carrying out further multiple sequence alignment to identify the one that deviated minimally from the consensus sequence. MultiAlign [19] software was used in the alignment to show how similar or dissimilar various sequences were.

3.2. Selecting a template

Selection of template structures for the three kinetoplastid rRNA was a rigorous exercise that is described below. These required a search of various structure libraries using the query sequence.

3.3. Obtaining and verifying template sequences and three-dimensional coordinate files

The templates for all the three kinetoplastids were again selected through an elaborate process that involved several steps. Firstly, by blasting individual query sequences in the gene bank (NCBI) and finding the sequences that are highly similar to the query and not in any way the query (we did not select the query if it was shown in the BLAST alignments) [37]. These sequences infer an evolutionary relationship with the query but not specifically the query if they cover a distinct region of the template to get a higher similarity score. Calculation of a local pair-wise alignment between the templates and their targets was performed. Secondly, checks and evaluations were done as the section above to check if these sequences were complete followed by a heuristic step, which intended to improve the alignment. Insertion and deletion placements in the template were considered for optimization. Of particular interest were the isolated residues in the alignment (Islands), which were moved to the flanks for the facilitation of loop building.

The next step was to find out if there was any crystal structure of the 18S rRNA template sequences that were available. This was done by checking the Research Collaborators for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) archive, which gives the 3D shapes of nucleic acids, proteins, and complex assemblies that help researchers and students understand all aspects of biology [38]. Coordinate files of the template structures of the 18S rRNAs obtained from the PDB website available online (http://www.rscb.org/pdb/explore.do) (accessed on 15 December 2020) were saved as PDB files on a text editor. Depending on the complexity of the rRNA homology, de novo modeling was done in parts by dividing it into the different parts after cleaning: 50major, central, 30minor, and 30major domains. An important point noted was one should align and cut the rRNA domains of both the template and the query sequences at similar points to achieve the best structure at the end. The structure was viewed in different software available that can read PDB files, such as pymol and Accelrys, among others. The crystal structures of the templates obtained had some challenges such as unresolved portions and gaps and were required to be further optimized.

3.4. Homology and de novo modeling

18S rRNA homology and de novo modeling were done using RNA123 version 2.0.1.3 and Genesilico software. RNA123 was able to predict the secondary and tertiary structure of the three kinetoplastids ribosomal RNA. RNA123 took three steps: Preprocessing, Alignment and Modeling [18]. Curation and validation of the built model was performed using several validation tools, such as PRO-CHECK [39], MATCHCHECK [39], and MOL-PROBITY [40]. These software help to understand the stereochemistry and geometry of the 3D structure of the modeled 18S rRNA. Ramachandran plot statistics were used to evaluate the best model. The outcome of simulation using Procheck and MolProbity provided valuable information on the quality and accuracy of a nucleotide structure determined through in silico simulation. The results of these analyses helped identify and correct errors in the structure, as well as provide insight into the structural features that may be important for the rRNA's function. Overall, these tools improved the reliability and confidence in the results of in silico simulation and aided in the interpretation of the outcome of the simulation. The built model was further superimposed on the experimental crystal structure of the template and the root mean square deviation (RMSD) was calculated [41].

3.5. 2D and three D superimposition and alignment of the kinetoplastid models

The modeled structures were superimposed both using the secondary and the three dimensional coordinates to be able to show the areas that shared potential homologs with each other and the emerging differences [64]. Keen to note the areas with homologs are much conserved sections that are important in the functional duties of the mRNA. Whereas the divergent section could be indicative of highly variable region which could not change the functionality of the mRNA [65].

4. Conclusion and Recommendations

In silico biological structures simulation is a powerful tool for understanding and predicting the behavior of biological systems. This method allows for the creation of virtual models of biological molecules and their interactions, which can be used to study a wide range of biological processes. The simulations can be used to predict the structure and function of proteins, nucleic acids, and other biomolecules, as well as to study the interactions between these molecules and other components of the cell.

Overall, in silico simulation is a valuable tool for advancing our understanding of biological systems and can be used to improve drug design, predict the effects of genetic mutations, and study the mechanisms of disease. It can also be used in many areas of biotechnology and medicine.

In conclusion, in silico biological structures simulation is an important tool for understanding kinetoplastids biological systems and can be used to improve drug design, predict the effects of genetic mutations, and study the mechanisms of disease. It is recommended to continue investment in this field and collaborate with other researchers and institutions to advance the capabilities of in silico simulation and improve its applications in biomedical research.

Authors' Contributions

Conceptualization, HNM and FJM; methodology, HNM, PWW and FJM; data analysis, HNM, AN and FJM; data curation, HNM, NK; PWW, AN and FJM; writing—original draft preparation, HNM, AN and FJM; writing—review and editing, HNM, NK, AN and FJM; project administration, HNM, NK, AN and FJM All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of University of Nairobi, Approval Date: 7 December 2020. Informed Consent Statement: Not applicable.

Data Availability Statement

Data is contained within the article and supplementary data available if required contact the corresponding author.

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