Journal of
Computer Science & Systems Biology

Fernandes et al., J Comput Sci Syst Biol 2014, 8:1
DOI: 10.4172/jcsb.1000164

Research Article Open Access

Molecular Modeling of Heat Shock Protein of 60-Kda from *Paracoccidioides Brasiliensis*: The First *in silico* Structural Model of a Fungal Hsp60

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Abstract

Paracoccidioides brasiliensis is a dimorphic fungus that causes paracoccidioido mycosis (PCM), an endemic mycosis in Latin America. PCM is a chronic, granulomatous, and progressive disease, which has a wide clinical spectrum of manifestations. Although it knows that the main clinical forms are consequences of fungus-host interaction, immune response in PCM is still an open field. The antigenic complexity of *P. brasiliensis* and the role of most antigens have been poorly explored, thereby decreasing the chances of finding vaccine and therapeutic targets for PCM. Recent results from our group have shown that heat shock protein of 60-kDa from *P. brasiliensis* strain 18 (Hsp60_Pb18) has a possible detrimental effect on the course of the PCM. Here, we show the molecular model of Hsp60_Pb18 that was generated with the program MODELLER9V8. The model validation was performed using PROCHECK and VERIFY3D. According to the results, the three-dimensional structure of Hsp60_Pb18 is a high reliability model, which displays a remarkable similarity with the three distinct domains of GroEL subunit-equatorial, intermediate and apical domains. This study will provide direction and continuity of studies to characterize Hsp60_Pb18 and their domains, and thus contribute to the knowledge of the fungus biology and to determine vaccines and/or therapeutic targets.

Keywords: *Paracoccidioides brasiliensis*; Vaccines target; Heat shock protein; Modeller, 3D structure

Introduction

Paracoccidioidomycosis (PCM) is a systemic mycosis of chronic granulomatous nature caused by the fungus *Paracoccidioides brasiliensis* [1]. This is the most prevalent systemic mycosis in Latin America with about 10 million of infected people, of which 1 to 2% will develop the disease manifestations. Although Brazil, Argentina, Colombia and Venezuela are responsible for almost all cases, Brazil contains the largest number of endemic areas, representing 80% of all reported cases of PCM in Latin America [2].

The infection with *P. brasiliensis* is initiated when the host inhales fragments or conidia from mycelium, which is the form of the *P. brasiliensis* at environmental temperature. Because of the host temperature, 35 to 37°C, primarily in the lungs, the mycelial form is converted to yeasts, which are the resistance form of the fungus [1]. *P. brasiliensis* infection can be restricted to the lungs or disseminated to other organs, leading to a wide spectrum of manifestations, i.e. the infection can range from asymptomatic to severe disseminated forms. PCM can be classified in two main clinical forms, acute/subacute and chronic ones. The acute/subacute disease has a rapid onset and affects the mononuclear phagocyte system of young people from both genders. The most common form of PCM is the chronic one, which has a slow evolution, involves lesions in few organs and mucosa mainly in adult men [3]. Some disseminated forms are associated with a less efficient immune response, a worse prognosis and more frequent relapses of the disease, as occurs in acute and subacute and chronic severe forms [4].

The antigenic complexity of *P. brasiliensis* has been little explored in terms of isolation and identification of new antigens and, consequently, elucidation of the role these molecules play in PCM. Our group has worked with identification of *P. brasiliensis* antigens [5] and studied the action of adjuvants or antigens in order to find important molecules for the development of immunotherapy for the PCM [6-9]. More recently, we identified heat shock protein of 60-kDa (Hsp60) from fungal fractions as a component that worsens the experimental PCM. Despite the Hsp60 presents a protective effect against PCM when administered prophylactically [10], our results show that this protein leads to a more severe experimental PCM with increased fungal burden and tissue injury when therapeutically administered (unpublished data). Therefore, this issue is of fundamental importance for the design of new vaccines and treatment, as well as to understand the biology of the fungus. Among potential vaccine candidates for infection diseases or cancer are the heat-shock proteins (Hsp), since they are associated with different phenomena of innate and adaptive immunity [11,12]. Initially, Hsp were seen as ubiquitous molecules produced by organisms or cells in response to exposure to elevated temperatures. In fact, Hsp are molecules involved in functions as molecular chaperones and co-chaperones by binding to intracellular and misfolded proteins, preventing the aggregation of these molecules and promoting their proper refolding and transport [13,14]. They are grouped in families based on their sequence homology and molecular weight, such as human Hsp110, Hsp90, Hsp70, Hsp60, Hsp40 and small Hsp [15].

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Received October 10, 2014; **Accepted** November 04, 2014; **Published** January 03, 2015

Citation: Fernandes FF, Rossetti RAM, Coelho-Castelo A, Panunto-Castelo A (2014) Molecular Modeling of Heat Shock Protein of 60-Kda from *Paracoccidioides Brasiliensis:* The First *in silico* Structural Model of a Fungal Hsp60. J Comput Sci Syst Biol 8: 241-244. doi:10.4172/jcsb.1000164

Hsp60 is a molecular chaperone known to assist protein folding in prokaryotes and in eukaryotic cell organelles [16]. In eukaryotes, Hsp60 typically resides in the organelles mitochondria and chloroplasts but it also occurs in the cytosol [17], mammalian cell surface [18], fungal cell wall [19] and extracellular space [20]. Extracellular Hsp60 can interact with a number of cell-surface receptors, such as CD14, CD40 and Toll-like-receptors (TLRs) [21], and induce pro- and anti-inflammatory effects [22].

Fungal Hsp60 has been identified acting as immunodominant antigens, resulting in humoral and cellular responses, including an induction of regulatory T cells through interaction with cells of the innate immune system [23,24]. Moreover, Raggam et al. [25] have shown that Hsp60 mRNA could be induced in eight fungal species under stress conditions and suggested that fungal Hsp60 might play a key coordinating role in the immune response of fungi-associated diseases.

Recent results from our group have shown that Hsp60 from *P. brasiliensis* has a potential detrimental effect on the course of PCM (unpublished data), probably because Hsp60 can modulate immune response by stimulating T regulatory cells [26], as well as other immune system components [27].

Bioinformatics tools have been applied to the identification of new molecular structures, relating them to their possible functions and thus directing future studies more objectively. In this study, we show the predicted three-dimensional heat shock protein of 60-kDa from *P. brasiliensis* strain 18 (Hsp60_Pb18) performed by molecular modeling and *in silico* evaluation of this proposed structure. According to these results, the Hsp60 model is highly reliable.

Methods

Sequence retrieval and alignment

The sequence of the Hsp60_Pb18 (PADG_08369) from *P. brasiliensis* was retrieved from the Broad Institute, in a FASTA format (http://www.broadinstitute.org/annotation/genome/*paracoccidioides_brasiliensis*/TranscriptDetails.html?sp=S7000001960871783). Search for similar proteins with determined three-dimensional structures was done in the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). After obtaining the similar protein, the alignment was made using the software ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Model building and evaluation

The MODELLER9V8 [28] was used to predict the three-dimensional structure of the protein. The obtained model was stereochemically validated by PROCHECK [29] and VERIFY3D [30]. The PROCHECK checked if the spatial properties were right, such as peptide bond planarity, nonbonded interactions, main chain hydrogen bond energy, and others. The VERIFY3D validated the refined model by analyzing the compatibility of an atomic model (3 Dimensional) with its own amino acid sequence (1 Dimensional), throughout a validated known score matrix built based on structures from the Protein Data Bank (PDB).

Results and Discussion

The complete protein sequence of Hsp60_Pb18 (PADG_08369.1) was used in the study. The length of Hsp60_Pb18 is 595 amino acids, with expected molecular weight of 62,389.21Da and isoelectric point (pI) of 5.51. Figure 1 shows the amino acid sequence of the Hsp60_Pb18 with the conserved chaperones domain Cpn60/TCP1. Proteins with these domains are induced under stress and work on the stabilization and protection of the polypeptides arrangement under conditions of heat shock (NCBI-http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

```
MQRAFTSSRALVLSSASSASSTRAPLSRFRSAGVGLQQQRFAHKELKFGVEARASLLKGIDT
LAKAVTTTLGPKGRNVLIESPYGSPKITKDGVTVAKAVNLQDKFENLGARLLQDVASKTNEV
AGDGTTTATVLARAIFSETVKNVAAGCNPMDLRRGIQSAVEAVVEYLQANKRDITTTEEIAQ
VATISANGDTHVGKLISNAMEKVGKEGVITVKDGKTIDDELEVTEGMRFDRGYVSPYFITDT
KAQKVEFEKPLILLSEKKISAVQDIIPALEASTSLRRPLVIIAEDIEGEALAVCILNKLRGQ
LQVAAVKAPGFGDNRKSILGDIGILTNATVFTDELDLKLEKATPDMLGSTGSITITKEDTII
LNGEGSKDAIAQRCEQIRSVISDPATSDYEKEKLQERLAKLSGGVAVIKVGGASEIEVGEKK
DRVVDALNATRAAVEEGILPGGGTALLKAAANGLTSLNPTNFDQKLGISIIKNAITRPARTI
VENSGLEGSVIVGKLTDDFASDFNRGFDSAKGEYVDMIGAGIVDPLKVVRTALVDASGVASL
LGTTEVAIVEAPEEKVPAGSGAGGMGGGMGGGMGGGMF
```

Figure 1: Amino acid sequence of the Hsp60_Pb18 protein obtained from the Broad Institute. The chaperone protein domain Cpn60/TCP1 is marked in blue.

```
Hsp60Pb18   MQRAFTSSRALVLSSASSASSTRAPLSRFRSAGVGLQQQRFAHKELKFGVEARASLLKGI
1WE3        ------------------------------------------MAKILVFDEAARRALERGV
                                                        * * *.  ** :* :*:

Hsp60Pb18   DTLAKAVTTTLGPKGRNVLIESPYGSPKITKDGVTVAKAVNLQDKFENLGARLLQDVASK
1WE3        NAVANAVKVTLGPRGRNVVLEKKFGSPTITKDGVTVAKEVELEDHLENIGAQLLKEVASK
            :::*:**..****:****:.*. :***.********** *:*:*:::**:**:**::****

Hsp60Pb18   TNEVAGDGTTTATVLARAIFSETVKNVAAGCNPMDLRRGIQSAVEAVVEYLQANKRDITT
1WE3        TNDVAGDGTTTATVLAQAIVREGLKNVAAGANPLALKRGIEKAVEAAVEKIKALAIPVED
            **:**************:**. * :******.**: *:***:.****.** ::*    :

Hsp60Pb18   TEEIAQVATISANGDTHVGKLISNAMEKVGKEGVITVKDGKTIDDELEVTEGMRFDRGYV
1WE3        RKAIEEVATISAN-DPEVGKLIADAMEKVGKEGIITVEESKSLETELKFVEGYQFDKGYI
             : * :******* *..*****::*********:***::.*::: **:..** :**:**:

Hsp60Pb18   SPYFITDTKAQKVEFEKPLILLSEKKISAVQDIIPALEASTSLRRPLVIIAEDIEGEALA
1WE3        SPYFVTNPETMEAVLEDAFILIVEKKVSNVRELLPILEQVAQTGKPLLIIAEDVEGEALA
            ****:*:.:: :. :*..:**: ***:* *::::* **  :.  :**:*****:******

Hsp60Pb18   VCILNKLRGQLQVAAVKAPGFGDNRKSILGDIGILTNATVFTDELDLKLEKATPDMLGST
1WE3        TLVVNKLRGTLSVAAVKAPGFGDRRKEMLKDIAAVTGGTVISEELGFKLENATLSMLGRA
            . ::***** *.***********.**.:* **. :*..**:::**.:***:** .*** :

Hsp60Pb18   GSITITKEDTIILNGEGSKDAIAQRCEQIRSVISDPATSDYEKEKLQERLAKLSGGVAVI
1WE3        ERVRITKDETTIVGGKGKKEDIEARINGIKKELET-TDSEYAREKLQERLAKLAGGVAVI
              : ***::* *:.*:*.*: *  * : *:. :.  : *:* :**********:******

Hsp60Pb18   KVGGASEIEVGEKKDRVVDALNATRAAVEEGILPGGGTALLKAAANGLTSLNPTNFDQKL
1WE3        RVGAATETELKEKKHRFEDALNATRAAVEEGIVPGGGVTLLRAISAVEELIKKLEGDEAT
            :**.*:* *: ***.*. **************:****.:**:* :     ::  : *:

Hsp60Pb18   GISIIKNAITRPARTIVENSGLEGSVIVGKLTDDFASDFNRGFDSAKGEYVDMIGAGIVD
1WE3        GAKIVRRALEEPARQIAENAGYEGSVIVQQILAETKNPR-YGFNAATGEFVDMVEAGIVD
            * .*::.*: .*** *.**:* ****** ::  :  .    **::*.**:***: *****

Hsp60Pb18   PLKVVRTALVDASGVASLLGTTEVAIVEAPEEKVPAGSGAGGMGGGMGGGMGGGMF
1WE3        PAKVTRSALQNAASIGALILTTEAVVAEKPEKKESTPASAGAGDMDF--------
             * **.*:** :*:.:.:*: ***..:.* **:* .: :.**. . .:
```

Figure 2: Amino acid sequence alignment of Hsp60_Pb18 protein with the 1WE3 protein sequence using Clustal W2. The displayed similarity between the sequences was 54%. The signals *, : and . indicate identical amino acids, conservative changes and semiconservative modifications, respectively, occupying the same position in both sequences.

As Hsp60_Pb18 did not have a three-dimensional structure available, we searched for other proteins that shared high amino acid sequence similarity with Hsp60_Pb18 and could be used as a template for protein modeling. We found three possible model proteins (1IOKA, 1KP8A e 1WE3A) by using the BLAST program and confirmed the high percentages of similarities between these sequences by ClustalW2 align software (data not shown). The modeling of protein Hsp60_Pb18 was performed with the three selected sequences, but the generated model was based on 1WE3A protein (54% similarity) (Figure 2), which provided the best stereochemical results (Figure 3). As expected, the obtained structure is quite similar to best-characterized chaperonin subunit GroEL (cpn60) from *Escherichia coli*, and comprises of three domains. The largest domain is the equatorial, which is high α-helical and well-ordered. The intermediate domain is the smaller and provides

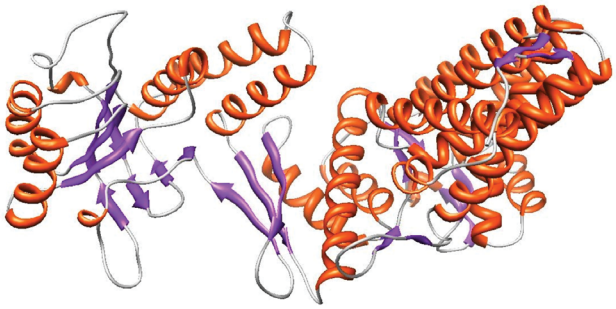

Figure 3: The best model of the 3D structure of the Hsp60_Pb18 protein obtained by Modeller9v2 based on the alignment with 1WE3A protein. In red are the α-helices and the β-sheets are in lilac.

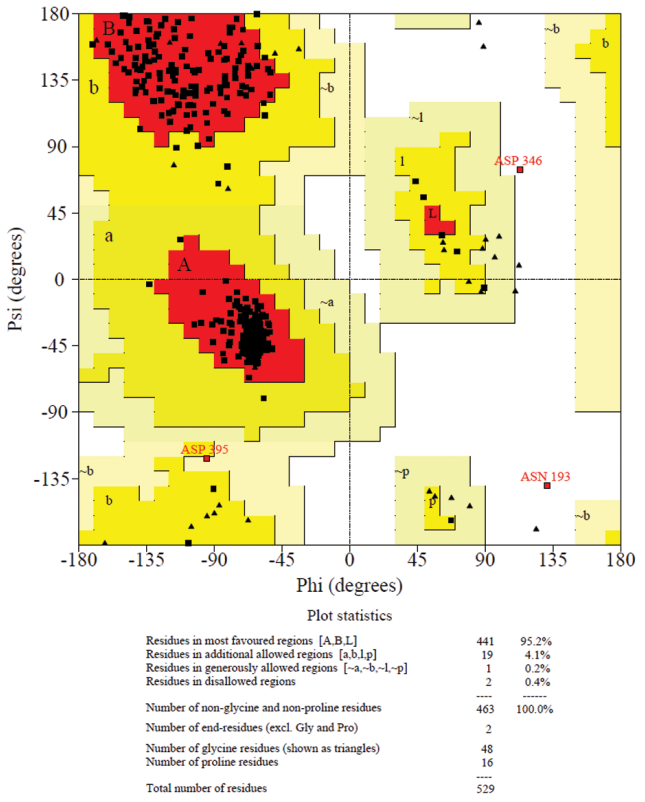

Figure 4: Ramachandran plot of the psi/phi distribution of the Hsp60_Pb18 model as obtained by PROCHECK.

connection between equatorial and apical domains. The apical domain is a mixture of α-helices and β-sheet. Once the modeled structure is quite similar to subunit cpn60 of GroEL family [31], we suggest that the domains of Hsp60_Pb18 have analogous functions, i.e., equatorial domain with ATPase function and apical domain with binding site for non-native proteins and GroES [31].

The level quality of a model generated by structural homology depends on a large number of properties, such as stereochemistry accuracy, quality, packaging and reliability of folding [32]. Thus, the generated model was subject to validation using PROCHECK [29] and VERIFY3D [30]. These two software applications encompass the analysis of the main properties to obtain a good molecular model.

The Ramashandran plot shows that the Hsp60_Pb18 structure has 95.2% residues in most favorable regions, 4.1% in additionally allowed regions and only 0.4% in disallowed regions. To be considered a good model, 90% of the residues should be located in favorable regions, so the model obtained can be considered a good representation of the 3D structure of the protein in question (Figure 4). Models with a high degree of reliability may be promising in the search for biologically active compounds as well as in optimizing prototypes, serving as a structural basis for the testing of hypotheses in medicinal chemistry, for example, in planning selective drugs for a particular therapeutic target [33].

The analysis performed by VERIFY3D evaluated the folding reliability, based on a statistical analysis involving the protein structures from the PDB. The results generated by this program showed that all residues are within acceptable range (between 0 and 0.62). Thus, it can be stated that the proposed model is consistent with the stereochemical parameters described above.

The three-dimensional structure generated in this work provides a good model for docking studies, mainly between this protein and the receptors of innate immunity cells, which could contribute to the knowledge of stimuli and receptors involved in the modulation of the immune response during PCM. Furthermore, this study will help to the development drugs that interact with important domains of this protein, specifically, and thus inhibiting its activity deleterious to the host.

Conclusion

According to this study, we can say that the Hsp60_Pb18 three-dimensional structure is a reliable model, but it is only predictive, and needs experimental confirmation. Therefore, these results will contribute to the direction and continuity of studies to characterize Hsp60_Pb18 and their domains, collaborating to the knowledge of the fungus biology and to determine vaccines and/or therapeutic targets.

Acknowledgement

We acknowledge the financial support of the São Paulo Research foundation –FAPESP (Grant numbers 2009/03235-3 and 2009/12278-3) and CNPq.

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