Biochimica et Biophysica Acta 1794 (2009) 655–662

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Stability improvement of the fatty acid binding protein Sm14 from *S. mansoni* by Cys replacement: Structural and functional characterization of a vaccine candidate☆,☆☆

Celso R.R. Ramos [a,b,c], Alberto Spisni [d,*], Sérgio Oyama Jr. [e], Mauricio L. Sforça [e], Henrique R. Ramos [a,b], Mônica M. Vilar [c], Adriana C. Alves [e], Rita C.R. Figueredo [e], Míriam Tendler [c], Nilson I.T. Zanchin [e], Thelma A. Pertinhez [d], Paulo Lee Ho [a,b,*]

[a] *Biotechnology Center, Butantan Institute, Av. Vital Brasil 1500, 05503-900, São Paulo, SP, Brazil*
[b] *Chemistry Institute, University of São Paulo, 05508-900, São Paulo, SP, Brazil*
[c] *Helminthology Department, Oswaldo Cruz Institute, Av. Brasil 4365, 21045-900, Rio de Janeiro, RJ, Brazil*
[d] *Department of Experimental Medicine, University of Parma, Via Volturno 39, 43100, Parma, Italy*
[e] *Structural Molecular Biology Center, Brazilian Synchrotron Light Laboratory, C.P. 6192, 13084-971, Campinas, Brazil*

ARTICLE INFO

Article history:
Received 24 September 2008
Received in revised form 21 November 2008
Accepted 4 December 2008
Available online 25 December 2008

Keywords:
Nuclear Magnetic Resonance
Sm14
Vaccine
Schistosoma mansoni
Schistosomiasis
Fatty-acid binding protein

ABSTRACT

The *Schistosoma mansoni* fatty acid binding protein (FABP), Sm14, is a vaccine candidate against, *S. mansoni* and *F. hepatica*. Previously, we demonstrated the importance of a correct fold to achieve protection in immunized animals after cercariae challenge [[10]. C.R.R. Ramos, R.C.R. Figueredo, T.A. Pertinhez, M.M. Vilar, A.L.T.O. Nascimento, M. Tendler, I. Raw, A. Spisni, P.L. Ho, Gene structure and M20T polymorphism of the *Schistosoma mansoni* Sm14 fatty acid-binding protein: structural, functional and immunoprotection analysis. J. Biol. Chem. 278 (2003) 12745–12751.]. Here we show that the reduction of vaccine efficacy over time is due to protein dimerization and subsequent aggregation. We produced the mutants Sm14-M20(C62S) and Sm14-M20(C62V) that, as expected, did not dimerize in SDS-PAGE. Molecular dynamics calculations and unfolding experiments highlighted a higher structural stability of these mutants with respect to the wild-type. In addition, we found that the mutated proteins, after thermal denaturation, refolded to their active native molecular architecture as proved by the recovery of the fatty acid binding ability. Sm14-M20(C62V) turned out to be the more stable form over time, providing the basis to determine the first 3D solution structure of a Sm14 protein in its apo-form. Overall, Sm14-M20(C62V) possesses an improved structural stability over time, an essential feature to preserve its immunization capability and, in experimentally immunized animals, it exhibits a protection effect against *S. mansoni* cercariae infections comparable to the one obtained with the wild-type protein. These facts indicate this protein as a good lead molecule for large-scale production and for developing an effective Sm14 based anti-helminthes vaccine.

1. Introduction

Schistosomiasis, is a disease caused by blood flukes belonging to the genus *Schistosoma*, and represents the most important human parasitic disease, after malaria, primarily for its magnitude and impact in young population living in endemic areas. More than 600 million people are at risk in those areas and nearly 200 million are infected [1]. The endemic zones are located in tropical and sub-tropical regions characterized by lack of sanitation, which favors its spread and maintenance. For the last decades, chemical therapy has been the major tool in control programs. However, in spite of the extensive and massive use of effective anti parasitic drugs, the epidemiological scenario has not changed. In the '90s, World Health Organization/Special Program for Research and Training in Tropical Diseases (WHO/TDR) created a special promotional program for the development of new and more effective vaccines against malaria, leishmaniasis and schistosomiasis [2]. In that context "push strategies" were initiated to foster innovative projects.

Abbreviations: CD, circular dichroism; DAUDA, 11-(dansylamino) undecanoic acid; FABP, fatty acid binding protein; *F. hepatica*, *Fasciola hepatica*; MD, molecular dynamics; MPL, monophosphoryl lipid A; PBS, phosphate-buffered saline; Rg, radius of gyration; RMSD, root mean square deviation; *S. mansoni*, *Schistosoma mansoni*; SDS-PAGE, sodium dodecyl sulfate poliacrylamide gel electrophoresis; TDM, trehalose dicoryno-mycolate; WHO/TDR, World Health Organization/Special Program for Research and Training in Tropical Diseases

☆ This research was partly supported by grants from the São Paulo State Research Foundation (FAPESP), Brazil, to AS (Proc. 99/11030-9, Proc. 99/07574-3) and to PLH (Proc. 97/13476-9). MLS (Proc. 01/08095-3) and SOJ (Proc. 01/08001-9) are recipients of Post-doctoral fellowships from FAPESP, TAP (Proc. 00/02026-7), is the recipient of a FAPESP (Brazil) Career Award Fellowship and HRR (Proc. 03/06011-2) is a recipient of a doctoral degree fellowship from FAPESP.

☆☆ The chemical shifts have been deposited in the BioMagResBank data base (http://www.bmrb.wisc.edu) under accession numbers 6150, and the atomic coordinates have been deposited in the in the Protein Databank (http://www.rcsb.org) under PDB code: 2POA.

* Corresponding authors. P.L. Ho is to be contacted at Tel.: +5511 37267222 ext. 2244; fax: +55 11 372261505. A. Spisni, Tel.: +39 0521033807; fax: +39 0521033802.
E-mail addresses: alberto.spisni@unipr.it (A. Spisni), hoplee@butantan.gov.br (P.L. Ho).

1570-9639/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbapap.2008.12.010

Schistosomes cannot synthesize sterols and fatty acids *de novo* and they are dependent on their hosts for obtaining those molecules. The Sm14 protein of *Schistosoma mansoni* (*S. mansoni*) belongs to the intracellular fatty acid-binding proteins (FABPs) family [3] and it plays a key role for the survival of schistosomes. Thus, it represents a good target for vaccine development [4].

As a vaccine antigen, Sm14 displays a good protection level against schistosomiasis and fasciolosis in mice and rabbits [5] and against fasciolosis in sheep [6]. The cellular immune response against this protein has been associated with the resistance to infection in humans living in Brazilian [7] and Egyptian [8] endemic regions. Overall, the demonstration of strong immunological cross reactivity with a shared antigen, provides this vaccine candidate with the potential to be used against more than one infection, i.e. *S. mansoni* in humans and *Fasciola hepatica* (*F. hepatica*), a liver fluke disease that causes annual losses estimated at around $3 billion US to rural agricultural communities [9], in animals.

In a previous work [10], we showed that the Sm14 protein family exists in two major isoforms presenting, at position 20, either a methionine (Sm14-M20) or a threonine (Sm14-T20). Thermal and urea unfolding studies indicated a higher structural stability for the Sm14-M20 isoform [10] and its crystal structure has been solved for two holo-forms containing oleic or arachidonic acid, respectively [11].

Recognizing the functional implication of the enhanced stability of Sm14-M20, we selected this isoform for schistosomiasis vaccine development. Nonetheless, during storage, Sm14-M20 exhibited a structural instability resulting in protein aggregation, precipitation and unreliable protection effect.

In order to design protein variants with a higher stability over time, an important step for the optimization of the large scale production of this antigen for schistosomiasis vaccine studies, we further investigated the Sm14 structural features.

These studies lead us to the selection of a better schistosomiasis vaccine candidate and to the determination of the first 3D structure of a Sm14 in its apo-form.

2. Experimental procedures

2.1. Molecular modeling

Energy minimization calculations, MD calculations and trajectory analysis were performed on a Compaq AlphaServer ES40 machine using the GROMACS suite of programs [12]. Molecular replacement and visualization procedures were carried out in the Insight II graphical environment (Accelrys Inc.) running on an Octane2 workstation (Silicon Graphics Inc.).

The X-ray structure for the holo-form of Sm14-M20(C62) (PDB 1VYF; *12*) was used as a template to obtain the initial coordinates of apo-Sm14-M20(C62), as well as of the mutants Sm14-M20(C62S) and Sm14-M20(C62V).

The resulting molecules were immersed in triclinic boxes filled with Simple Point Charge water molecules [13]. Each box size was adjusted in a way to assure a minimum distance of 0.8 nm between the protein and the periodic box edges. The N- and C-termini were treated as charged ammonium and carboxylate groups. Each system was neutralized with the necessary number of Cl-counter ions. The MD calculations were performed with the standard GROMACS force field using periodic boundary conditions. A cut off radius of 0.8 nm for short-range interactions was used and the neighbor pair list was updated every 10 steps. Long-range electrostatic interactions were treated with the Particle Mesh Ewald method [14]. The LINCS algorithm [15] was used to constrain all bond lengths and the SETTLE algorithm [16] to constrain water geometries. A time step of 2 fs was chosen for integrating the equations of motion. Initially, for the simulation protocol, relaxation of solvent and ions was achieved through 500 steps of the steepest descent energy minimization algorithm, while keeping the protein backbones fixed. After freeing the structures, the systems were submitted to another short minimization step with position restraints applied to the backbone atoms, with a force constant of 1000 kJ/mol/nm^2. Thereafter, the systems were equilibrated through a 200 ps step of MD calculation, at 300 K, with equal position restraints. After equilibration, two consecutive 5 ns MD calculations were carried out without the imposition of any restraint, the first being performed at 300 K and the second at 550 K.

For the analysis of the data, the RMSD of the backbone atoms of each structure collected along the MD calculations was determined with respect to the initial conformation of each run. The DPSS algorithm [17] was used to monitor the behavior of the secondary structure elements during each MD calculation.

2.2. DNA mutagenesis, cloning, protein expression and purification

The presence of the EcoRI restriction site near the cysteine 62 codon was used in the site-directed mutagenesis using PCR. The forward primer (5′-ACCTCGAGGATATCCATATGTCTAGTTTCTTGG-3′) and the pAE-Sm14-M20 plasmid used as template were previously described [10]. Two reverse primers were designed to carry out the changes to the targeted cysteine 62 codon (TGT) for serine (TCT) or valine (GTT), named RS62 (5′ tcgaattcctcgccgaacttgaacgtaGaagaaag 3′) and RV62 (5′ tcgaattc ctcgccgaacttgaacgtaAC agaaag 3′), respectively (underlined is the EcoRI site and in uppercase, the nucleotides responsible for the codon changes). After PCR amplification, the DNA fragments were inserted in the corresponding region of the pAE-Sm14-M20(C62) plasmid by replacement, using the XhoI and EcoRI restriction enzymes. Both constructions, pAE-Sm14-M20(C62S) and pAE-Sm14(C62V), express a His_6-tagged Sm14 protein in the N-terminus. The success of each mutagenesis was confirmed by DNA sequencing. The expression and purification procedures used for these mutants are the same as described for Sm14 (C62) [10].

For the NMR studies, the Sm14-M20(C62V) cDNA was re-cloned in order to remove the His_6-tag coding sequence. The recombinant protein was expressed in the ^{15}N labeled or $^{13}C/^{15}N$ double labeled form and purified as indicated previously [18].

2.3. Protein stability studies

Equilibrium unfolding as a function of temperature was monitored by circular dichroism (CD). CD measurements were carried out on a J-810 Circular Dichroism Spectropolarimeter (Jasco Inc.) coupled to a Peltier Jasco PFD-425S system for temperature control. Far-UV spectra were recorded in the spectral range 190–260 nm and averaged over five scans at 20 °C, using a 1 mm path length quartz cell, 0.5 nm step resolution, 50 nm/min speed, 8 s response time, and 1 nm bandwidth. Protein concentration was 10 μM in 10 mM Na-phosphate buffer, pH 7.4. Spectral correction was performed by subtracting the blank. The measured ellipticity, θ (mdegree), was converted to molar mean residue ellipticity, $[\theta]$ (degree.cm^2.dmol^{-1}).

For thermal unfolding/refolding studies, the temperature range tested was between 15 °C and 80 °C. Temperature scan was 0.5 °C/min in order to maintain thermal equilibrium. The loss of secondary structure was followed by measuring $[\theta]$ at 216 nm. The T_m values were calculated as the inflection point of the unfolding curves, fitted according to the single step (two-state) transition model [19].

For the urea unfolding studies, the protein concentration was 2 μM in 10 mM phosphate-buffered saline (PBS), pH 7.4. After each addition of urea the sample was equilibrated at 20 °C before measurements. The intrinsic fluorescence of the recombinant Sm14 proteins, mainly due to the two Trp residues, was recorded setting the excitation wavelength at 285 nm and monitoring the shift of the emission maximum in the range 330–355 nm. The analysis of the denaturation curves was carried out using the method described by Prinsen and Veerkamp [20].

The binding ability of the proteins before and after thermal denaturation was determined by fluorescence spectroscopy using the