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NMR structure of *Plasmodium falciparum* malaria peptide correlates with protective immunity

Jindra Purmova, Luz Mary Salazar, Fabiola Espejo, Mary Helena Torres, Marcia Cubillos, Elizabeth Torres, Yolanda Lopez, Raul Rodríguez, Manuel Elkin Patarroyo*

Fundación Instituto de Inmunología de Colombia (FIDIC), Carrera 50, No. 26-00, Bogotá, Colombia

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Abstract

Apical membrane antigen-1 is an integral *Plasmodium falciparum* malaria parasite membrane protein. High activity binding peptides (HABPs) to human red blood cells (RBCs) have been identified in this protein. One of them (peptide **4313**), for which critical binding residues have already been defined, is conserved and nonimmunogenic. Its critical binding residues were changed for amino acids having similar mass but different charge to change such immunological properties; these changes generated peptide analogues. Some of these peptide analogues became immunogenic and protective in ***Aotus*** monkeys. Three-dimensional models of peptide **4313** and three analogues having different immune characteristics, were calculated from nuclear magnetic resonance (NMR) experiments with distance geometry and restrained molecular dynamic methods. All peptides contained a β-turn structure spanning amino acids 7 to 10, except randomly structured **4313**. When analysing dihedral angle ϕ and ψ values, distorted type III or III′ turns were identified in the protective and/or immunogenic peptides, whilst classical type III turns were found for the nonimmunogenic nonprotective peptides. This data shows that some structural modifications may lead to induction of immunogenicity and/or protection, suggesting a new way to develop multicomponent, subunit-based malarial vaccines.

Keywords: Malaria; Peptide; Conformation; Nuclear magnetic resonance; Apical merozoite antigen-1 protein

1. Introduction

Several targets have been suggested as objectives for development of vaccines against malaria (mainly that caused by *Plasmodium falciparum*) to control the death of more than 2.5 million people and the infection of another 300 million annually throughout the world [1].

P. falciparum causes the most lethal form of malaria and several merozoite proteins have been identified in red blood cell (RBC) invasion, seeming to play a critical role in this interaction [2]. One of them, the apical merozoite antigen-1 (AMA-1), an 83-kDa integral membrane protein, located in rhoptry necks, seems to be transported to the merozoite membrane where it may be involved in merozoite reorientation [3]. AMA-1 presents high homology between the different *P. falciparum* strains [4]. Thirty-one nonoverlapping 20-residue-long peptides spanning this protein's entire sequence were synthesised to identify peptides relevant to the human RBC binding and invasion process [5]. These peptides were used in RBC binding screening assays, eight binding with high specificity and affinity.

The binding assays showed that the **4313** conserved binding peptide (DAEVAGTQ***YRL***PSGK***C***PVFG), located between this protein's 134–153 residues, have a 120 ± 12 nM affinity constant and that residues critical for binding to RBCs were **Y**, **R**, **L** and **C**.

Evidence reported elsewhere [6,7] has shown that conserved peptides do not induce immune responses during natural infection (therefore being nonantigenic), nor when used as vaccines (being not immunogenic) [8,9]. The objective of this work was to modify the conserved high activity binding peptides (HABPs) to induce immune responses and to find correlation with their tridimensional structure determined by ^{1}H nuclear magnetic resonance (NMR).

The positive and negative immunological assay results for peptide **4313** and its **10022**, **13480**, **13766** and **13768** analogues were studied in this work.

* Corresponding author. Fundación Instituto de Immunología de Colombia (FIDIC), Carrera 50, No. 26-00, Bogotá, Colombia. Tel.: +57-1-481-5219; fax: +57-1-481-5269.

E-mail address: mepatarr@mail.com (M.E. Patarroyo).

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2. Materials and methods

2.1. Synthetic peptides

The peptides were synthesised by the multiple solid phase peptide synthesis method, using the *tert*-butoxycarbonyl strategy [10]. The peptides were numbered according to those serial numbers used in FIDIC, purified and characterised by RP-HPLC and mass spectrometry (MS). Peptide polymers for immunisation studies were obtained after CG addition at the N and C termini. Cysteine oxidation methodology was used for polymerisation, with oxygen at pH 7.4.

Peptides from Table 1 (modifications shown in bold) were synthesised, the priority being the replacement of critical residues by amino acids having different polarity and similar mass, to identify those replacements significant in protective immune response induction.

2.2. Animals

Spleen-intact *Aotus nancymaae* monkeys (KI karyotype, $2n = 54$), captured in the Colombian Amazon jungle and kept in our monkey colony in Leticia, were used for this trial. This species proved to be susceptible to experimental infection with *P. falciparum* using different FVO strain inocules [11]. All monkeys included in this report were phenotypically characterised, having an average 700 g weight and haematocrit higher than 45%. Members of the same family were randomly selected into different experimental groups to avoid immunological phenomena attributable to immune response genes. The animal handling was performed according to USA National Institute of Health animal handling criteria.

2.3. Immunisations

Groups of 5 to 10 *Aotus* monkeys each received three 125 μg subcutaneous injections of polymerised peptide on days 1, 20 and 40, homogenised in the first dose with complete Freund's adjuvant, whilst incomplete Freund's adjuvant was used in the second and third doses. Controls received only Freund's adjuvant on the same days.

2.4. Challenge

The FVO *P. falciparum* strain was kept by successive passages in nonimmune, spleen-intact owl monkeys. Erythrocytes parasitised with this strain was stored frozen in glycerolised solution in liquid nitrogen according to described techniques [12] and passed in a group of two or three monkeys prior to the challenge in order to have a fresh, stable and quantifiable accurate infection dose [11].

Protection was defined as being the complete absence of parasites in immunised monkey blood throughout 15 days following an intravenous inoculation of 200,000 infected RBC from the FVO *P. falciparum* strain. This dose was 100% infective for *Aotus* monkeys. Nonprotected, as well as control monkeys, developed parasitaemia $\geq 6\%$ by days 8 to 10 and were treated with quinine [11].

2.5. Parasitaemia assessment

Fresh blood samples (0.05 ml/monkey), obtained from saphenous veins, were employed. Parasitaemia was measured daily for each monkey starting on day 5 after challenge. Twenty-five microliters of each sample were diluted in 25–50 μl of 0.06% acridine orange solution in 0.86%

Table 1
Humoral immune response and protective efficacy induced by **4313** derived peptides in ***Aotus*** monkeys

Polymerized peptide Nr	Peptide Sequence																						IFA TITERS ≥320 II 15	III 20	PROT	
4313			D	A	**E**	V	A	G	T	Q	**Y**	**R**	**L**	P	S	G	K	**C**	P	V	F	G	0	0	0/5	
10098			-	-	-	-	-	-	-	-	**Y**	**M**	**H**	-	-	-	-	**S**	-	-	-	-	0	**1(5120)**	**1/5**	
10022			-	-	-	-	-	-	-	-	**Y**	**F**	**H**	-	-	-	-	**S**	-	-	-	-	0	**1(5120)**	**1/5**	GROUP
22780	G	E	-	-	-	-	-	-	-	-	**Y**	**F**	**H**	-	-	-	-	**V**	-	-	-	-	**2(2560)**	ND.	**2/10**	1
22782	G	E	-	-	-	-	-	-	-	-	**W**	**F**	**V**	-	-	-	-	**V**	-	-	-	-	**1(2560)**	ND.	**1/10**	
13480			-	-	-	-	-	-	-	-	**W**	**F**	**L**	-	-	-	-	**S**	-	-	-	-	**2(640)**	**2(320)**	0/6	
13766			-	-	-	-	-	-	-	-	**W**	**F**	**D**	-	-	-	-	**S**	-	-	-	-	**1(640)**	**2(640)**	0/5	GROUP
14022			-	-	-	-	-	-	-	-	**W**	**F**	**T**	-	-	-	-	**T**	-	-	-	-	0	**1(320)**	0/5	2
15508			-	-	-	-	-	-	-	-	**Y**	**R**	**H**	-	-	-	-	**T**	-	-	-	-	0	**1(320)**	0/2	
13768			-	-	-	-	-	-	-	-	**W**	**F**	**N**	-	-	-	-	**S**	-	-	-	-	0	0	0/6	
14026			-	-	-	-	-	-	-	-	**W**	**F**	**H**	-	-	-	-	**T**	-	-	-	-	0	0	0/5	
15998			-	-	-	-	-	-	-	-	**W**	**R**	**L**	-	-	-	-	**T**	-	-	-	-	0	0	0/6	
10024			-	-	-	-	-	-	-	-	**Y**	**F**	**D**	-	-	-	-	**S**	-	-	-	-	0	0	0/5	
13808			-	-	-	-	-	-	-	-	**W**	**F**	**T**	-	-	-	-	**S**	-	-	-	-	0	0	0/6	GROUP
10026			-	-	-	-	-	-	-	-	**Y**	**F**	**N**	-	-	-	-	**S**	-	-	-	-	0	0	0/5	3
12694			-	-	-	-	-	-	-	-	**W**	**F**	**H**	-	-	-	-	**S**	-	-	-	-	0	0	0/4	
17924			-	-	-	-	-	-	-	-	**Y**	**R**	**H**	-	-	-	-	**S**	-	-	-	-	0	0	0/7	
17922			-	-	**M**	-	-	-	-	-	**Y**	**R**	**H**	-	-	-	-	**S**	-	-	-	-	0	0	0/7	
CONTROLS																							0	0	0/50	

PROT. = fully protected monkeys.

saline solution. Parasites were seen by reading the proportion (%) of parasitised RBC on the slide by fluorescence.

2.6. Indirect immunofluorescence assays (IFA)

IFA was performed 15 days after the second (II-15) and 20 days after the third immunisations (III-20) to asses humoral immune response.

Late-stage schizonts from a continuous *P. falciparum* culture (FCB-2 strain), presenting a 10% parasitaemia, synchronised according to Lambros and Vandenberg's [13] method, collected and washed in sterile PBS (phosphate buffer 0.15 M, containing 0.15 M NaCl, pH 7.2) were used. The infected cell pellet of was suspended in foetal bovine serum (FBS)/PBS (1:1 v/v) and left to dry on the slide. The parasites were blocked for 10 min with 1% nonfat milk and incubated for 30 min with preimmune or immune sera. Reactivity was seen by fluorescence microscopy using goat anti-monkey IgG/FITC conjugate F(ab′)2 fragment at 1/100 dilution. Preimmune *Aotus* sera were used as negative controls.

2.7. Parasites

The FCB-2 *P. falciparum* strain from Bogotá, Colombia, was cultured in vitro with human group Orh+ erythrocytes using a modification [14] of the Trager and Jensen [15] culture method.

2.8. Parasite lysate for Western blotting

Late stage schizonts from continuous *P. falciparum* cultures, exhibiting 20% parasitaemia, were collected, washed in sterile PBS and lysed in 0.2% saponine solution with vigorous vortexing for 20 s. The pellet was washed twice with large volumes of PBS to remove haemoglobin and erythrocyte debris. Seven volumes of lysis buffer (5% SDS, 1 mM EDTA, 1 mM PMSF) were added to the pellet and vortexed for 10 min; the supernatant was further centrifuged at 22,500 $\times g$ for 30 min. This lysate was kept frozen in liquid nitrogen until use. The noninfected RBC lysate was prepared in the same way.

2.9. Immunoblotting

The proteins were electrophoretically separated, transferred to nitrocellulose paper and incubated with preimmune serum at 1/100 dilution. The reaction was revealed with affinity-purified goat anti-*Aotus* IgG alkaline phosphatase conjugate; the nitroblue tetrazolium (NBT) and 5-bromo, 4-chloro, 3-infonilphosphate (BCI) substrate was used as the developing reagent, according to Blake et al. [16].

2.10. NMR analysis

NMR experiment samples were prepared by dissolving 10 mg of peptide in 500 μl trifluoroethanol-d_3/H_2O 30:70 [17] for the structure analysis. ^{1}H spectra were run in a BRUKER DRX-500 spectrometer. Proton spectra were assigned by using Double Quantum Filter COrrelation SpectroscopY (DQF-COSY), TOtal Correlation SpectroscopY (TOCSY) and Nuclear Overhauser Enhancement SpectroscopY (NOESY) experiments [18]. The 2D NMR data were processed with XWIN-NMR software. The NOESY spectra recorded at different temperatures (285–315 K) were used to obtain amide temperature coefficients ($-\Delta\delta/\Delta T$). Spin coupling constant ($^3J_{NH-C\alpha H}$) on DQF-COSY spectra were measured.

2.11. Structure calculations

Peptide structure was determined by using Molecular Simulations Inc. (MSI) software. The NOEs were grouped into three categories (strong, medium and weak) then converted into distance restraints (1.8–2.7, 2.7–3.5 and 3.5–5.0 Å). Hydrogen bond constraints were introduced for the slow exchange rate peptide NH, distance ranges involving these likely hydrogen bond NH...O were set at 1.8–2.5 Å. The ϕ angle constraints derived from $^3J_{NH-C\alpha H}$ were restricted to $-70 \pm 30°$ if $^3J_{NH-C\alpha H} < 6$ Hz and to $-120 \pm 30°$ if 9 Hz $> {}^3J_{NH-C\alpha H} >$ 8 Hz. The Distance Geometry (DGII) programme was used to generate 50 starting structures. These structures were refined using a restrained simulated annealing protocol.

3. Results and discussion

3.1. Immunological studies

It has been shown that *P. falciparum* parasite protein conserved sequences are poorly antigenic [6,7] and poorly immunogenic [8,9]. In this, and a previous paper [9], we show that these proteins' conserved HABPs to human erythrocytes are poorly immunogenic in *P. falciparum* malaria susceptible *Aotus* monkeys.

Therefore, for the development of a logical and rational approach to a multicomponent, subunit-based malarial vaccine, systematic modifications of these HABPs' amino acid sequences in the critical binding residues were undertaken to render these nonimmunogenic, nonprotectogenic HABPs immunogenic and protective.

Critical binding residue (to host cells) were replaced by amino acids having similar mass but different polarity, and different immune responses were obtained when these were polymerised and used as vaccines in the experimental monkey model.

The modified peptides' immunogenicity was assessed in immunised monkey sera by three of the most reliable methodologies: (a) antibody induction against nonfixed, nondenatured, native proteins, as determined by the ImmunoFluorescence Antibody assay (IFA). (b) Western blot recognition of the denatured protein from which the amino

acid sequence was derived and (c) *Aotus* monkey protection against experimental infection with a 100% infective *P. falciparum* malaria strain dose.

Three types of immune response (named 1 to 3, Table 1) were observed based on these methodologies (including the most important one, i.e. protection against experimental challenge). In Group 1, two patterns were seen: those for peptides **10098** and **10022**, having $\mathbf{R_{10}M}$, $\mathbf{L_{11}H}$ and $\mathbf{R_{10}F}$, $\mathbf{L_{11}H}$ modifications, respectively (three-dimensional structure was determined for the latter). Three doses of these modified peptides, used for ***Aotus*** immunisation, induced very high antibody titres (1:5120) as assessed by IFA, protecting one out of five monkeys from each group. Further immunisation with peptide **10022**, repeated twice, with a total of 20 monkeys, fully protected four of them (20%) with 1:1280 antibody titres. In the second set, peptides **22780** and **22782** with $\mathbf{R_{10}F}$, $\mathbf{L_{11}H}$ and $\mathbf{Y_9W}$, $\mathbf{R_{10}F}$ and $\mathbf{L_{11}H}$ modifications, respectively, had the GE sequence added at the N-terminal. These additions, plus modifications observed in Table 1, induced very high antibody titres (>1:2560) with only two doses, protecting 2 and 1 monkeys, respectively, in each group of 10. Therefore, modifications of peptides in Group 1 made them highly immunogenic and protective.

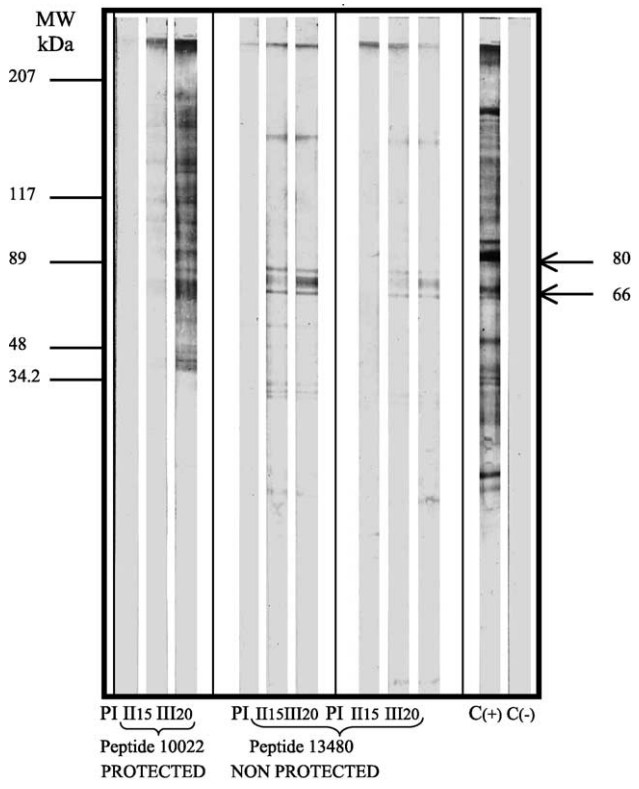

Fig. 1. Western blot analysis. Western blot analysis of solubilised antigens from *P. falciparum* schizonts shows reactivity with the *Aotus* sera 83- and 66-kDa doublet taken 15 days (II-15) and 20 days (III-20) after the second and third immunisations, respectively. Preimmune sera (PI) are negative. C(+) and C(−) are positive and negative controls. Note the intensity of band reactivity associated with the antibody titre in the fully protected (1:5120) and the two nonprotected (1:640), (1:320) monkeys.

Peptides from Group 2 (having those modifications shown in Table 1) induced antibody levels between 1:320 and 1:640 with two or three doses, but they were not protective. This could be due to the smaller amount of antibodies produced, *Aotus* IgG subclass variations, or to a difference in their antibody affinities. Peptides **13480** and **13766** are found within this group, having the $\mathbf{Y_9W}$, $\mathbf{R_{10}F}$ and $\mathbf{Y_9W}$, $\mathbf{R_{10}F}$ and $\mathbf{L_{10}D}$ modifications, respectively. The three-dimensional structure of both was determined.

In Group 3, those modifications made to **4313** peptide are found, being neither immunogenic nor protective. The structure of peptide **13768** was determined for comparison.

With the second methodology to asses immunogenicity, peptide Groups 1 and 2 induced antibodies recognising structures in AMA-1 denatured proteins, as determined by Western blot, recognising the 83- and 66-kDa doublet molecules, characteristic of AMA-1 with different intensities, corresponding to the antibody levels. This data shows that immunisation with these modified peptides does induce antibodies identifying similar structures to the modified peptide present in the native protein as assessed by IFA, or in the denatured protein as determined by Western blot (Fig. 1).

3.2. Structural analysis

MS analysis showed that all synthesised monomers had the expected theoretical mass.

The peptides included in this study presented only signals from one major component. The presence of strong cross-peaks between αH or NH for the amino acid preceding proline and its δH indicate that these peptides are *trans* isomers. All peptides' NOESY spectra showed αHi–NH$i+1$ sequence signals stronger than intraresidue cross-peaks. Medium or weak intensity NH–NH cross-peaks were also observed. In addition to these sequential cross-peaks, some medium-range αHi–NH$i+2$ cross-peaks were found and NHi–NH$i+2$ cross-peaks in the case of peptide **13768**.

NMR correlation reveals that peptides are present in some types of turn (αHi–NH$i+2$, NHi–NH$i+1$, NH slow exchange residue 10). The **4313** peptide has a totally extended form due to absence of medium range signals; it was thus not possible to determine its 3D structure. Peptides **10022**, **13480**, **13766** and **13768** sequential medium range NOEs are summarised in Fig. 2A.

Two hundred thirty-five NOEs (26 backbone interresidues), four angular constraints and one hydrogen bond constraint between the main chain C=O of residue i and the NH of residue $i+3$ were used in peptide **13480** structure calculations. The hydrogen bond corresponds to F10 NH and T7 C=O because of its concordance with NOE signals (αNH$\boldsymbol{i}+2$ of Q8-F10) and the distance between α carbons Ci(T7) to C$i+3$(F10) in previous

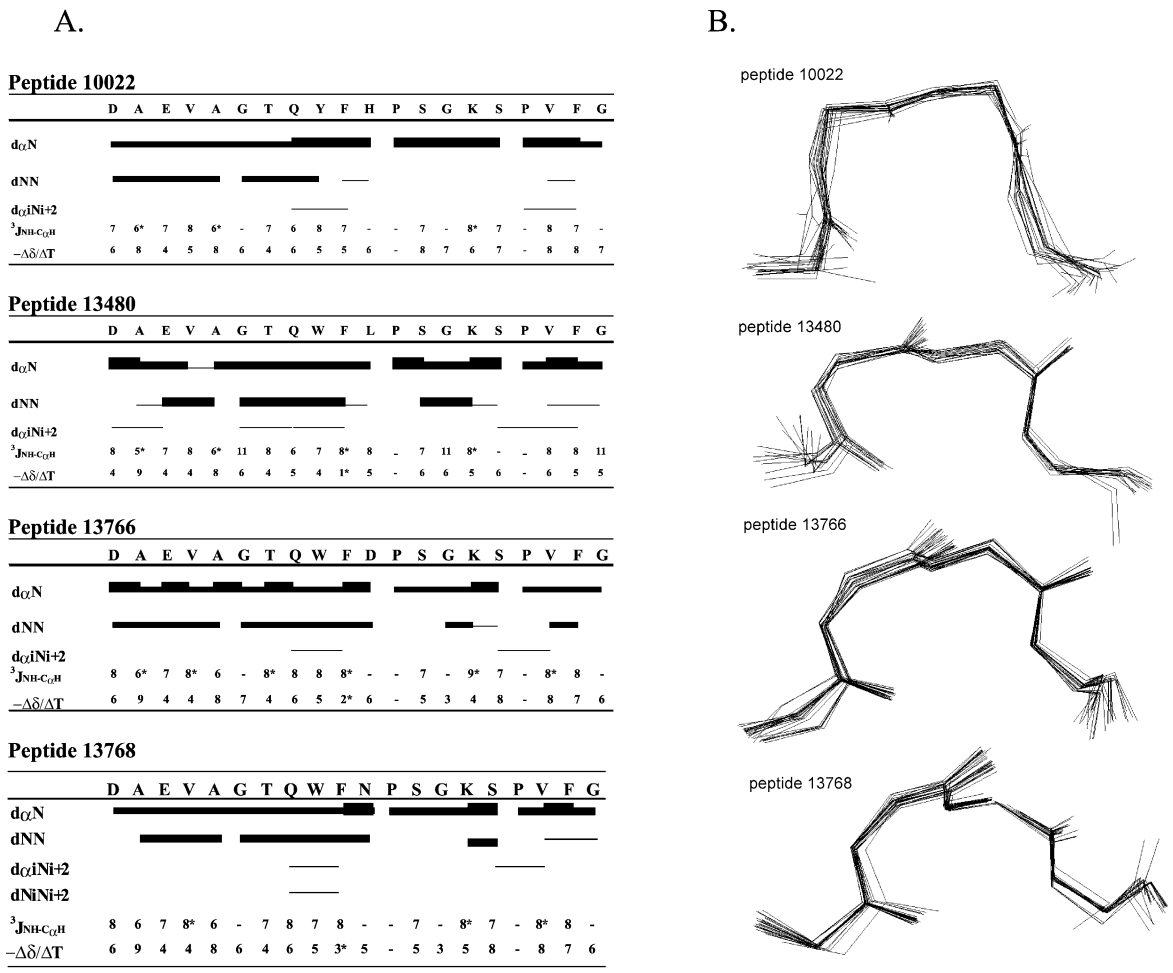

Fig. 2. Summary of sequential medium range NOEs and peptide structures. (A) Summary of sequential medium range NOE connectives represented by different line thickness. $^3J_{NH-C\alpha H}$ and NH protons with slow exchange used in the calculation are indicated by asterisks. (B) Structures of the **10022**, **13480**, **13766**, and **13768** peptides. Residues 7 to 10 backbone representation. **13768**'s classical β-turn type III is shown for comparison with immunogenic peptide **10022**, **13480**, **13766** distorted β-III structures. **10022** was also protective.

calculation run without hydrogen bond data around 7 Å corresponding to the turn-like structure. Sets of 50 independently generated structures were obtained satisfying the experimental constraints. A family of 17 low-energy conformers, having root mean square deviation (RMSD) equal to 0.52 Å superimposing backbone atoms from T7 to F10

Table 2
Results of structure calculations for peptides **10022**, **13480**, **13766** and **13768**

Peptide	Type of turn, $\phi\psi$ average values of amino acids $i+1$ and $i+2$	NOEs used	Superimposed structures of 50		RMSD [Å]	Residues in the most favored region of Ramachandran plot [%]	Immunogenicity	Protectivity
10022	distorted III′ $\phi_{Q8}=57°$; $\psi_{Q8}=72°$ $\phi_{Y9}=50°$; $\psi_{Y9}=68°$	197 (22)	22	42*	0.37	96.8	+	+
13480	distorted III′ $\phi_{Q8}=55°$; $\psi_{Q8}=7°$ $\phi_{W9}=47°$; $\psi_{W9}=55°$	235 (36)	17	41*	0.52	91.2	+	–
13766	distorted III $\phi_{Q8}=-61°$; $\psi_{Q8}=-17°$ $\phi_{W9}=-81°$; $\psi_{W9}=-10°$	251 (32)	29	10*	0.25	98.5	+	–
13768	III $\phi_{Q8}=-49°$; $\psi_{Q8}=-13°$ $\phi_{O8}=-49°$; $\psi_{O8}=-13°$	239 (31)	35	36*	0.27	98.8	–	–

Interresidue NOEs are in parenthesis and consensus structure numbers are marked with asterisks.

was finally selected. The final structures had 91.20% residues in the Ramachandran plot's most favourable region, determined by Procheck [19].

This data shows that peptide **13480** contains a turn spanning the T7 to F10 amino acids (Fig. 2B), being the area where modified critical residues are found. The rest of the peptide is highly flexible, probably in extended or β-strand conformation. According to ϕ and ψ angle values, the turn is *distorted* type III′ . The ideal values for a type III′ turn are $\phi_{i+1}=60°$, $\psi_{i+1}=30°$, $\phi_{i+2}=60°$ and $\psi_{i+2}=30°$. The average values of ϕ and ψ angles for the amino acids present in the **13480** peptide structures superimposed on the consensus structure were $\phi_{Q8}=55°$, $\psi_{Q8}=7°$, $\phi_{Q9}=47°$, $\psi_{Q9}=55°$.

Structure calculations and results for peptides **10022**, **13766**, **13768** are shown in Table 2. The average Cαi–C$\alpha i+3$ distance was <7 Å in all peptides; there was no distance violation greater than 0.27 Å or dihedral angle violation greater than 0.90°.

3.3. Structural and immunological associations

Immunogenic but nonprotective peptides **13480** and **13766** had either a type III′ or III ***distorted*** β-turn structure. Peptide **10022**, which was highly immunogenic and protective after the third dose in several monkeys (5/25 in three experiments), also has the same III′ ***distorted*** conformation. For comparison, the nonimmunogenic nonprotective **13768** peptide presented a ***classical*** type III β-turn structure. This data shows that distortions in the type III or III′ β-turn structures are associated with immunogenicity and/or protection. One has to keep in mind that it is probable that monomeric forms show the same conformational configurations as polymeric peptides (which were used in immunisations), although there is the possibility of new epitope induction in polymeric forms.

Major histocompatibility complex (MHC) class II molecules present peptide antigens to T cells in the generation of immune responses mediated by antibodies [20]. This suggests that these modifications may permit a better orientation of amino acids to interact with immune system molecules. Therefore, on theoretical grounds, classical type III β-turn structures may not fit well into MHC class II molecules, the T cell receptor αβ heterodimer (TCRαβ) or the TCRαβ–MHC molecules, whilst distorted Type III or III′ β-turn structures do, thereby activating the immune system. Supporting this idea, it has been very recently reported that it is feasible to repair MHC, class I [21] or the TCR–peptide–MHC interaction defect with modified peptides [22], thus improving anti-tumour immunity by converting a poorly immunogenic peptide or an antagonist into an agonist peptide [23].

Work published very recently by our institute has shown that specific modifications of another conserved HABP (named **1585**, derived from merozoite surface protein 1 (MSP-1)) rendered it immunogenic and protective. In that paper, we also showed that these modifications induced specific distortions of a completely helical configuration from the lead peptide, making it more flexible in the modified portion.

The present paper confirms the previous data related to the need for specific modifications to induce immunogenicity and protectivity with HABPs and adds that *P. falciparum* protein type III′ or distorted type III β turn (but not classical βIII type turn) structures could represent excellent possibilities for testing as components in a subunit-based, malaria vaccine candidate. Alternatively, specific modifications to these HABPs able to induce configurational changes (such as those described here, or increase peptides flexibility) could make them suitable peptides for use as vaccine components.

Hypothetically then, what we are doing when conserved, nonimmunogenic, nonprotective peptides are modified is to induce distortions into these peptides' 3D structures to facilitate better interaction with immune system molecules, either the MHC class II molecules, the TCR or both.

The understanding of this correlation between the 3D structure of native as well as modified peptides may lead to a rational approach for the development of multicomponent subunit-based peptide malaria vaccines.

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