Structure, Immunogenicity, and Protectivity Relationship for the 1585 Malarial Peptide and Its Substitution Analogues

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Malaria continues to be one of the greatest public health problems in the world. Between 300 and 500 million people are affected annually, which leads to 1.5 to 2.5 million deaths, mainly in children less than 5 years old.[1, 2] Immunoprophylactic method development is the only cost-effective control alternative[3] and currently several malarial vaccines including our SPF-66 have been tested and shown to have limited (but significant)[4] or no efficacy in humans.[5]

The *Plasmodium falciparum* merozoite surface protein-1 (MSP-1) is one of this parasite's most abundant merozoite membrane molecules. It plays a key role in merozoite invasion of human erythrocytes, and is therefore regarded as a promising vaccine candidate.[6] Our objective is to induce an immune response able to block the receptor–ligand interaction responsible for human red blood cell (RBC) invasion.

Our first step was to identify MSP-1 sequences involved in the invasion process; such sequences were then used (unmodified or with single or multiple substitutions) in immunization studies. The changes induced in the lead peptide three-dimensional structure (determined by using NMR spectroscopy) were analyzed by correlating the substitution with the degree of protective immunity in *Aotus* monkeys.

Relevant MSP-1 sequences have previously been identified by their interaction with human RBCs.[7] One of these sequences, located in the N-terminal portion of the 42-kDa MSP-1 fragment (peptide 1585: EVLYLKPLAGVYRSLKKQLE), binds to RBC with an affinity constant of 180 nM. We have chosen the peptide 1585 (which has a conserved sequence in all parasite strains) to avoid the wide genetic variability of *P. falciparum*.[8, 9]

Evidence to date suggests that the conserved protein sequence in *P. falciparum* has poor or no antigenicity (capacity to induce immune response after infection) nor immunogenicity (capacity to induce immune response when used as a vaccine) in humans.[7]

The first step in the design of analogues was to recognize those amino acids critical to the RBC binding process and to determine if residue substitution induced immunogenicity and protectivity. Determination of these residues[10, 11] showed that the L5, K6, P7, A9, and S14 residues of peptide 1585 (EVLY**LKP**L**A**GVYR**S**LKKQLE) are essential to RBC binding (data not shown).

All peptides used in this study were synthesized by a solid-phase multiple peptide synthesis procedure using the *tert*-butoxycarbonyl (Boc) strategy.[12] The final product was characterized by analytical high performance liquid chromatography (HPLC) and mass spectrometry.

Table 1 shows sequences for peptide 1585 and its substitution analogues that were used in immunization studies of *Aotus* monkeys highly susceptible to *P. falciparum* malaria. Humoral immune response and protective efficacy induced by peptide 1585 and its analogues are also shown in Table 1. Humoral immune response was quantified by measuring the level of parasite-specific antibodies in immunized monkey sera by an immunofluorescence assay (IFA).[13]

Immunized and control monkeys were challenged on day 60 by intravenous inoculation of 200 000 freshly infected erythrocytes obtained from a nonimmune donor previously infected with the FVO strain of *P. falciparum*. This dose is known to be 100 % infective for these monkeys.[13]

All control and nonprotected monkeys developed patent parasitaemia (determined by using acridine orange staining and fluorescence microscopy) by day 5; they were treated with quinine when 6 % levels of parasites were reached (usually by days 8–10).

The data presented in Table 1 shows how some amino acid (AA) substitutions render the lead peptide 1585 immunogenic. Group A comprises glycine analogues of peptide 1585: substitution of AAs critical to RBC binding made peptides 5187, 5188, and 5189 immunogenic and protective, whilst noncritical residue substitution (peptides 6187 and 6177) failed to induce sero-conversion and protection in immunized monkeys.

Critical binding residues were substituted by AAs with similar mass but different polarity in Group B analogues. This group presents very high antibody levels associated with full protection for 16 to 50 % of the immunized monkeys. Experiments repeated three times with peptide 11860 resulted in 30 % protective efficacy in 31 monkeys (data not shown).

Group C shows that 12 out of 30 substitution analogues, as well as the lead peptide 1585, did not induce *P. falciparum* parasite antibodies nor protection against experimental challenge.

Sera from monkeys vaccinated with the protective analogues induced parasite-specific antibodies that recognized the denatured 195-kDa MSP-1 protein and its 83-kDa, 42-kDa, 33-kDa, and 21-kDa cleavage products, among them peptide 10014. Monkeys immunized with nonprotective peptides (13454) did not produce such antibodies, as determined by using Western Blott analysis (Figure 1). Table 1 clearly demonstrates that modifications induced by some AA substitutions render the nonimmunogenic peptide 1585 immunogenic and protective.

NMR structure determinations were carried out with the lead peptide 1585 (*EVLY**LKP**L**A**GVYR**S**LKKQLE*), protective analogues 10014 (*EVLY**HV**PLAGVYRSLKKQLE*) and 11860 (*EVLY**HM**PL**G**GVYR**A**LKKQLE*), and the nonpro-

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  1433-7851/01/4024-4654 $ 17.50+.50/0

Table 1. Humoral immune response and protective efficacy induced by analogues of peptide 1585 in *Aotus* monkeys.[a]

Polymerized peptide No.	Peptide sequence																			IFA titers ≥320			Group
																				II_{15}	III_{20}	Prot	
1585	E	V	L	Y	**L**	**K**	**P**	L	**A**	G	V	Y	R	**S**	L	K	K	Q	L	E	0	0	0/5	
5187	–	–	–	–	**G**	**K**	**P**		**A**	–	–	–	–	**S**	–	–	–	–	–	–	ND.[b]	1(320)	1/4	A
5188	–	–	–	–	**L**	**G**	**P**	–	**A**	–	–	–	–	**S**	–	–	–	–	–	–	ND.[b]	1(320)	1/4	A
5189	–	–	–	–	**L**	**K**	**G**	–	**A**	–	–	–	–	**S**	–	–	–	–	–	–	ND.[b]	1(1280)	1/4	A
6187	–	–	–	–	**L**	**K**	**P**	**G**	**A**		–	–	–	**S**	–	–	–	–	–	–	0	0	0	A
6177	–	–	–	–	**L**	**K**	**P**	–	**A**	–	–	–	–	**G**	–	–	–	–	–	–	0	0	0	A
13450	–	–	–	–	**L**	**L**	**D**		**A**	–	–	–	–	**S**	–	–	–	–	–	–	2(2560)	1(2560)	2/4	B
22806				–	**L**	**L**	**D**	–	**A**	–	–	–	–	**S**	–	–	–	–			3(1280)	1(1280)	2/9	B
10014	–	–	–	–	**H**	**V**	**P**	–	**A**	–	–	–	–	**S**	–	–	–	–	–	–	2(640)	1(640)	2/4	B
22768				–	**H**	**L**	**P**	–	**A**	–	–	–	–	**A**	–	–	–	–			0	1(5120)	1/10	B
11860	–	–	–	–	**H**	**M**	**P**	–	**G**	–	–	–	–	**A**	–	–	–	–	–	–	1(1280)	1(2560)	2/6	B
22770				–	**H**	**L**	**P**		**G**	–	–	–	–	**A**	–	–	–	–			1(1280)	1(280)	2/9	B
13454	–	–	–	–	**L**	**M**	**S**	–	**A**	–	–	–	–	**S**	–	–	–	–	–	–	0	0	0/5	C
14448	–	–	–	–	**H**	**M**	**D**	–	**G**	–	–	–	–	**V**	–	–	–	–	–	–	0	0	0/3	C
14496	–	–	–	–	**H**	**M**	**D**	–	**S**	–	–	–	–	**V**	–	–	–	–	–	–	0	0	0/4	C
13724	–	–	–	–	**H**	**V**	**T**	–	**S**	–	–	–	–	**S**	–	–	–	–	–	–	0	0	0/5	C
12896	–	–	–	–	**H**	**V**	**P**	–	**S**	–	–	–	–	**A**	–	–	–	–	–	–	0	0	0/5	C
12898	–	–	–	–	**H**	**V**	**P**	–	**G**	–	–	–	–	**A**	–	–	–	–	–	–	0	0	0/5	C
15954	–	–	–	–	**L**	**M**	**D**	–	**A**	–	–	–	–	**S**	–	–	–	–	–	–	0	0	0/3	C
15956	–	–	–	–	**L**	**M**	**P**	–	**A**	–	–	–	–	**S**	–	–	–	–	–	–	0	0	0/4	C
15958	–	–	–	–	**L**	**K**	**D**	–	**S**	–	–	–	–	**S**	–	–	–	–	–	–	0	0	0/6	C
15960	–	–	–	–	**L**	**M**	**D**		**S**	–	–	–	–	**S**	–	–	–	–	–	–	0	0	0/4	C
13728	–	–	–	–	**N**	**L**	**D**	–	**G**	–	–	–	–	**S**	–	–	–	–	–	–	0	0	0/5	C
13452	–	–	–	–	**L**	**I**	**D**	–	**A**	–	–	–	–	**S**	–	–	–	–	–	–	0	0	0/5	C
controls																					0	0	0/50	C

[a] Each monkey received three 125-μg subcutaneous polymerized peptide injections[11] on days 1, 20, and 40, homogenized in the first dose with complete Freund's Adjuvant. Incomplete Freund's Adjuvant was used in the second and third doses. Controls received only Freund's Adjuvant on the same days. IFA was carried out to assess humoral immune response 15 days after the second (II_{15}) and 20 days after the third (III_{20}) immunization. Protection was defined as complete parasite absence in immunized monkey blood for 15 days following challenge. [b] Not determined.

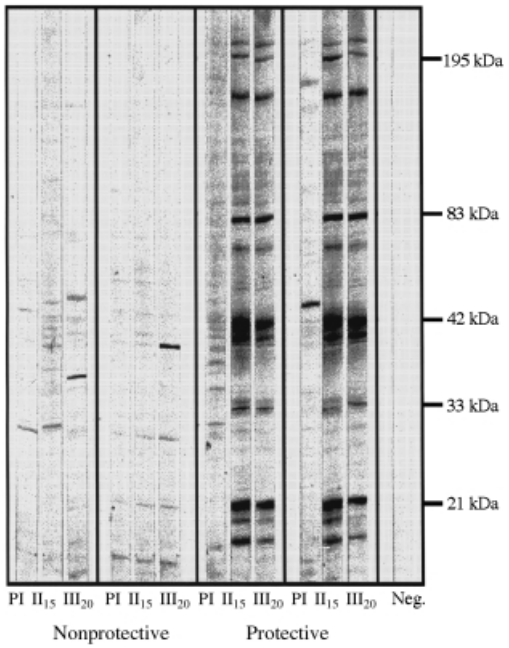

Figure 1. Western Blott analysis for solubilized antigens from late-stage *P. falciparum* schizonts. Sera from *Aotus* monkeys immunized with 13454 nonprotective (left) and 10014 protective (right) peptides were used. PI = Preimmune sera; II_{15}, III_{20} = sera taken 15 or 20 days after the second and third immunization, respectively.

tective 13454 analogue (*EVLYL**MS**LAGVYRSLKKQLE*) to establish the relationship between the three-dimensional structure of relevant substitution analogues and immunogenicity and protective efficacy observed in monkeys.

Peptide samples (8 mM) were dissolved in 25% 2,2,2-trifluoroethanol/water (TFE/H_2O) at pH 4–5.[14] A Bruker DRX-600 spectrometer was used for standard ^1H-^1H two-dimensional experiments (DQF-COSY, TOCSY, and NOESY) using Xwinnmr software. Sequence-specific assignments were made following standard procedures;[15] NOESY spectra recorded at different temperatures (285–315 K) were used to obtain amida temperature coefficients for predicting hydrogen bonds ($-\Delta\delta/T < 4$).

A structural calculation that was consistent with experimental data was carried out using a Molecular Simulations Inc. Software package (Insight II). NOE intensities were partitioned 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). The ϕ angular constraints were derived from $^3J_{NH\text{-}C\alpha H}$ coupling constants and were constrained to $-70^\circ \pm 30^\circ$ if $^3J_{NH\text{-}C\alpha H} < 6$.

We determined the structure of peptide 1585 in solution by using connectivities (Figure 2) and a database with a total of 251 NOE constraints, 12 ϕ dihedral angle constraints, and 9 hydrogen bond constraints. The 18 best (lowest energy) structures which fulfilled the constraints were selected from the 50 resulting conformers in the refined set and had 0.2 Å maximal distance and 2.4° maximal angular constraint violation. Procheck[16] was used on the refined structures. The root-mean-square deviation (rmsd) for the 18 structures (backbone atoms) versus the consensus structure was 0.17 Å. The results for the other analogues are presented in Table 2.

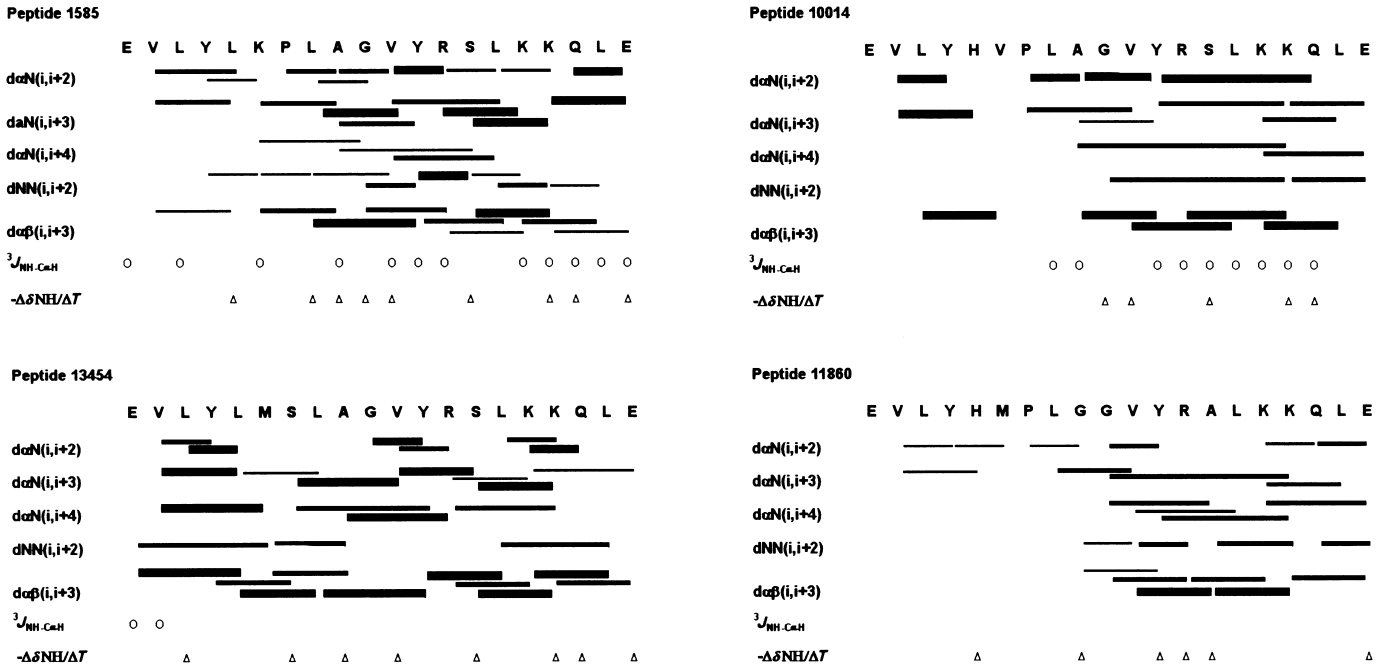

Figure 2. Summary of sequential medium range NOE connectivities (NOE intensities are represented by the line thickness). $^3J_{NH\text{-}C\alpha H}$ coupling constants are indicated by ○ if lower than 6 Hz. NH protons with slow exchange rates (involved in H-bonds $NH_i \cdots O_{i-4}$) are indicated by △.

Table 2. Summary of structure calculation results with the α-helical regions shaded.

Peptide	Helical structure residues	NOE connectivities	Number of structures superimposed out of 50 (helical segments shaded)	Rmsd [Å]	Max NOE violation [Å]	Protection
1585	4–12	251	18 EVL YLKPLAGVY RSLKKQLE	0.17	0.20	nonprotective
13454	5–16	279	33 EVL YLMSLAGVYRSLKKQLE	0.59	0.20	nonprotective
10014	9–18	198	15 EVLYHVPLAGVYRSLKKQLE	0.48	0.30	protective
11860	8–17	244	16 EVLYHMPLGGVYRALKKQLE	0.47	0.40	protective

Figure 3 shows the three-dimensional structures of lead peptide 1585, the nonprotective analogue 13454, as well as the protective 10014 and 11860 analogues.

All the peptides have a partial α-helical structure in 25% TFE, which indicates that each peptide has a strong helix-forming region; however, significant differences were observed in the location and extent of this structural element. In peptide 1585 the α-helical structure extends from residue 4 to 12 and in nonprotective peptide 13454 from residue 5 to 16, so in both cases it includes areas in which critical binding residues are located.

By contrast, immunogenic and protective peptides 10014 (L5 →H and K6 →V modifications) and 11860 (with L5 →H, K6 →M, A9 →G and S14 →A changes) possess a more flexible structure in critical binding residue areas (L5, K6, P7, A9). In these peptides the α-helical portion comprises residues 9–18 and 8–17, respectively, which leaves the N-terminal portion more flexible. These protective peptides may thus access a higher number of possible conformers.

Side chain conformation reliability is limited by relatively high conformational freedom. Nevertheless, a dramatic change in the relative spatial arrangement of the helical central region AA side-chain of peptide 1585 was clearly seen when critical RBC-binding residues were substituted.

Increased flexibility of the critical binding residue in peptides 10014 and 11860 (located next to the relatively rigid helical core) may allow these analogues to fit better into immune system molecules, which explains the induction of protective immunity. Such modifications may increase the

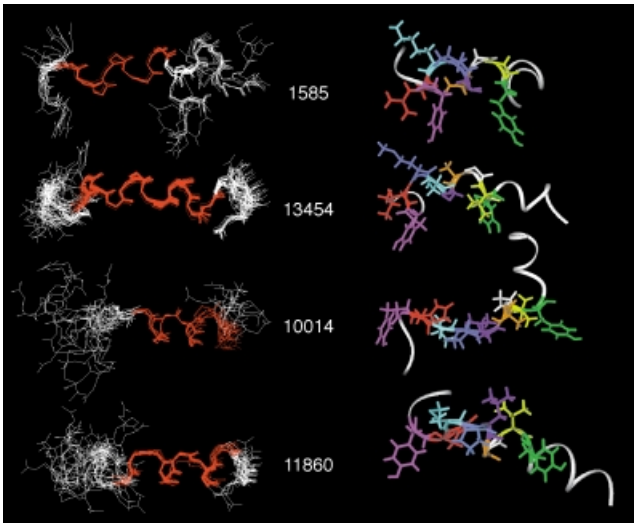

Figure 3. Structures of the nonprotective peptides 1585 and 13454, as well as the protective peptides 10014 and 11860. Left: modified amino acid backbones are shown in red. Right: ribbon representations. Color code: Y4 fuchsia; L5 (1585, 13454), H5 (10014, 11860) red; K6 (1585), M6 (13454, 11860), V6 (10014) pale blue; P7 (1585, 10014, 11860), S7 (13454) dark blue; A9 (1585, 10014, 13454), G9 (11860) brown; V11 yellow; Y12 green.

  1433-7851/01/4024-4656 $ 17.50+.50/0

stability of the complex formed by major histocompatibility complex molecules, altered peptide ligands and T-cell receptors (MHC – altered-peptide-ligand – TCR complex) as recently shown for the altered AH1 tumor peptide, for which increased MHC – peptide – TCR complex stability was correlated with improved immune response.[17] Alternatively, perhaps a T-cell antagonist is converted into an agonist by repair of TCR – peptide – MHC interface defects, as suggested by other research groups.[18]

Herein we were able to correlate immunogenicity (particularly protective efficacy of a synthetic peptide in vivo) with three-dimensional structural features. Understanding the structure-protection relationship will lead to a more rational design of a *P. falciparum* malaria vaccine in the near future and any one of the reported modified peptides could be used as part of a multicomponent subunit malaria vaccine.

Received: June 12, 2001
Revised: October 10, 2001 [Z17274]

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β-Fibrillogenesis from Rigid-Rod β-Barrels: Hierarchical Preorganization Beyond Microns**

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Fibrillogenesis of β-sheet peptides is attracting considerable scientific attention because of its importance in medicine and nanoscience.[1–12] Recent structural studies with synthetic models go beyond pure peptide chemistry.[5–9] These studies were consistently in support of repeated "β-dyads" of polar and apolar amino acid residues as the minimal structural requirement for β-fibrillogenesis[4] (if any),[3] and also supported a universal "cross-β" motif in β-fibrillar quaternary structures. The term cross-β is used for layered β-strands that are oriented perpendicular to the long fibril axis. In this case, one fibril (or protofilament) dimension is defined by the length of the β-strand, the other by the number of stacked, amphiphilic β-bilayers.[1–12] Further self-assembly of β-fibrils produces a quite remarkable, in part transient, polymorphism including formation of helices, ribbons, tubes, and sheets.[1–12] Whereas elegant strategies to inhibit random β-fibril self-assembly[8, 9] and to control two-dimensional organization of β-sheet monolayers[13] have been conceived recently, little is known about how to direct fibril self-assembly toward higher order structures. However, the difficulties with preorganizing β-sheet tertiary structures can be bypassed by using *p*-oligophenyl rods instead of β-sheets as β-barrel "staves".[14–16] The objective of this study was to expand this powerful preorganization strategy from tertiary β-barrel structures to quaternary β-fibril structures. We report, for the first time, the direct observation of "rigid-rod" β-barrels by atomic force microscopy as well as their controlled transformation into unidirectional self-assembled "rigid-rod" β-fibrils of exceptional beauty.

The formation of tetrameric rigid-rod β-barrels **1** is preorganized by rigid-rod "staves" in the monomers **2** (Figure 1). Barrel – stave supramolecule **1** further contains 32 interdigitating β-dyad-pentapeptides that produce hydrophobic outer and hydrophilic inner β-barrel surfaces (Figure 1).[14–16] Structural and functional studies implied that tetramer **1**

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[**] We thank J. Buffle and J. Dubochet for their fruitful discussions and the Swiss NSF (21-57059.99 (S.M.), 2000-050629.97 (K.J.W.), and the National Research Program "Supramolecular Functional Materials" 4047-057496 (S.M.)) for their financial support.

  1433-7851/01/4024-4657 $ 17.50+.50/0