Orientating Peptide Residues and Increasing the Distance between Pockets to Enable Fitting into MHC−TCR Complex Determine Protection against Malaria

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ABSTRACT: The erythrocyte binding antigen EBA-175 is a 175-kDa *Plasmodium falciparum* protein, which has been shown to be involved in the process of invasion of erythrocytes. It has been found that conserved peptide 1818 belonging to this protein has high red blood cell binding capacity and plays an important role in the invasion process. This peptide is neither immunogenic nor protective. Peptide 1818 analogues had some of their previously recognized critical red blood cell binding residues substituted for amino acids having similar volume or mass but different polarity to make them fit into HLA-DRβ_1*1101 molecules; these 1818 peptide analogues were then synthesized and inoculated into *Aotus nancymaae* monkeys, generating different immunogenic and/or protective immune responses. Short structures such as 3_{10}-helix, classical, or distorted type-III β-turns were found in the immunogenic and protective peptides once the secondary structure had been analyzed by NMR and its structure correlated with its immunological properties. These data suggest that peptide flexibility may lead to better fitting into immune system molecules, therefore making them excellent candidates for consideration as components of a subunit-based, multicomponent synthetic antimalarial vaccine.

A vaccine is urgently needed to protect humankind against the *Plasmodium falciparum* parasite which causes the most lethal form of malarial infection in humans, responsible for more than 90% of malaria cases in Africa, meaning around 250 million new cases and two million deaths annually. Because of this parasite's increased resistance to drugs, much attention is being focused on developing vaccines based on either single or multiple parasite derived antigens. Because of the *P. falciparum* parasite's tremendous complexity (*1*), we believe that effective malaria vaccines must contain a variety of immunogens selected to induce different immune responses against antigens from the different stages of the *P. falciparum* life cycle (*2*, *3*). The first synthetic multiantigenic malaria vaccine (SPf66) was developed within this context 17 years ago. This vaccine underwent preclinical (*4*), clinical (*5*), and field trials in different ethnic (*6*−*9*), age (*10*), and transmission conditions (*11*), showing clear but limited protective efficacy in these studies. Our institute has continued searching for a rational approach toward identifying these putative targets and their action mechanisms in the attempt to improve the efficacy of SPf66. We have found that high activity binding peptides (HABPs)[11] from proteins involved in invasion represent the best targets.

The *P. falciparum* malaria parasite uses a 175-kDa protein ligand known as erythrocyte binding antigen (EBA-175) for erythrocyte invasion (*12*). EBA-175 is located in the micronemes and has been identified as ligand for merozoite invasion into human erythrocytes that binds to glycophorin A in a sialic acid-dependent manner (*13*). It therefore acts as a bridge between parasite and host. This molecule has been divided into a number of domains based on structural characteristics including three cysteine-rich regions (F1, F2, and C). The F1 and F2 domains located at the molecule's N-terminal are responsible for glycophorin-A binding to the erythrocyte membrane (*13*). The two cysteine-rich regions (often referred to as regions II and VI) bear numerous conserved cysteine residues possessing the erythrocyte binding function (*14*). Antibodies raised against EBA-175 have been shown to block in vitro invasion of red blood cells (RBC) (*15*); immunization with EBA-175 gives some protection in nonhuman primate experimental challenge models (*16*).

The entire EBA-175 protein has been synthesized in our institute in 20-mer long peptides to identify high activity RBC binding peptides. Several conserved HABPs were identified; one of them, coded **1818** having the amino acid

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[1] Abbreviations: HABPs, high activity binding peptide; RBC, red blood cells; NMR, nuclear magnetic resonance; MHC, major histocompatibility complex; TCR, T-cell receptor; HLA, human leukocyte antigen; HPLC, high-pressure liquid chromatography; CD, circular dichroism; IFA, immunofluorescence assay; BOC, *tert*-butyloxycarbonyl; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; TFE, 2,2,2-trifluoroethanol; DQF-COSY, double-quantum-filtered correlated spectroscopy; TOCSY, total correlation spectroscopy; NOESY, nuclear overhauser enhancement spectroscopy.

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Table 1: Humoral Immune Responses and Protective Efficacy Induced by **1818**-Derived Peptides in *Aotus nancymaae* Monkeys[a]

Polimerized Peptide Nr.	Peptide Sequence (1 5 10 15 20)	IFA >1:320 Post 2nd	IFA >1:320 Post 3rd	Protected Nr. of monkeys	Group
1818	**N N** N F **N** N I P S R Y N L **Y** D K **K** L D L	0	0	0/5	
22690	———————— **M**——— D	2/(320)	1/(1280)	2/10	A
24166	———————Y——**Y**—K **M**—P— D D	1/(320)	2/(640)	2/5	A
23390	———————N——D— **M**——L D D L	2/(320)	9/(320)	0/9	B
24084	——————————**V**—— **M**— P—	0/10	0/10	0/10	
23762	———————————— **M**— P— D L	0/9	0/9	0/8	
27006	——————— I—— **I**— Q **M**— P— D D	0/8	0/6	0/6	C
27608	———————N——D— **M**— E L D D	0/8	0/8	0/7	C
27996	—————D A————— **M**——— D D	0/8	0/8	0/8	C
27998	—————D A————— **M**— P— D D	0/10	0/8	0/8	C
CONTROLS		0/50	0/50	0/50	

[a] The putative binding residues for P1, P4, P6, P9 pockets of the respective Class-II molecules 24166 (HLA-DRβ_1*-1101) 23390 and 27608 (HLA-DRβ_1*-0301) are shown shaded from left to right.

sequence ^{1280}NNNFNNIPSR**Y**NLY**D**K**K**LDL^{1299}, was located in the 3′ cysteine-rich region. This peptide bound to erythrocytes with 146 nM affinity (*17*). Amino acids important in the process of binding to erythrocytes (Y11, D15, and K17), underlined above, were determined by glycine analogue scan binding inhibition (*17*).

Since *P. falciparum* conserved sequences are poorly antigenic and poorly immunogenic (*18*−*20*), a series of peptide analogues were synthesized in which critical binding residues were replaced by amino acids having a similar mass but different polarity. Immunizations were then performed on *Aotus nancymaae* monkeys which are highly susceptible to malaria infection to identify their immunogenic and protective capacity. The 3D structures of lead peptide **1818** and modified protective peptides were determined by ^{1}H nuclear magnetic resonance (NMR) to try to find a correlation between this immunological function and their structure.

In previous papers (*18*−*20*), we have launched the hypothesis that modifications performed on these peptides to render them immunogenic and protection-inducing may allow them to fit perfectly to conform to the major histocompatibility complex Class-II molecules (MHC-II)−peptide−T-cell receptor (TCR) complex (MHC-II−peptide−TCR). Some of these peptides were thus analyzed to test this hypothesis concerning their capacity to bind specifically to some purified HLA-DRβ1* molecules, trying to correlate their biological, immunological, and structural characteristics in the search for a more rational and logical approach for developing multiantigenic subunit-based synthetic *P. falciparum* malaria vaccines.

MATERIALS AND METHODS

Synthetic Peptides. Synthetic peptides included in this study were synthesized by the multiple-solid-phase technique, using previously described *tert*-butoxycarbonyl (Boc) strategy (*21*). They were then purified by reverse-phase HPLC on a C18 LiChrospher column (Merck, Germany). The products were assessed by analytical high performance liquid chromatography (HPLC) and mass spectrometry (MALDI-TOF). Peptide polymers were obtained for immunization studies after Cys and Gly had been added at the N- and C-termini. Our Institute's carefully defined cysteine oxidation method ology was used to obtain high molecular weight polymers (oxygen, at pH 7.4), usually in the 8−24 kDa range. Ten peptides were synthesized (Table 1), numbered according to our laboratory's sequential numbering system showing modifications made.

Circular Dichroism (CD) Analysis. The CD spectra of the peptides in 50 mM phosphate buffer at pH 7.0, 30% TFE/water solution were measured using a JASCO J810 spectropolarimeter using a 1-mm path-length cell. The peptides' CD spectra were recorded at 20 °C in the 190−260 nm range using a 0.2-nm spectra bandwidth and 10 nm/min scan speed. Peptide concentration was 0.2 mM. CD data were expressed as mean residue ellipticity [θ] given in deg cm^2 dmol^{-1}.

Animals and Immunization. Spleen-intact *A. nancymaae* monkeys, kept at our Primate Station in Leticia (Colombia) in the Amazon region according to National Institute of Health guidelines for animal handling, were analyzed by immunofluorescence (IF) antibody assay for the presence of *P. falciparum* schizont parasite antibodies in their sera, at 1:20 serum dilution. The few proving positive were returned to the jungle without being submitted to further procedures. Those monkeys proving negative were distributed in random groups of 5−9 to be immunized with the synthesized polymeric peptide analogues shown in Table 1 to induce humoral immune response as well as protection against experimental challenge with the *P. falciparum* malaria parasite.

Each monkey subcutaneously received 125 μg of the polymerized peptide homogenized with Freund's complete adjuvant for the first dose on day 0, and Freund's incomplete adjuvant for the second dose on day 20.

Blood was drawn for immunological analysis on 1 day prior to the first vaccination and 20 days after each immunization.

Challenge and Parasitaemia Assessment. Immunized as well as control *A. nancymaae* monkeys were infected with 200 000 *P. falciparum* FVO-strain infected RBC via femoral vein for challenge 20 days after the last immunization (*22*). Protection was defined as being the total absence of parasites in blood during the 15 days of the experiment. Nonprotected monkeys developed patent parasitaemia from day 5 or 6, reaching ≥6% levels between days 8−10. After receiving treatment with antimalarial drugs in pediatric doses, they

were kept in quarantine to ensure cure and returned to the jungle later on.

Each monkey's parasitaemia was measured daily, starting on day 5 after challenge. Immunofluorescence was used for reading parasites in terms of the percentage of parasitized RBC on the slide following acridine orange staining.

IFA and Western Blot. Late-stage schizonts from a continuous *P. falciparum* culture (FCB-2 strain) were synchronized according to Lambros and Vandenberg's method (*23*). They were washed and treated as described earlier (*22*). The slides with the dry parasites were blocked for 10 min with 1% nonfat milk and incubated for 30 min with appropriate dilutions of monkey sera (starting at 1:40 dilution) for antibody analysis. Reactivity was observed by fluorescence microscopy using the F (ab′) 2 fragments from a 1:100 diluted goat anti-*Aotus* monkey IgG:FITC conjugate. Preimmune sera from all monkeys were used as negative controls.

The 20% late parasitaemia RBCs were washed with PBS (pH 7.2) and lysed with 0.2% saponin (Merck) for Western blot analysis. The parasite proteins were extracted with lysis buffer (1 mM PMSF, 1 mM EDTA, and 5% SDS); the lysate was then centrifuged. A 10% resolving gel was used for SDS−PAGE. The ensuing product was transferred onto nitrocellulose paper and incubated with 1:100 diluted preimmune or immune sera for Western blot analysis. The reaction was revealed with affinity purified goat anti-*Aotus* IgG-alkaline phosphatase conjugate (*24*).

HLA-DR Molecule Affinity Purification. Human molecules were purified from DR1 WT100BIS (DRβ1*0101), DR3 COX (DRβ_1*0301), DR4 BSM (DRβ_1*0401), and DR11 BM21 (DRβ_1*1101) homozygous EBV-B cell lysates by affinity chromatography (*25*) using anti-HLA-DR mAb L-243 cross-linked to protein-A sepharose CL-4B (Amersham Pharmacia Biotech AB) as affinity support. Cells were lysed at 10^8 cells per milliliter density on ice for 60 min in 1% (v/v) Nonidet P-40, 25 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg/mL for each of the following: soybean trypsin inhibitor, antipain, pepstatin, leupeptin, and chymostatin in 0.05 M sodium phosphate buffer, 0.15 M NaCl, pH 7.5. Lysates were cleared of nucleid acids and debris by spinning at 27000*g* for 30 min. After 0.2 volumes of 5% sodium deoxycholate (DOC) had been added to the supernatant and mixed for 10 min, the lysate was centrifuged at 100000*g* for 2 h and subsequently filtered through a 0.45 μm membrane. Lysates were first passed over a sepharose CL-4B precolumn and subsequently over the Sepharose-A mAb L243 protein column for Class-II molecule affinity purification. The affinity column was then washed with (i) 20 column volumes of 50 mM Tris-HCl pH 8, 0.15 NaCl, 0.5% NP-40, 0.5% DOC; (ii) 5 column volumes of 50 mM Tris-HCl pH 9, 0.5 M NaCl, 0.5% NP-40, 0.5% DOC; and (iii) 5 column volumes of 2 mM Tris-HCl pH 8, 1% octyl-β-D-glucopyranoside (Sigma, St. Louis). HLA-DR molecules were eluted with 0.05 M diethylamine in 0.15 M NaCl, pH 11.5, containing 1% octyl-β-D-glucopyranoside, 1 mM EDTA. The eluate was immediately neutralized with 1/20 volume of 1 M Tris/HCl, pH 6.8. Preparations were kept in aliquots at −70 °C until use.

Competition Binding Assays. Peptide binding competition assays were conducted to measure unlabeled peptide ability to compete with biotinylated indicator peptides for binding to purified HLA-DR molecules, as previously described (*26*). Biotinylated-labeled hemagglutinin HA 306−318 (PKYVKQNTLKLAT) peptide was used as control peptide for DRβ_1*0101, DRβ_1*0301, DRβ_1*0401, and Gly-Phe-Lys-$(Ala)_7$ ($GFKA_7$) for DRβ_1*1101. Both peptides were +N-terminally labeled with sulfo-NHS-LC−Biotin (Pierce Chemical, Rockford, III). Purified HLA-DR molecules were diluted in freshly prepared binding buffer containing 100 mM citrate/phosphate buffer (pH 7), 0.15 mM NaCl, 4 mM EDTA, 4% NP-40, 4 mM PMSF, and 40 μg/mL for each of the following: soybean trypsin inhibitor, antipain, leupeptin, and chymostatin. Ninety microliters of HLA-DR molecules (0.1 μM) were added to Eppendorf tubes together with 30 μL of biotinylated-labeled peptide (5 μM) in DMSO: PBS (1:4) for direct binding assay. An extra 30 μL (250 μM) was added to the competition assay. After 24 h of incubation at room temperature, the peptide/Class-II complexes were transferred to ELISA-well plates (Nunc-Immuno Modules Maxisorp Loose Brand product, Denmark) which had been coated with a 10 μg/mL anti-HLA-DR mAb-L-243 solution and subsequently blocked with PBS containing 5% bovine serum albumin. After 2 hours' incubation at room temperature, plates were washed with PBS and 0.05% Tween-20. After incubation with alkaline phosphatase-labeled streptavidine (Vector Laboratories, Burlingame, Calif), labeled peptide/HLA-DR complexes were revealed with 4-nitrophenyl phosphate substrate (Kirkegaard and Perry Laboratories. Maryland, USA). A Titertek MC Multiscan ELISA reader (Labsystems, Franklin, Mass) was used with a 405-nm filter for determining peptide binding to HLA-DR molecules by measuring the optical densities (OD). The amount of bound peptide was normalized to the maximum observed binding.

Relative binding affinities were determined for other peptides by competition assay. According to this assay, a good competitor was a peptide that was capable of inhibiting more than 50% of the indicator peptide's binding to the HLA molecule being tested.

NMR Analysis. NMR experiment samples were prepared by dissolving 10 mg of peptide in 500 μL of TFE-d_3 (Cambridge Isotope, 99.94%)/H20 mixture (30/70 v/v). Studies have shown that nascent structures have an inherent propensity for native structure formation; in the presence of solvents such as 2,2,2-trifluoroethanol (TFE), they can be stabilized into well-ordered conformations (*27*).

^{1}H spectra were run on a BRUKER DRX-500 spectrometer. Proton spectra were assigned by using double quantum filter correlation spectroscopy (DQF-COSY) (*28*), total correlation spectroscopy (TOCSY) (*29*) and nuclear Overhauser enhancement spectroscopy (NOESY) experiments (*30*). The 2D NMR data were processed using XWIN NMR software. The NOESY spectra recorded at different temperatures (285−315 K) were used to obtain amide temperature coefficients for predicting hydrogen bonds ($-\Delta\delta H^N/\Delta T$ ppm/K). Spin coupling constants ($^3J_{HN-H\alpha}$) from DQF−COSY spectra were measured.

Structure Calculations. Peptide structure was determined by using Molecular Simulations Inc. (MSI) software. The NOEs were grouped into three categories (strong, medium, and weak) and then converted into distance restraints (1.8−2.8, 2.8−3.5, and 3.5−5.0 Å). TOCSY spectra recorded at different temperatures (285−315 K) were used to obtain

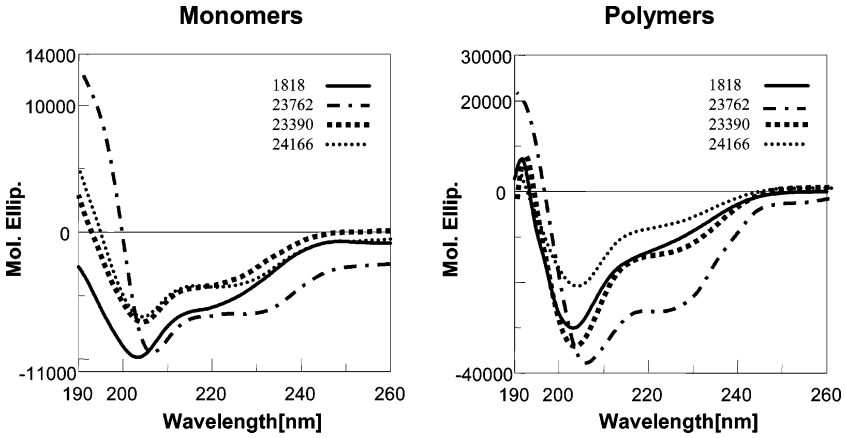

FIGURE 1: Circular dichroism for peptide **1818** and its monomer and polymer analogues.

amide temperature coefficients for predicting hydrogen bonds; distance ranges involving these likely NH···O hydrogen bonds were set at 1.8−2.5 Å. The ϕ angle constraints derived from $^3J_{HN-H\alpha}$ were restricted to $-70° \pm 30°$ if $^3J_{HN-H\alpha} < 6$ Hz and to $-120° \pm 30°$ if 9 Hz $> {}^3J_{HN-H\alpha} > 8$ Hz. The Distance Geometry (DGII) program was used to generate 50 starting structures. These structures were refined using a restrained simulated annealing protocol from the Discover program. The interatomic distances given in angstroms for the molecular models were obtained by using Accelrys Inc's InsightII Distance Measurement software.

Iterative calculations (with violated constraint correction) were done from original data, and the final representative family of low energy conformers (satisfying the set of experimental distance restraints) was determined by energy and root-mean-square deviation (rmsd).

RESULTS AND DISCUSSION

Because of the difficulty of adding sequential asparagines (N) during synthesis (a well-known chemical peptide synthesis problem), analogues were synthesized without the N-terminal asparagines (N). To compensate for these truncations, aspartic acids (D) and or leucine (L) present in EBA-175 protein amino acid sequence were added at the C-terminal to try to conserve their sequences and length. The peptides which had their 3D structure determined were those that were representative of any important immunological function such as just immunogenicity, immunogenicity, and protection and those peptides having no immunological function.

Peptide Characterization. Analytical chromatography results of peptides shown in bold in Table 1 revealed that peptide purity after semipreparative HPLC was sufficiently high to be analyzed by NMR. The experimental masses (followed by theoretical mass in parentheses) shown by mass spectroscopy (MS) were 2,454.50 (2,456.60) for peptide **1818**; 2,218.60 (2,222.80) for **23762**; 2,316.69 (2,355.90) for **23390**; and 2,456.9 (2,371.90) for peptide **24166**. The difference was probably due to dehydration of asparagine in peptide **23390** [M-36] and the addition of an extra serine [M+85] in peptide **24166**.

The polymers used for immunization had molecular masses in the 8−24 kDa range as assessed by size exclusion chromatography (SEC). Their structural conformation was similar to their monomeric counterparts as determined by CD.

The CD spectra of monomeric and polymeric peptides, in 30% TFE, are shown in Figure 1. Peptide **1818** exhibited a minimum wavelength of around 200 nm, indicative of a random conformation. Peptide **23762** showed a shift in minimum toward 208 nm, with some ellipticity also developing at 222 nm. This is consistent with a shift toward an α-helical conformation. Peptides **23390** and **24166** presented two minimums in the same wavelength (being a little deformed around 225 nm), indicating the possibility of presenting a tendency toward shorter structures. Spectra were quite similar for monomers used for structural and biological studies as well as for those polymers used for immunological studies.

Immunological Studies. As conserved HABPs are nonantigenic, nonimmunogenic, and nonprotection-inducing, our institute undertook the task of recognizing those structural changes needed to render these HABPs immunogenic and capable of inducing the appropriate immune responses. These responses should block parasites' receptor−ligand interaction with their host cells rendering them immunogenic and protective. Therefore, critical binding residues were replaced by amino acids having similar mass but different polarity (*18*−*20*).

Amino acid changes made to peptide **1818**, their capacity to induce antibodies as determined by IFA, and their ability/inability to induce protection are shown in Table 1.

Three groups of peptide analogues can be clearly seen. Group A shows those modifications capable of inducing ≥ 1: 320 IFA antibody titers and complete protection for *Aotus* against experimental challenge, as parasites were not found in their blood during the 15 days the experiment lasted. The change of polarity (K17M and D19P) in this group of critical binding residues having similar mass and volume but different polarity and the addition of one Asp at the C-terminus in peptide **22690** and two Asp in **24166** (for which the 3D structure was then determined) improved immunogenicity and protective efficacy. Four out of 15 monkeys immunized with these two modified peptides were fully protected against a 100% *P. falciparum* parasite infective dose.

Modifications made to peptide **1818** in group B induced 1:320 antibody titers following the three doses, as in peptide **23390** (for which the 3D structure was then determined) which, besides having had two aspartic acids (D) and one leucine (L) added at the C-terminal, did not improve its immunogenicity or protectivity, since antibody titers did not

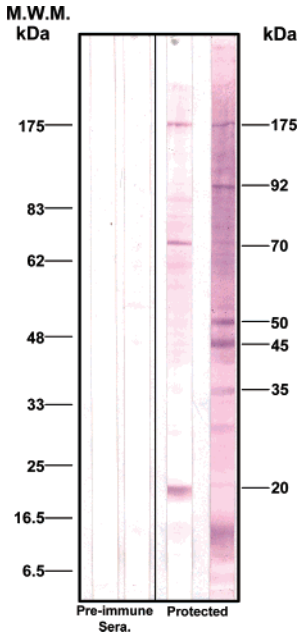

FIGURE 2: Western blot analysis of solubilized antigens obtained from late stage *P. falciparum* schizonts using sera from *A. nancymaae* monkeys immunized with peptide **24166** and **22690**, respectively.

increase and monkeys were not protected, showing that these antibody levels, their affinity, or those subclasses involved were not enough to lead to a protective immune response. Therefore, elongation of this peptide does not improve its immunological capacity. Modifications inducing neither antibodies nor protection were found in Group C; analogue **23762** was found in this group and its structure was then determined.

Western blot analysis of monkeys sera representative of peptides **22690** and **24166** showed recognition of 175-kDa native protein as well as its 70, 45, 35, and 20 kDa process products (Figure 2), as has been shown by others (*31*). All these data show that specific modifications can make a conserved, nonimmunogenic peptide (like lead peptide **1818**) become immunogenic and/or protective, recognizing proteins by IFA, Western blot, and inducing protection against a lethal dose of this parasite.

Purified HLA-DRβ_1 Molecule Binding Studies.* Molecule **1818** did not bind to any of the HLA-DRβ_1* molecules studied (0101, 0301, 0401, and 1101); this could partly explain its lack of immunogenicity and protectivity in *Aotus* (Table 2).

Modifications were made to immunogenic and protective Group A peptides **24166** and **22690** based on a priori assignments of motifs (*32*) previously determined experimentally as specific for the HLA-DRβ_1*-1101 molecule. In this motif, if amino acid Y11 is considered to be the most appropriate for fitting into Pocket 1 (as amino acids **Y**, W, F, I, L, V are the experimentally identified residues for fitting into this pocket) then Y14 will fit into Pocket 4, since this is one of those amino acids (Y, F, L, I, M, V) experimentally found to fit into this HLA-DRβ_1*-1101 pocket. K16 is one of the amino acids that has been described as being specific for DRβ_1*1101 in Pocket 6 (R, **K**, H) and the same holds true for P19 for Pocket 9 (where A, G, S, and **P** have been described as being specific for HLA-DRβ_1*1101). These modifications allow the peptide **24166** to fit into HLA-DRβ_1*-1101, rendering it immunogenic and protective. The presence of D21 in peptide **22690** did not allow it to bind to any one of the molecules here studied, suggesting that it could be binding to another molecule not included in this analysis or to *Aotus*-specific Class-II molecules as described by Niño et al. (*33*). Peptides **23390** (immunogenic but not protective) and **27608** (nonimmunogenic, nonprotective) bound with high affinity to the HLA-DRβ_1*-0301 molecule, but this suggests that the binding has shifted to amino acids N12, D15, M17, and L20, being HLA-DRβ_1*-0301 binding motifs (excluding N12), which could partly explain the differences in immunological behavior. This suggest that the peptide register has shifted to fit now into the HLA-DRβ_1*0301 groove. Peptide **23762** (nonimmunogenic, non-protection-inducing) deserves special consideration as it is different from peptide **24166**, being one residue shorter (F) at the N-terminal and having had the D22L replacement made (Table 2). This peptide did not bind to any Class-II molecule studied here, suggesting that small changes can modify not only the 3D structure of the molecule as shown, but also its biological and immunological behavior as it will be shown later on.

NMR Studies. The **1818** peptide has a totally extended form due to the absence of medium-range signals; it was thus not possible to determine its 3D structure. Peptide **23762** showed, $d_{NN}(i,i+1)$ $d_{\alpha\beta}(i,i+3)$, $d_{\alpha N}(i,i+3)$, $d_{\alpha N}(i,i+4)$ NOE connectivity, lowered amide proton chemical shift temperature coefficients for some of the amino acids and < 6 Hz $^3J_{HN-H\alpha}$ coupling constant values, suggesting the presence of a partial α-helical structure between P8 and M17 in 30% TFE (Figure 3). Sequential-, short-, and medium-range $d_{NN}(i,i+1)$, $d_{\alpha N}(i,i+2)$ NOE connectivity and the low-temperature dependence ($-\Delta\delta H^N/\Delta T$) values observed for some of the amino acids revealed the presence of some type of turn for peptides **23390** and **24166** (Figure 3). The presence of strong cross-peaks between Hα or HN for the amino acid preceding proline and its δH indicates that these peptides are trans isomers.

A set of 50 structures was calculated for peptide **23762** (neither immunogenic nor protective) using 300 NOEs (214 intraresidue, 69 sequential) together with four angle restraints and three hydrogen bond restraints. Twenty peptide **23762**

Table 2: Binding Profile for Peptide 1818 Analogue to the HLA-DRβ_1* Alleles

Peptide	Amino-acid sequence	Percentage of binding to HLA - DRβ_1* molecules 0101	0301	0401	1101	Group
1818	N N N F N N I P S R Y N L Y D K K L D L	2	33	11	6	
24166	F - - - - - - - - - - - - M - P - D D	45	5	18	**69**	A
22690	N - - - - - - - - - - - - M - - - D	0	8	13	24	A
23390	N - - - - - - - - - - - - M - - - D D L	-6	**52**	15	24	B
23762	N - - - - - - - - - - - - M - P - D L	27	29	10	29	C
27608	F - - - - - - - - - - - - M - E - D D	38	**63**	23	24	C

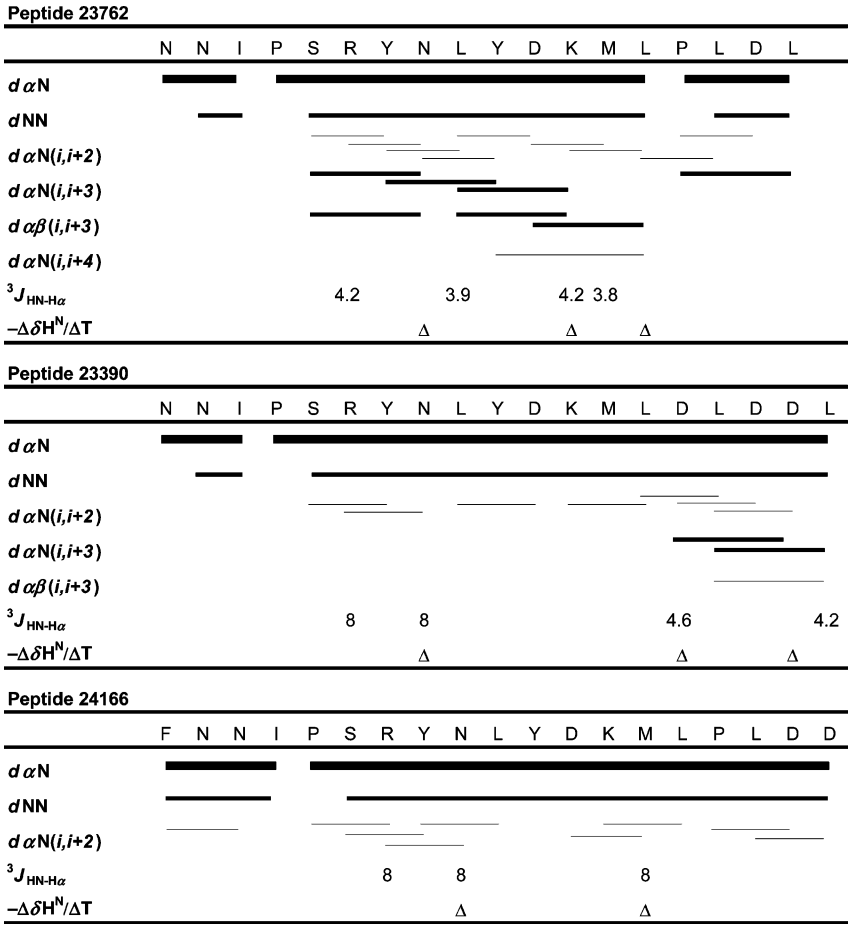

FIGURE 3: Summary of sequential medium range NOE connectives (NOE intensities are represented by different line thickness). $^3J_{HN-H\alpha}$ coupling constants and protons with slow exchange used in the calculation are indicated by Δ.

Table 3: Summary of Structure Calculation Results[a]

peptide number	structural features	NOEs used	no. of superimposed structures out of 50	RMSD Å[b]	maximal NOE violations Å	maximal angular violations (°)	I	P
23762	α-helix P8 to M17	300	20	0.21	0.28	1.00	−	−
23390	distorted type III β-turn	292	39	0.19	0.17	1.10		
	$\Phi_{R10} = -76°$ $\Psi_{R10} = -39°$							
	$\Phi_{Y11} = -137°$ $\Psi_{Y11} = -37°$						+	−
	3_{10} helix D19 to L23		24	0.23	0.37	1.70		
24166	classical type III β-turn	278	42	0.07	0.27	1.20		
	$\Phi_{R10} = -48°$ $\Psi_{R10} = -47°$							
	$\Phi_{Y11} = -65°$ $\Psi_{Y11} = -39°$							
	classical type III β-turn		30	0.21	0.33	1.80	+	+
	$\Phi_{D15} = -52°$ $\Psi_{D15} = -41°$							
	$\Phi_{K16} = -65°$ $\Psi_{K16} = -37°$							

[a] I = immunogenicity; P= protection. [b] The RMSD values are specifically for each peptide's structured region.

conformers, best satisfying the given constraints, were then selected. These structures had no angle constraint violation larger than 1.00° (degrees) nor distance constraint violations larger than 0.28 Å. The root-mean-square deviation for the 20 structures' backbone atoms versus average structure was 0.21 Å between P8 to M17 residues (Table 3). According to Kabsch & Sander (*34*), peptide **23762** (nonimmunogenic, nonprotective) has a well-defined α-helix structure between residues P8-M17, confirming our previous findings (*35*−*37*) that highly helical structures located in those regions where the critical binding residues were located are neither immunogenic nor protective. The structured part of the peptide can be observed in red ribbon (Figure 4a, left-hand panel).

Peptide **23390** (immunogenic and nonprotective) showed two main secondary structure elements that could be identified on the basis of the observed backbone NOEs (Figure 3). Medium-range NOEs between the Hα of residue *i* and Hβ proton of residue *i*+*3*, together with NOEs between Hα-amide protons from residue *i* and *i*+*2* indicate the presence of a 3_{10}-helix between D19-L23 amino acids (Figure 4c, left-hand panel) and a distorted type III β-turn between S9 to N12 (Figure 4b, left-hand panel). NOE signal $d_{\alpha N}(i,i+2)$ R10−N12 was concordant with the hydrogen bond corresponding to N12 NH−S9 C═O; the distance between C^{α} (*i*) and $C^{\alpha}(i+3)$ was less than 7 Å corresponding to the β-turn structure. The values observed for this turn were $\phi_{i+1} = -76°$, $\psi_{i+1} = -39°$, $\phi_{i+2} = -137°$, $\psi_{i+2} = -37°$ (the ideal

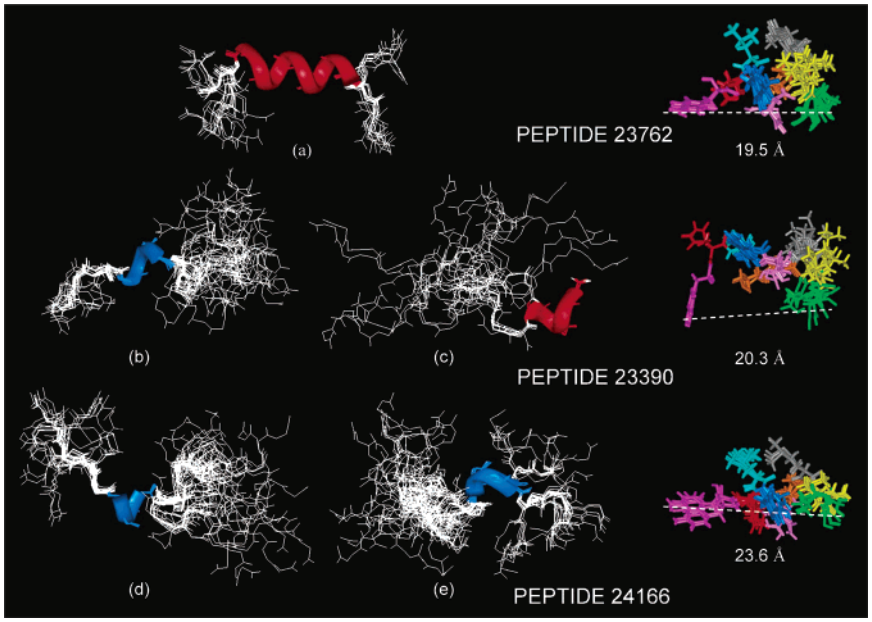

FIGURE 4: Structural features for peptide **1818** and its analogues. Left-hand panel: representation of peptide backbone: **23762** (nonprotective, nonimmunogenic), **23390** (immunogenic, nonprotective), and **24166** (immunogenic, protective). The structured region is shown in color: (a) α-helix of **23762** from P8 to M17 in red. (b) For peptide **23390** distorted type III β-turn from S9 to N12 in blue and (c) 3_{10} helix from D19 to L23 in red. (d) For peptide **24166** classical type III β-turn from S9 to N12 in blue and (e) classical type III β-turn from Y14-M17 in blue. Right-hand panel: lateral chains of residues 11−19. Color code: fuchsia Y11; red N12; pale blue L13; dark blue Y14; rose D15; light-brown K16; gray M17; yellow L18; green P19 (**24166** and **23762**), D19 (**23390**). The distances between the OH of Y11 (Pocket 1) and γCH2 of P19 (**24166** and **23762**) or the γC═O of D19 (23390) (Pocket 9) are shown by a dashed white line.

values for a type III turn being $\phi_{i+1} = -60°$, $\psi_{i+1} = -30°$, $\phi_{i+2} = -60$ $\psi_{i+2} = -30°$), showing distortion in the ϕ_{i+2} angle (Table 3). A flexible region from L13 to L18 connected these two structures.

Peptide **24166** (immunogenic and protective) showed two classical type III β-turn structures between S9−N12 and Y14-M17 (Figure 4, panels d and e, left-hand panel). The structure calculations and results for this peptide are shown in Table 3. These data suggest that modifications such as inducing short helixes or β-turn formation in random configuration peptides are associated with inducing immunogenicity and/or protection, suggesting in turn that these modifications may increase modified peptides' capacity to interact in conforming the MHC-II−peptide−TCR complex, which may then lead to appropriate activation of the immune system, as suggested in previous papers (*18*−*20*, *38*, *39*).

Crystallographic evidence has shown that peptides binding to Class-II molecules have a polyprolinyl type II-like configuration *(40)*; however, this type of structure is very difficult to recognize by NMR analysis. Since both methodologies have their pros and cons, we believe that shortened α-helical structures or distorted β-turns determined by NMR in solution may also fit into the Class-II molecule groove to property activate the immune system.

Biological, Immunological, and Structural Consideration. When analyzing the immunogenic and protective **24166** peptide's structure in the area where changes were made, starting with amino acid Y11, this protection inducing peptide could theoretically have been fitting into Pocket 1 (fuchsia), Y14 into Pocket 4 (dark blue), K16 into Pocket 6 (light-brown), and P19 into Pocket 9. All pointed downward to fit properly into the theoretical pockets of HLA-DRβ_1*1101 (Figure 4, right-hand panel). The other residues pointed upward, suggesting that modifications to residues which theoretically interact with the TCR were appropriate and might have allowed a better fit into HLA-DRβ_1* 1101 molecules and the TCR to conform the appropriate MHC II−peptide−TCR complex.

Peptide **1818** had a random configuration as shown before by both CD spectra analysis and NMR; therefore, a 3D structure could not have been determined. Nonimmunogenic, nonprotective peptide **23762** has a very helical structure between P8-M17 that might not allow it to fit very well into Class-II molecule pockets since there is a distance of 19.5 Å between putative Pocket 1 binding residue Y11 and putative Pocket 9 binding residue P19. This is too short to fit into the 24.5 ± 3.0 Å distance experimentally determined for these pockets (*41*, *42*). The same could have been happening to immunogenic but nonprotective peptide **23390** that bound to HLA-DRβ_1*0301 but in a different and not so strongly interacting register, where the distance between the same residues was 20.3 Å. By contrast, immunogenic and protective peptide **24166** had the appropriate 23.6 Å distance and appropriate amino acid sequence and amino acid orientation that should have allowed it to bind to HLA-DRβ_1*1101, as found experimentally in HLA-DRβ_1* binding studies.

Homologous molecules have been found in *A. nancymaae* monkeys by genomic analysis of their DRβ_1 exon 2 genes. D19P modification is essential in peptide **24166** for fitting into HLA-DRβ_1*1101 molecule, since this molecule only accepts small apolar amino acids such as A, S, G, and P in Pocket 9. As stated before, **22690** could be binding to molecules not included in the present study or to *Aotus*-specific Class-II molecules (*33*).

Although side conformation reliability has been found to be limited by relative high conformational freedom, there was a striking similarity between nonimmunogenic, nonpro-

tection-inducing peptide **23762** (which did not bind to any Class-II molecule) and immunogenic and protection-inducing HLA-DRβ_1*1101 binding peptide **24166**. However, it can be clearly seen that there is a 3 Å distance difference between these two peptides (between putative Pocket 1 and 9 binding residues) and such structural difference might account for the difference in biological and immunological functions. Therefore, the later can fit well into Class-II molecules, as found experimentally. The difference between these two peptides could lie in the D22L substitution at the C-terminal due to differences in their polarity, charge, and volume. Although these two peptides now bind to HLA-DRβ_1*-0301, there is a change in D19E in **27608** which can explain in part the difference in immunological activity of these two peptides.

Peptide binding in the Class-II molecule groove has been thoroughly studied during the past decade and represents the first step in MHC−peptide−TCR complex conformation appropriately activating the immune system.

What is shown in this paper for the first time is that modifications made to critical binding residues induce strong structural changes in nonstructured, nonimmunogenic, non-protection inducing peptides making them fit into specific Class-II molecule grooves such as HLA-DRβ_1*1101 for which a homologous molecule has been found in the *A. nancymaae* monkey. These modifications render some of these peptides immunogenic and protection-inducing. Therefore, the modifications we are making to them could allow these peptides to fit better into the MHC Class-II−peptide−TCR complex. These findings may allow a better design of multiantigenic, multistage, subunit-based synthetic malaria vaccines.

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