

Antimalarial Activity of Cupredoxins

THE INTERACTION OF PLASMODIUM MEROZOITE SURFACE PROTEIN 1₁₉ (MSP1₁₉) AND RUSTICYANIN*

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Isabel Cruz-Gallardo^{†1}, Irene Díaz-Moreno[‡], Antonio Díaz-Quintana[‡], Antonio Donaire^{§2}, Adrián Velázquez-Campoy^{¶3}, Rachel D. Curd^{||4}, Kaveri Rangachari^{||}, Berry Birdsall^{**}, Andres Ramos^{**5}, Anthony A. Holder^{||6}, and Miguel A. De la Rosa^{†7}

From the [†]Instituto de Bioquímica Vegetal y Fotosíntesis (IBVF), cicCartuja, Universidad de Sevilla-CSIC, Avenida Américo Vespucio 49, Sevilla 41092, Spain, the [§]Departamento de Química Inorgánica, Facultad de Química, Universidad de Murcia, Campus Universitario de Espinardo, Murcia 30100, Spain, the ^{||}Instituto de Biocomputación y Física de Sistemas complejos (BIFI), Universidad de Zaragoza, c/Mariano Esquillor, Zaragoza 50018, Spain, the ^{||}Parasitology Division and ^{**}Molecular Structure Division, Medical Research Council (MRC) National Institute for Medical Research, The Ridgeway, Mill Hill, London W7 1AA, United Kingdom

Background: The interaction of MSP1₁₉ with the cupredoxin azurin inhibits the growth of *Plasmodium falciparum* in red blood cells.

Results: Rusticyanin forms a well defined complex with MSP1₁₉ upon binding at the same surface area than inhibitory antibodies.

Conclusion: Rusticyanin becomes an excellent therapeutic agent for malaria.

Significance: Knowing the rusticyanin-MSP1₁₉ interface will allow the design of novel antimalarial drugs.

The discovery of effective new antimalarial agents is urgently needed. One of the most frequently studied molecules anchored to the parasite surface is the merozoite surface protein-1 (MSP1). At red blood cell invasion MSP1 is proteolytically processed, and the 19-kDa C-terminal fragment (MSP1₁₉) remains on the surface and is taken into the red blood cell, where it is transferred to the food vacuole and persists until the end of the intracellular cycle. Because a number of specific antibodies inhibit erythrocyte invasion and parasite growth, MSP1₁₉ is therefore a promising target against malaria. Given the structural homology of cupredoxins with the Fab domain of monoclonal antibodies, an approach combining NMR and isothermal titration calorimetry (ITC) measurements with docking calculations based on BiGGER is employed on MSP1₁₉-cupredoxin complexes. Among the cupredoxins tested, rusticyanin forms a well defined complex with MSP1₁₉ at a site that overlaps with the surface recognized by the inhibitory antibodies. The addition of holo-rusticyanin to infected cells results in parasitemia inhibition, but negligible effects on parasite growth can be observed for apo-rusticyanin and other proteins of the cupredoxin family. These findings point to rusticyanin as an excellent

therapeutic tool for malaria treatment and provide valuable information for drug design.

Malaria is a widely spread disease causing morbidity and mortality throughout a large part of the world. The increasing resistance of *Plasmodium falciparum*, the causative agent of the most deadly form of the disease, to current drugs has only increased the urgency for finding new antimalarial agents (1, 2), including an effective vaccine and new drug therapies (3, 4). There are five *Plasmodium* species that infect humans, whereas others infect other primates or rodents. Among this latter group, rodent parasites such as *Plasmodium yoelii* provide useful laboratory models for the study of malaria. The disease is caused by the replication and multiplication of the asexual blood stages in red blood cells. The merozoite form of the parasite invades the host cell, where it develops and replicates to form several new merozoites that then burst out of the cell to continue the cycle of invasion and multiplication. The invasion of red blood cells requires an initial recognition and binding mediated by parasite surface ligands, followed by reorientation and the formation of a moving junction between the erythrocyte and merozoite surfaces as the parasite enters the cell. Merozoite surface protein-1 (MSP1)⁸ has been implicated in this initial binding between parasite and host cell.

Located on the surface of the asexual blood-stage schizont and merozoite, MSP1 is one of the most frequently studied molecules of the parasite (5). It is synthesized as a ~200-kDa

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⁷ To whom correspondence should be addressed. Tel.: 34-954489506; Fax: 34-954460065; E-mail: marosa@us.es.

⁸ The abbreviations used are: MSP1, merozoite surface protein-1; Az, azurin; BiGGER, bimolecular complex generation with global evaluation and ranking; HSQC, heteronuclear single-quantum coherence; ITC, isothermal titration calorimetry; mAb, monoclonal antibody; MSP1₁₉, MSP1 19-kDa C-terminal fragment; PC, plastocyanin; PDB, Protein Data Bank; Rc, rusticyanin.

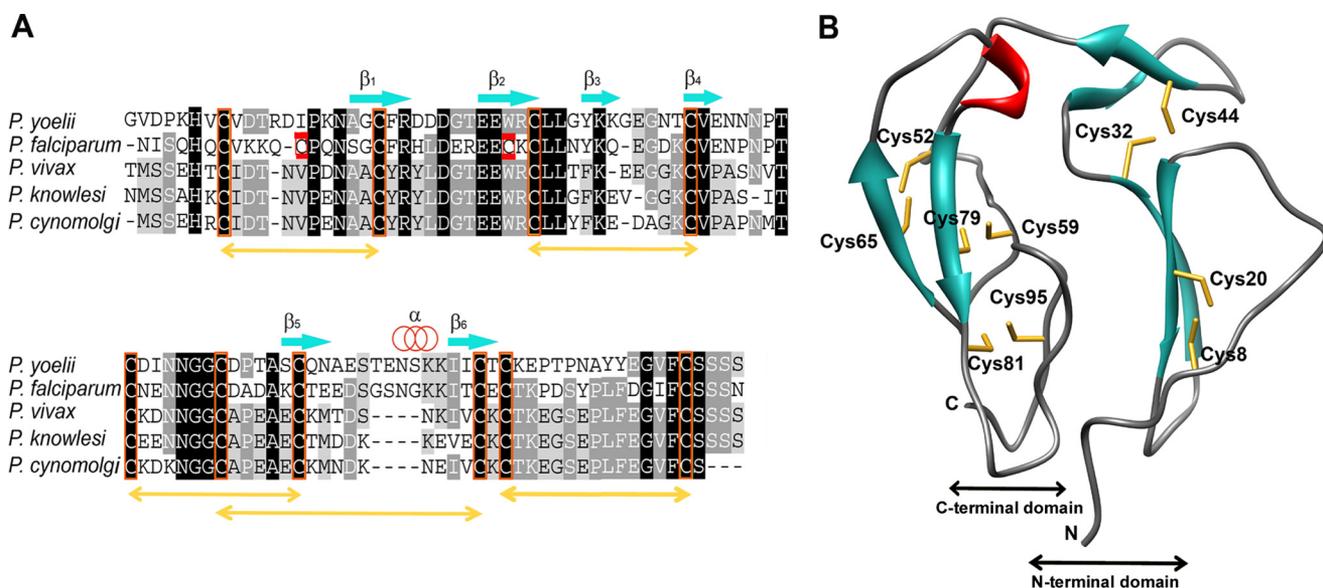


FIGURE 1. **MSP1₁₉ protein.** A, multiple sequence alignment of MSP1₁₉ with ClustalW2 (18) from a number of *Plasmodium* species. Sequences are colored by percent identity: black (100%), dark gray (80%), light gray (60%), and white (<50%). Secondary structure elements of *P. yoelii* MSP1₁₉ are shown. Orange boxes highlight cysteine residues that are conserved across different *Plasmodium* species, whereas red boxes highlight those present only in *P. falciparum*. Gold arrows stand for disulfide bonds formed by cysteine residues. The main difference between *P. falciparum* MSP1₁₉ and homologs from other *Plasmodium* species characterized to date lies in the disulfide bond pattern of the protein from the other species: one of the cysteine pairs in the first EGF domain is substituted by a tryptophan and a nonpolar or aliphatic residue. B, ribbon representation of the lowest energy NMR structure of *P. yoelii* MSP1₁₉ (BMRB accession number: 19233). Side chains of cysteine residues forming disulfide bridges are represented in gold.

precursor attached to the surface of the parasite via a glycosylphosphatidylinositol anchor, which undergoes a two-step proteolytic process: first, at merozoite release and then at erythrocyte invasion (6). As a result of this processing, the MSP1 is cleaved into several polypeptides that are shed from the surface in the final processing step, save a 19-kDa C-terminal fragment (MSP1₁₉). MSP1₁₉ is retained on the parasite surface by the glycosylphosphatidylinositol anchor and taken into the red blood cell at invasion (7–9). The role of MSP1₁₉ in the subsequent intracellular development of the parasite is poorly understood, although it is transferred to the developing food vacuole, where it remains until the end of the intracellular cycle and is discarded in the residual body together with products of hemoglobin digestion such as hemozoin (10). MSP1₁₉ is considered a promising malaria vaccine candidate due to the abundant evidence of specific antibodies inhibiting erythrocyte invasion and parasite growth, for instance, via the disruption of MSP1 proteolytic processing and intracellular parasite development (11).

At the structural and functional levels, MSP1₁₉ is particularly well conserved among *Plasmodium* species (Fig. 1) (12–17), and its three-dimensional structure has been shown to consist of two epidermal growth factor (EGF)-like domains in close contact. A characteristic disulfide-bridge pattern (Fig. 1) makes MSP1₁₉ highly resistant to proteases (19) and may explain why MSP1₁₉ remains intact in the digestive food vacuole up to the end of the intracellular cycle (10).

MSP1-specific immunoglobulins react with conformational epitopes of MSP1₁₉. Some of these antibodies inhibit parasite invasion of erythrocytes, whereas others do not. Fine structure epitope mapping of different monoclonal antibodies (mAbs) and the use of NMR methods indicates the binding of two inhibitory antibodies to epitopes on one side of the molecule

near the interface between the two EGF domains, including residues from both domains (20, 21). By contrast, non-inhibitory neutral mAbs bind elsewhere on the molecule (15, 20). Here, we have used MSP1₁₉ from *P. yoelii*, a rodent malaria parasite used as a laboratory model for vaccine studies (22) and for which both inhibitory and neutral antibodies have been partially mapped on the structure (23, 24).⁹ Independently of the immunoglobulin class, complexes involving MSP1₁₉ are kinetically rather stable with dissociation constants in the micromolar to subnanomolar range (15, 25).¹⁰ Because the binding affinity is similar in all cases, it has been assumed that the inhibitory effect depends mainly on steric factors, namely, epitope location, rather than the nature of the antibody.

The present study has been based on the structural homology of cupredoxins with the Fab fragment of an antibody, as well as on reports of a protein from this family interacting directly with MSP1₁₉ and blocking the increase of parasitemia in human red blood cells infected by *P. falciparum*, suggesting a promising treatment (26, 27). We first used the DaliLite pairwise comparison program to identify structural similarities between cupredoxins and the Fab fragment (28). A screening was then performed combining NMR and ITC measurements with docking calculations using BiGGER which indicated that, among the cupredoxins tested, rusticyanin (Rc) provided the most effective binding to MSP1₁₉. The two proteins form a well defined complex where Rc interacts at the interface between the two MSP1₁₉ subdomains, at a site that overlaps with the surface recognized by the inhibitory antibodies. Further, *P. falciparum* growth is inhibited by the presence of Rc in red blood cell cul-

⁹ R. D. Curd and A. A. Holder, unpublished data.

¹⁰ M. J. Lock and A. A. Holder, unpublished data.

MSP1₁₉-Rusticyanin Complex

tures. Interestingly, the copper site plays a key role in complex formation, because apo-Rc is not only unable to interact with MSP1₁₉, but also to inhibit parasite invasion and development in infected red blood cells.

EXPERIMENTAL PROCEDURES

Expression and Purification of Proteins—*P. yoelii* MSP1₁₉ (¹⁵N-labeled or unlabeled) was produced (essentially as described previously (29)) from a synthetic gene optimized for *Pichia pastoris* expression using as nitrogen source either ¹⁵NH₄Cl or (NH₄)₂SO₄ for labeled and unlabeled protein, respectively. The 99-amino acid sequence corresponds to residues 1656–1754 of the UniProtKB entry P13828 containing the N-terminal tag HHHHHHIEGR that has little effect on the NMR spectrum (20). Secreted His₆-tagged MSP1₁₉ was purified from the culture medium by nickel affinity chromatography (Ni-Sepharose 6 Fast Flow; GE Healthcare) and according to a previously elaborated protocol (12).

All recombinant metalloproteins were expressed in *Escherichia coli* cultures in LB medium and purified according to previously elaborated procedures, namely *Acidithiobacillus ferrooxidans* Rc (30, 31), *Nostoc* sp. PCC 7119, *Phormidium laminosum* and poplar plastocyanins (Pc) (32–34) and *Pseudomonas aeruginosa* azurin (Az) (35).

NMR Spectroscopy—All protein samples were concentrated in 10 mM potassium phosphate (pH 6.5) using Millipore 3000 NMWL centricons and microcons. MSP1₁₉ samples ranged in concentration from 0.5 to 2 mM, whereas cupredoxins were used in the range of 2–5 mM. All NMR samples contained 10% D₂O to adjust the lock signal. Reduction of the metal center in samples of copper(I) cupredoxins was achieved by adding sodium ascorbate, whereas oxidation of the metal center in samples of copper(II) proteins was achieved using sodium ferricyanide for Pc and Az, and sodium hexachloroiridate(IV) for Rc. In all cases, the proteins were washed extensively to remove the excess of the reducing/oxidizing agent.

NMR experiments were performed in a Bruker Avance 600 MHz spectrometer at 25 °C. The sequence-specific assignment of the backbone amide groups of ¹⁵N MSP1₁₉ (BMRB accession number: 19233) was achieved using standard backbone experiments (HNCACB, HNCA, etc.) and was confirmed using three-dimensional ¹H-¹⁵N NOESY-HSQC and three-dimensional ¹H-¹⁵N total correlation spectroscopy-HSQC spectra. The interaction of MSP1₁₉ with cupredoxins was followed by acquiring two-dimensional ¹H-¹⁵N HSQC spectra during the titration of 0.5 mM ¹⁵N-MSP1₁₉ solutions with an increasing amount of oxidized or reduced cupredoxins up to a final cupredoxin:MSP1₁₉ molar ratio of 4:1. The pH value of the sample was verified after each titration step. Prolines, which are invisible resonances in ¹⁵N HSQC spectra, are located at the positions 4, 15, 50, 61, 84, and 86, whereas Gly-1, Val-2, Glu-69, and Asn-73 are unassigned residues. All data processing was performed with Bruker TopSpin 2.0, and NMR analysis of line broadening perturbations of the cupredoxin-bound MSP1₁₉ with respect to free malarial protein was performed in the SPARKY program (36).

NMR Line Width Analysis—To estimate line widths, the peaks were fitted to a Gaussian function for the ¹⁵N and ¹H

dimensions using the program SPARKY with a 10,000 steps minimization and a 0.05% tolerance. In the analysis of the line widths ($\Delta\nu_{1/2}$), the overall broadening ($\Delta\Delta\nu_{1/2}$) obtained from signals displaying only minor line broadening was first subtracted from the line width of the corresponding signal. Then, for each residue, the differences of line widths between free and interacting MSP1₁₉ were calculated in every titration series ($\Delta\Delta\nu_{1/2 \text{ Binding}}$). The threshold value, used to identify a specifically broadened residue when data from the titration series were analyzed together, was defined as the average $\Delta\Delta\nu_{1/2 \text{ Binding}}$ for the system plus 2 standard deviations ($2S_{n-1}$). The average $\Delta\Delta\nu_{1/2 \text{ Binding}}$ and standard deviation were calculated for all amides with values ≤ 10 Hz on the basis that data > 10 Hz clearly indicated a specifically broadened residue, and their inclusion would bias the average to a higher value. Some assigned signals of the free MSP1₁₉ HSQC spectrum overlap (Val-9, Asn-87, and Cys-95) or exhibit very low intensity (Gly-41 and Asn-42), so they could not be properly integrated to include them in the line width analysis.

ITC—All ITC experiments were performed using VP-ITC and Auto-ITC200 instruments (Microcal; GE Healthcare) at 25 °C titrating Rc with MSP1₁₉. The reference cell was filled with distilled water. The experiments consisted of 10- μ l or 2- μ l injections of 0.3 mM MSP1₁₉ solution in 10 mM potassium phosphate buffer (pH 6.5) into the sample cell, initially containing 6.67 μ M Rc solution (reduced, oxidized, and apo forms) in the same buffer. All of the solutions were degassed before the titrations were performed. Titrant was injected at appropriate time intervals to ensure the thermal power signal returned to the base line prior to the next injection. To achieve homogeneous mixing in the cell, the stirring speed was kept constant at 1000 rpm in the Auto-ITC200 and at 450 rpm in the VP-ITC. The data, specifically the heat per injection normalized per mol of injectant versus molar ratio, were analyzed with Origin 7 (Microcal) using a single-site binding model. Calibration and performance tests of the calorimeter were carried out conducting CaCl₂-EDTA titrations with solutions provided by the manufacturer.

Molecular Docking Simulations—A soft docking algorithm implemented in the BiGGER software package (37) was used to determine *in silico* a model of the complexes MSP1₁₉-Az, MSP1₁₉-Pc, and MSP1₁₉-Rc. The PDB coordinates files of cupredoxins were 1JZG for Az (38), 1NIN for Pc (39), and 1A3Z for Rc (40). For each run, 5000 docking geometric solutions were generated based on the complementarity of the protein surfaces. These solutions were evaluated and ranked according to their “global score” and different interaction criteria including electrostatic energy of interaction, relative solvation energy, and the relative propensity of side chains to interact. For the MSP1₁₉-Rc adduct, NMR restraints were introduced in the docking calculations. All complexes graphic images were generated using the UCSF Chimera package (41).

***P. falciparum* Cultures**—Synchronized *P. falciparum* 3D7 late stage trophozoites at 33–36 h were used. The final parasitemia and hematocrit were between 0.1–0.2 and 2%, respectively. Red blood cells used for the assay were centrifuged to remove the buffy coat and washed twice in RPMI 1640 medium so that no white blood cells were present. The cul-

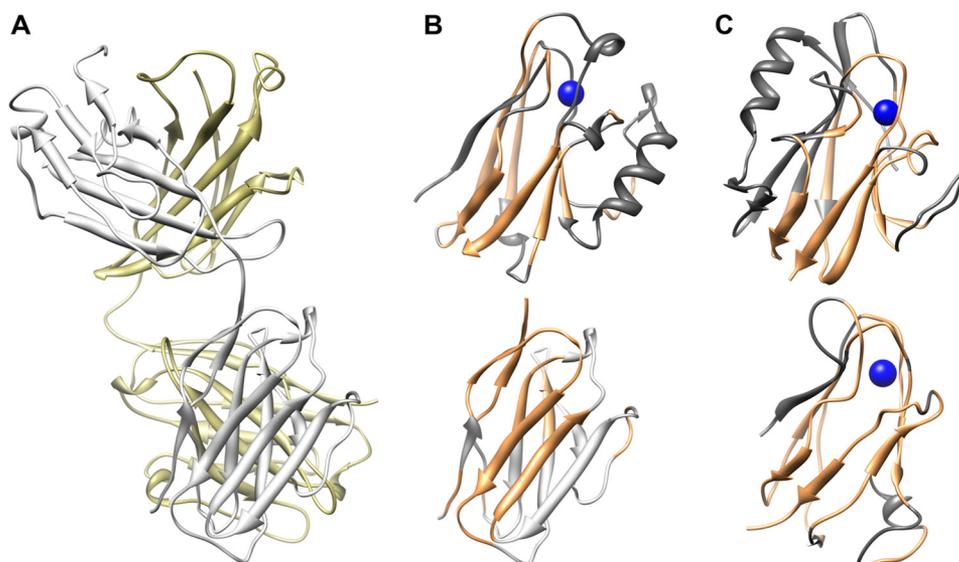


FIGURE 2. **Conserved structural motifs.** A, ribbon representation of the Fab fragment of mAb G17.12 (PDB ID code 1O81). Domain A is depicted in *light gray* and domain B in *gold*. B, structural alignment of Az (*upper*, PDB ID code 1JZG) and A1 fragment of Fab (*lower*) built by DaliLite pairwise comparison server. Matching regions, with a Dali Z-score of 2.9, appear in *orange*. C, ribbon representation of Rc (*upper*, PDB ID code 1A3Z) and Pc (*lower*, PDB ID code 1N1N). Dali Z-scores for Rc and Pc are 2.6 and 3.1, respectively. Copper atoms are colored in *blue*.

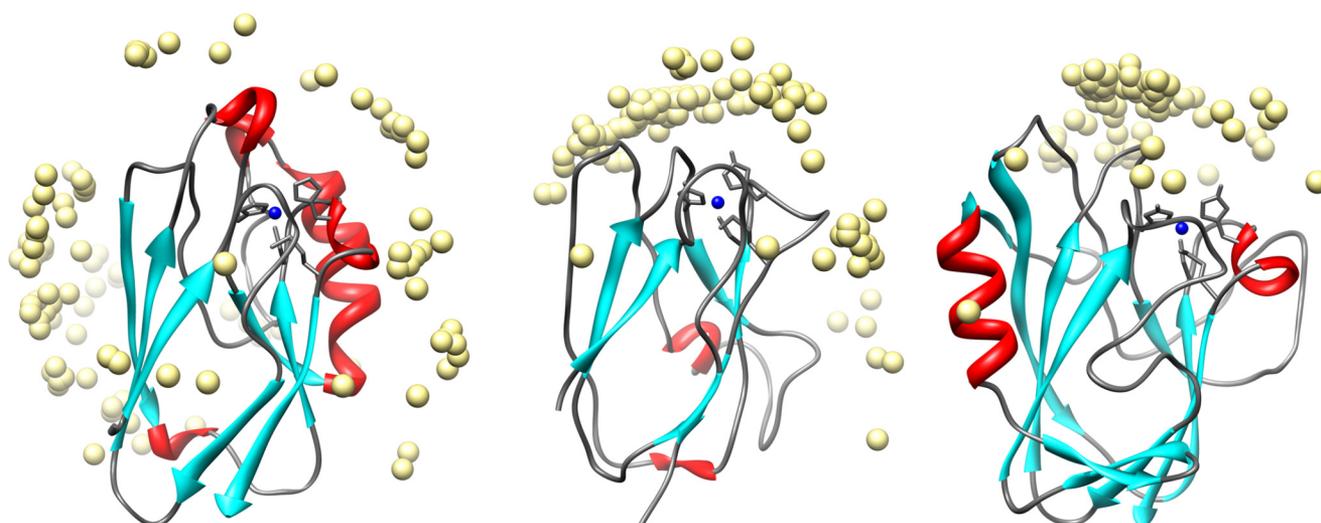


FIGURE 3. **Ab initio docking of cupredoxins with MSP1₁₉, performed by BiGGER.** Left, Az. Center, Pc. Right, Rc. Light yellow spheres represent the geometry centers of MSP1₁₉ in the 100 best solutions generated for each complex. All cupredoxins are oriented with respect to their copper center. Robertson diagrams of the cupredoxins are colored according to secondary structures: α -helices in *red* and β -strands in *blue*. Copper atoms are represented as *blue spheres* and the residues bonding to them are in *gray sticks*.

ture medium contained RPMI 1640 medium supplemented with 5 g/liter albumax, 0.025 g/liter gentamycin, and 0.292 g/liter L-glutamine.

***P. falciparum* Growth Inhibition Assay**—Sterile 96-well black tissue culture plates (Costar) were used routinely for every assay. The holo- or apo-species of Rc and *Nostoc* Pc were diluted in culture medium and used in duplicate wells for each dilution (200, 100, 50, and 25 μ M, respectively) in a final volume of 100 μ l/well. Two control sets were used in duplicate wells, one set with no added cupredoxin (positive control) and one with uninfected red blood cells (negative control). The plates were incubated at 37 °C for 48 h in a gas chamber flushed with 5% CO₂, 5% O₂, and 90% N₂. After 48 h, supernatants were removed from each well, replaced with fresh medium containing protein, and incubated for a further 48 h in the same man-

ner. At the end of the 96-h incubation, 25 μ l of SYBR Green I dye (SYBR Green I nucleic acid gel stain 10,000 \times , in dimethyl sulfoxide from Invitrogen) in lysis buffer (1 μ l dye to 1 ml lysis buffer), was added to each well and stored overnight at -20 °C. The lysis buffer contained Tris-HCl (20 mM, pH 8.0), EDTA (2 mM), Saponin (0.16% w/v) and Triton X-100 (1.6% v/v). Plates were thawed at room temperature, and fluorescence intensity was measured with a FLUO Star Omega microplate fluorescence reader (BMG Labtech). Values were expressed in relative fluorescence units. Binding of SYBR Green is specific for parasite DNA as mature erythrocytes lack DNA and RNA. Fluorescence intensity unit was converted to percentage (%) of growth as follows: % growth = (culture under Rc or Pc) – (uninfected RBC)/(culture with no Rc or Pc) – (uninfected RBC) \times 100, where RBC are red blood cells.

RESULTS

Structural Similarities between Cupredoxins and the Fab Fragment of a mAb—The Fab fragment crystal structure of mAb G17.12 (15), shown to bind MSP1₁₉, is composed of two domains, A and B, containing two regions, variable (symbolized by 1) and constant (symbolized by 2), with the canonical β -sandwich fold of the immunoglobulin superfamily (Fig. 2A). Consistent with previous reports, the DaliLite pairwise comparison program (28) identified significant structural similarities between the A1 fragment of the mAb and copper-containing redox proteins with immunoglobulin fold, such as Az, Pc, and Rc. In particular, the structural alignment between the A1 fragment and Az reveals a matching region, which is localized mainly at the two antiparallel β -sheets, as described previously (26; Fig. 2B), and with a Dali Z-score of 2.9. For the two other cupredoxins, Pc and Rc, this structural match is extended to the loops connecting β -strands yielding Z-scores of 3.1 and 2.6, respectively (Fig. 2C).

Given the structural similarity between various cupredoxins and the Fab fragment, *ab initio* docking approaches were performed with no experimental restraints (Fig. 3) to explore the capability of the metalloproteins to interact with MSP1₁₉ from *P. yoelii*. MSP1₁₉ is well conserved among the species with >50% sequence identity (Fig. 1A), with conserved three-dimensional structure and common functional features (17). We used the BiGGER rigid docking algorithm to generate sets of possible orientations for the different cupredoxin probes around MSP1₁₉ (the target). Fig. 3 shows the distribution of MSP1₁₉ mass centers resulting from the 100 best solutions from each computation around the corresponding copper protein. For Az and Pc, we observe a remarkably broad dispersion of the MSP1₁₉ geometry centers, suggesting the lack of specific surface complementarity. By contrast, molecular docking for the Rc-MSP1₁₉ interaction indicates that MSP1₁₉ explores a well defined area of Rc surrounding its copper center.

MSP1₁₉ Interactions with Cupredoxins by NMR—The MSP1₁₉-cupredoxin interaction was monitored by recording two-dimensional ¹⁵N HSQC NMR spectra on ¹⁵N-MSP1₁₉, both free and following the addition of Az, Pc, or Rc. The absence of changes in MSP1₁₉ resonances, either chemical shift perturbations or line broadening, indicates no detectable binding to Az and Pc in any of their oxidation states (Fig. 4, *top* and *middle* panels). By contrast, line width changes of certain MSP1₁₉ amide signals upon addition of Rc(Cu⁺) suggest a specific MSP1₁₉-Rc interaction (Fig. 4, *bottom* panel). Such observations coincide with *ab initio* docking simulations performed (Fig. 3) which suggested a well defined complex only between Rc and MSP1₁₉.

To probe in greater detail the interaction of MSP1₁₉ with either oxidized or reduced Rc, we analyzed the line widths of MSP1₁₉ resonances from ¹⁵N HSQC spectra across several titrations. Binding to Rc results in general signal broadening due to the increase in the rotational correlation time of MSP1₁₉ when interacting with Rc. In addition, several MSP1₁₉ backbone amides clustered in one area of the structure undergo larger changes in line widths ($\Delta\Delta\nu_{1/2}^{\text{Binding}}$) upon Rc binding (Figs. 5 and 6). These resonances are expected to be at or in the

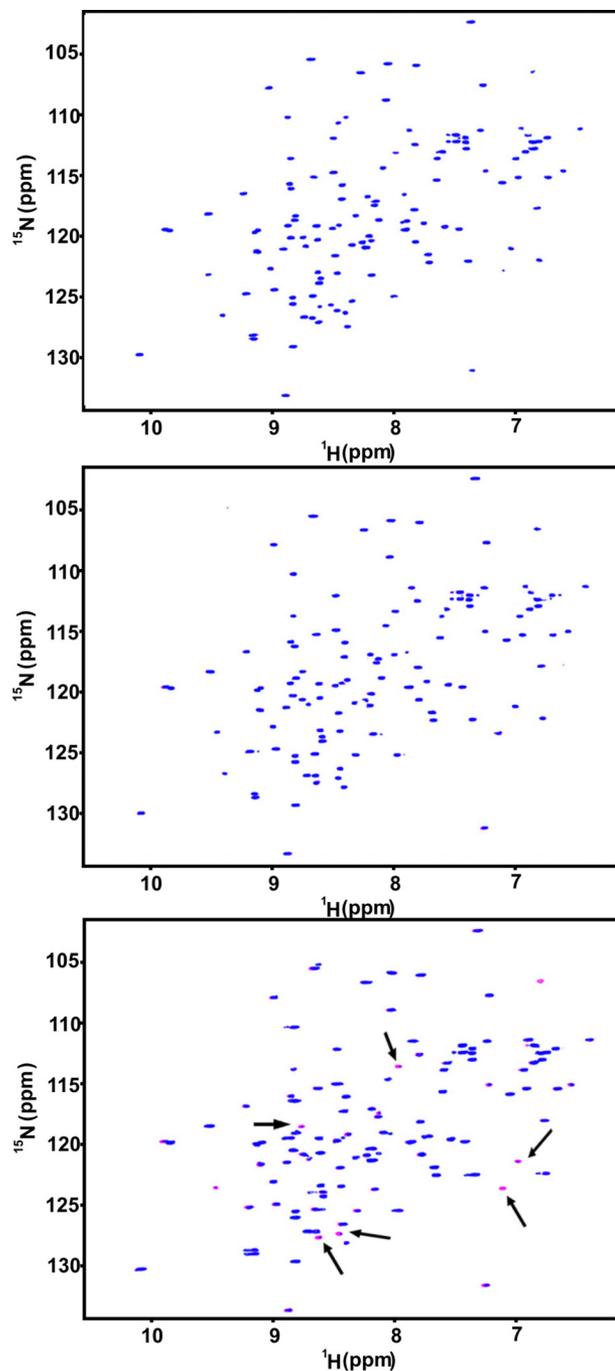


FIGURE 4. NMR titrations of ¹⁵N MSP1₁₉ with reduced cupredoxins. *Top*, Az from *P. aeruginosa*. *Middle*, Pc from *Nostoc* sp. PCC 7119. *Bottom*, Rc from *A. ferrooxidans*. Superimposition of ¹⁵N HSQC spectra of free MSP1₁₉ (magenta) and after adding one of three cupredoxins (blue) at a cupredoxin:MSP1₁₉ ratio of 4:1 is shown. Arrows point out those residues that experience substantial broadening. Negligible binding to MSP1₁₉ was observed upon adding aliquots of Pc and Az.

proximity of the area of MSP1₁₉ interacting with Rc. To selectively define resonances most likely to be part of the interface, threshold values (specifically, $\Delta\Delta\nu_{1/2}^{\text{Binding}} \geq 5\text{Hz}$ for ¹⁵N and $\geq 11\text{Hz}$ for ¹H dimension) were set (see “Experimental Procedures”). As expected, the distribution of line width changes ($\Delta\nu_{1/2}$) becomes broader as the Rc:MSP1₁₉ ratio increases (data not shown).

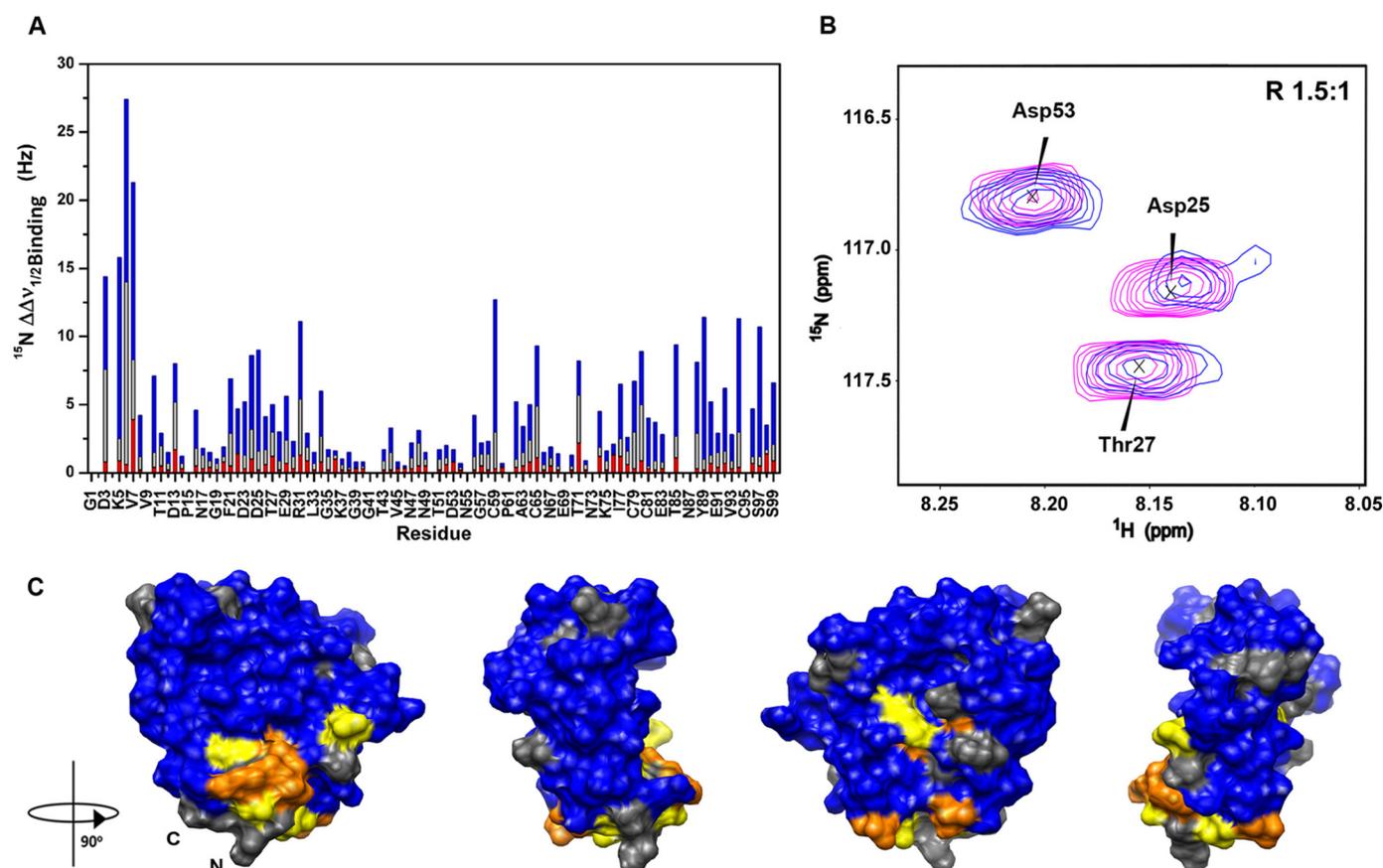


FIGURE 5. NMR titration of ^{15}N MSP1₁₉ with reduced Rc. *A*, ^{15}N line width differences ($^{15}\text{N} \Delta\Delta\nu_{1/2\text{Binding}}$) between free and Rc-bound MSP1₁₉. The Rc:MSP1₁₉ ratios are 0.5:1 (red), 1:1 (gray), and 1.5:1 (blue). *B*, superposition of ^{15}N HSQC spectra of free MSP1₁₉ (magenta) and bound to Rc (blue) in a Rc:MSP1₁₉ ratio of 1.5:1. A subset of three representative resonances is labeled in black. *C*, map of MSP1₁₉ interface upon binding to Rc. MSP1₁₉ surface is rotated 90° around the vertical axes in each view. Residues are colored according to their $^{15}\text{N} \Delta\Delta\nu_{1/2\text{Binding}}$ (Hz): the resonances that undergo the largest broadening (≥ 5 Hz) are orange, and the signals with a significant line width over the detection limit < 5 Hz are yellow. The limit of 5 Hz corresponds to a threshold value relative to the average plus 2-fold the S.D. ($\Delta\Delta\nu_{1/2\text{Binding}} \geq \langle \Delta\Delta\nu_{1/2\text{Binding}} \rangle + 2S_{n-1}$). Residues with no line width perturbation are marked in blue, whereas prolines are in gray.

At the Rc(Cu^{1+}):MSP1₁₉ ratio of 1.5:1, 14 amino acids show considerable ^{15}N line width changes in MSP1₁₉ HSQC spectra (Fig. 5, *A* and *B*). These residues are distributed mainly along two regions of MSP1₁₉, namely, at the beginning of N-terminal EGF domain involving Asp-3, Lys-5, His-6, Val-7, Asp-10, and at the end of C-terminal domain comprising Thr-85, Ala-88, Tyr-89, Phe-94, and Ser-97. In addition, Asp-24, Asp-25, and Arg-31 at the two first antiparallel β -strands, along with Cys-59, are also altered. Fig. 5*C* shows the map of MSP1₁₉ residues affected by Rc addition, with colors corresponding to line broadening in the ^{15}N dimension. The sequential stretches of residues detailed above form the main cluster on the MSP1₁₉ surface, which surrounds the two EGF domain interface. Interestingly, some of these affected residues (Asp-24, Asp-25, Arg-31 and Cys-59) are located at the rear of the protein (Fig. 5*C*). Whereas Asp-24 and Asp-25 are close to amino acids at the N terminus, Arg-31 and Cys-59 lie near Phe-94. Because these four residues are adjacent to others placed at the EGF domain interface and involved in direct contact with Rc, these line width perturbations are probably a secondary effect of binding. Similar conclusions may be inferred from ^1H line width analysis, although protons are more sensitive to broadening (data not shown).

NMR titration of oxidized Rc on MSP1₁₉ results in significant MSP1₁₉ line width perturbations at a Rc(Cu^{2+}):MSP1₁₉ ratio of 4:1, at which some signals broaden beyond the detection limit (Fig. 6*A*). The MSP1₁₉ resonances affected by Rc(Cu^{2+}) binding involve both N-terminal (Asp-3, Lys-5, Asp-10, and Asp-13) and C-terminal (Cys-79, Thr-85, Tyr-89, Gly-92, Phe-94, Ser-97, and Ser-98) regions, as described for the reduced system at a Rc(Cu^{1+}):MSP1₁₉ ratio of 1.5:1. An additional stretch (Phe-21 to Asp-24, Gly-26, Glu-29, Arg-31, and Cys-59) is also perturbed whereas His-6, Val-7, Asp-25, and Thr-27 were residues over the detection limit (Fig. 6*A*). Notably, the strength of the broadening observed for a 4:1 Rc(Cu^{2+}):MSP1₁₉ ratio is comparable with the one observed at a 1.5:1 Rc(Cu^{1+}):MSP1₁₉ ratio, indicating that MSP1₁₉ binds more weakly to the oxidized Rc. However, the MSP1₁₉ interacting surface involved in Rc recognition at a Rc:MSP1₁₉ ratio of 4:1 is independent of the cupredoxin redox state (Fig. 6).

Binding of MSP1₁₉ to Holo-Rc by ITC—ITC measurements reveal that MSP1₁₉ binds to Rc either in its reduced or oxidized state with a 1:1 stoichiometry at 25 °C (Fig. 7). Notably, the interaction of MSP1₁₉ with Rc(Cu^{1+}) is exothermic with a dissociation affinity constant (K_d) of 2 μM , whereas that with Rc(Cu^{2+}) is an endothermic process with lower binding affinity

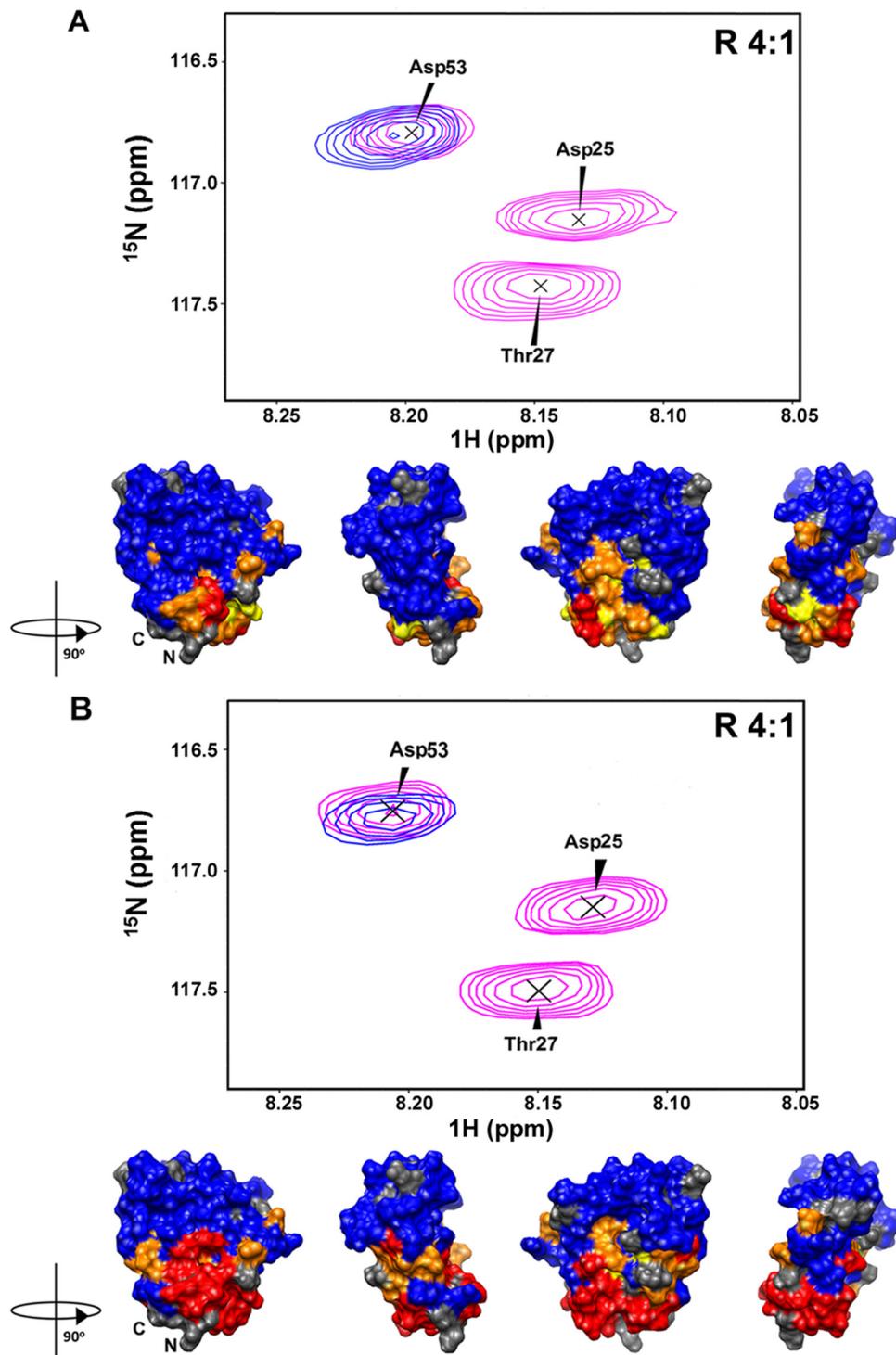


FIGURE 6. Comparison between NMR titrations of ¹⁵N MSP1₁₉ with either oxidized or reduced Rc at Rc:MSP1₁₉ ratio of 4:1. Most of the MSP1₁₉ signals in binding to Rc(Cu¹⁺) are broadening over the detection limit at the Rc:MSP1₁₉ ratio of 4:1, suggesting that MSP1₁₉ binds reduced Rc with a higher affinity. A, upper, overlap between ¹⁵N HSQC spectra of free MSP1₁₉ (magenta) and oxidized Rc-bound MSP1₁₉ (blue). A, lower, map of MSP1₁₉ in the presence of oxidized Rc. B, the same as A with reduced Rc. The 90°-rotated surface representations of MSP1₁₉ show residues colored according to their ¹⁵N $\Delta\Delta\nu_{1/2}^{\text{Binding}}$ following the same color code as in Fig. 5C. Those residues broadened beyond the detection limit are highlighted in red.

($K_d = 25 \mu\text{M}$) (Table 1). Such differences in K_d values are in agreement with NMR titrations and suggest that MSP1₁₉ binds to reduced Rc with a greater affinity than to oxidized Rc. Further experiments need to be performed to explain the opposite sign in the enthalpy of both processes as it could be related to (i) changes in the protonation/deprotonation

equilibrium of ionizable groups of the interacting proteins; (ii) variations in the hydrogen-bonding networks; (iii) differences in water arrangement in the vicinity of oxidized or reduced cofactors; and (iv) slight conformational modifications altering the number of solvent molecules excluded from the protein interface.

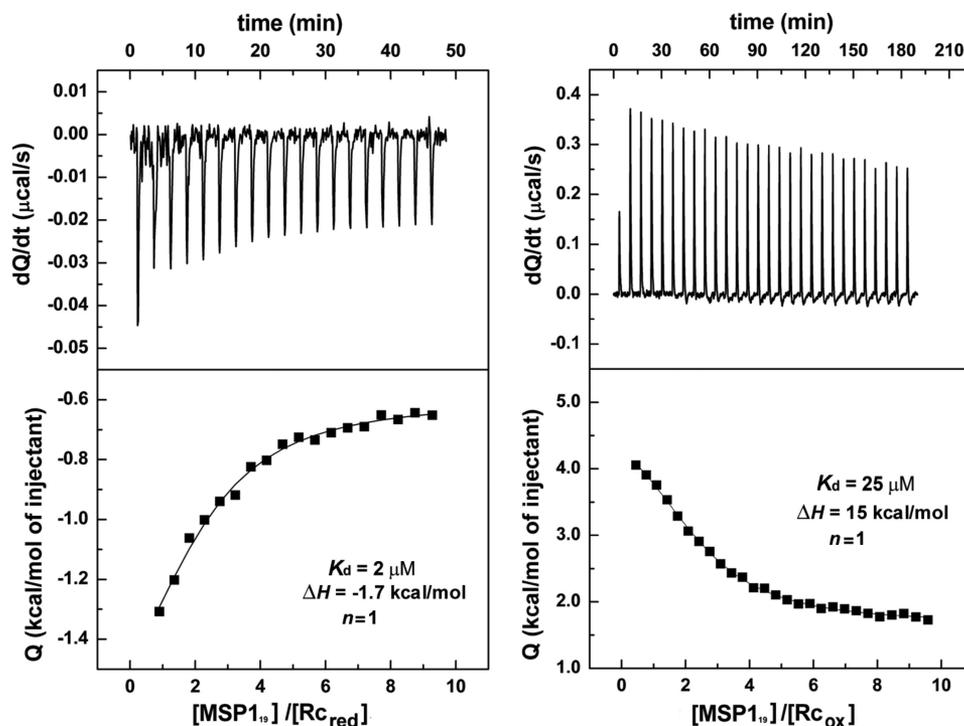


FIGURE 7. ITC titrations of MSP1₁₉ with both redox states of Rc. Binding assays of the complexes between MSP1₁₉ and reduced (*left*) or oxidized Rc (*right*) are shown. Thermograms are shown at the *top* and binding isotherms at the *bottom*, along with the dissociation constant (K_d expressed in μM), enthalpy (ΔH in kcal/mol). The stoichiometry value (n) was fixed to avoid degeneracy in the nonlinear regression data analysis. Typical relative errors are 20–25% for the dissociation constant and 5–10% for the stoichiometry.

TABLE 1

Thermodynamic values inferred from ITC experiments

The affinity of a protein-protein interaction is defined by the Gibbs energy of the binding: $\Delta G = -RT \ln K_d$. ΔG has two different contributions, ΔH and ΔS , according to the equation: $\Delta G = \Delta H - T\Delta S$, thus several combinations of those values could yield similar binding affinities. The complex formation is entropically driven in both cases, being the enthalpic contribution to the binding unfavorable (15 kcal/mol with oxidized Rc) or only slightly favorable (−1.7 kcal/mol with reduced Rc).

MSP1 ₁₉ complex	ΔG	ΔH	$-T\Delta S$	K_d
	kcal/mol	kcal/mol	kcal/mol	μM
Oxidized Rc	−6.3	15.0	−21.3	25.0
Reduced Rc	−7.8	−1.7	−6.1	2.0

The Case of Apo-Rc—To determine the role of the copper center, NMR and ITC titrations were carried out using MSP1₁₉ and apo-Rc. Surprisingly, NMR titrations showed no substantial line width changes even at an apo-Rc:MSP1₁₉ ratio of 4:1 (Fig. 8). The finding was further corroborated by ITC measurements, as the weak calorimetric profile suggests a lack of interaction (Fig. 8). Altogether, these data indicate the relevance of the copper center in the binding to MSP1₁₉. Despite the small structural differences in cupredoxins reported previously in solid state, there exists a high degree of mobility of the metal-binding loops in solution in the apo form, as recently demonstrated by NMR (42, 43). The different pattern between apo- and holo-Rc *versus* MSP1₁₉ could be related with the found differences in the dynamics of this site in the two forms.

Docking Simulations with BiGGER—Along with the *ab initio* docking calculations run on the MSP1₁₉-Rc(Cu⁺) complex, revealing how the MSP1₁₉ mass center docks on the Rc metal crevice (Fig. 3), an NMR-restrained docking with BiGGER was also performed. Line width data for those MSP1₁₉ residues in contact with Rc at a Rc:MSP1₁₉ ratio of 1.5:1 were included in

the run. The output is a set of docked solutions that can be ranked according to the BiGGER global score or individual scores, such as hydrophobic criteria, electrostatics, and geometrical parameters. Fig. 9 shows the best 100 solutions, as represented by Rc geometry centers, according to the global and hydrophobic scores from the program (Fig. 9A). The best scoring models predicted by restrained docking reveal how MSP1₁₉ leans its EGF domain interface to approach Rc (Fig. 9A). Indeed, the proximity of the surfaces on both proteins in the complex is shown in the space-filling representation (Fig. 9A). In addition to the copper center, loops connecting Rc β -strands are in close contact with MSP1₁₉. A deep analysis of the complex interface predicted by docking points to the N-terminal and C-terminal regions of the MSP1₁₉, as driving complex formation as some NMR restraints are satisfied (Fig. 9B). In the model reported, some electrostatic interactions occur (Fig. 9C) because Rc residues Lys-81 and Lys-116 are close to Glu-91 and Glu-83 in MSP1₁₉. Furthermore, Lys-5 of MSP1₁₉ can interact with Glu-9 of Rc, although the Glu is partially buried. The Rc interaction surface is mainly hydrophobic with the exception of a single positive spot, Lys-81 and Lys-116, surrounded by Phe-83, Gly-82, and Trp-7 and the copper center, which, in turn, is enclosed by Met-99, Val-98, and Pro-141. This interaction mode resembles those of the copper protein Pc and cytochrome *c*₆ with their photosynthetic partners, cytochrome *f* and photosystem I. In such complexes, Pc and cytochrome *c*₆ use the hydrophobic site surrounding the copper center and the heme group (site 1) as well as their charged patch (site 2; 44, 45).

Inhibition of P. falciparum Growth by Holo-Rc—To assess the physiological relevance of the MSP1₁₉ binding to Rc, the growth of *P. falciparum* within red blood cells was followed in

MSP1₁₉-Rusticyanin Complex

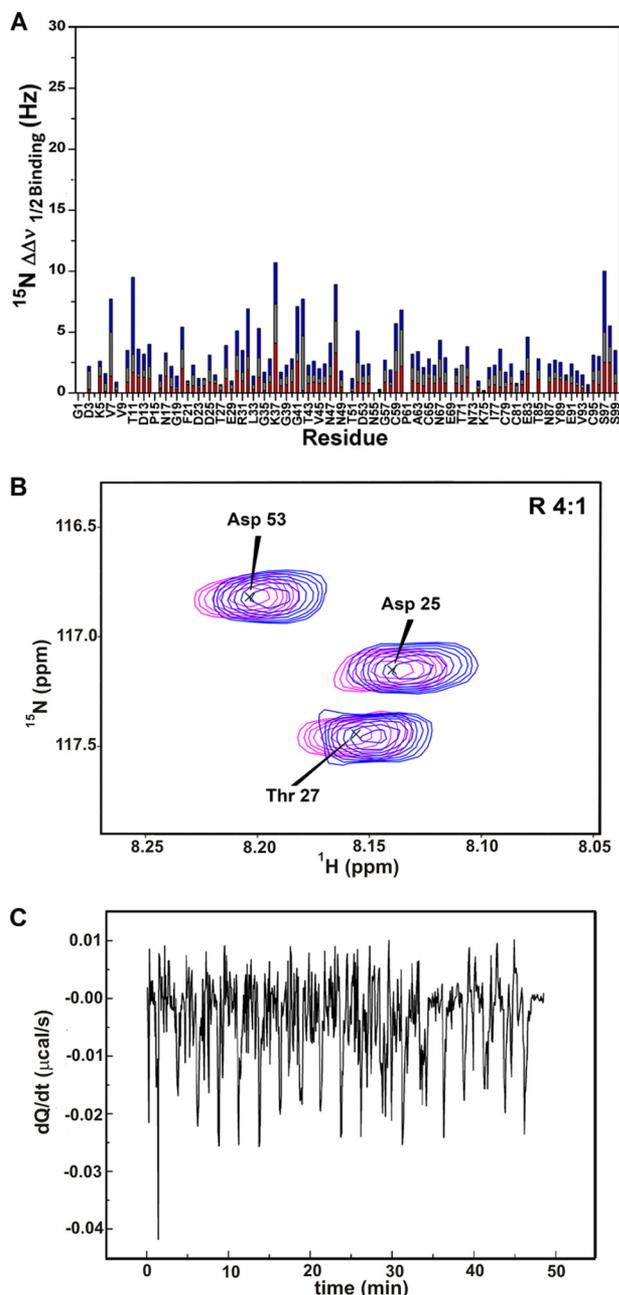


FIGURE 8. NMR and ITC titrations of MSP1₁₉ with apo-Rc. *A*, ¹⁵N line width differences (¹⁵N ΔΔν_{1/2Binding}) between free MSP1₁₉ and in the presence of apo-Rc. The apo-Rc:MSP1₁₉ ratios were 1:1 (red), 1.5:1 (gray), and 4:1 (blue). *B*, overlap between ¹⁵N HSQC spectra of free MSP1₁₉ (magenta) and in the presence of apo-Rc (blue) at an apo-Rc:MSP1₁₉ ratio of 4:1. *C*, ITC thermogram obtained from the apo-Rc-MSP1₁₉ titration, revealing the lack of binding between both proteins because of the flat calorimetric profile.

the presence of either holo- or apo-Rc (see “Experimental Procedures”). The addition of holo-Rc to infected cells resulted in parasitemia inhibition, as culture growth decreased significantly (Fig. 10) at a concentration of 100 μM until the point of complete inhibition at 200 μM holo-Rc. In contrast, the apo form even at high concentrations did not have a significant effect on *P. falciparum* growth. These data fully corroborate previous observations using NMR or ITC as much as apo-Rc is unable to bind to MSP1₁₉.

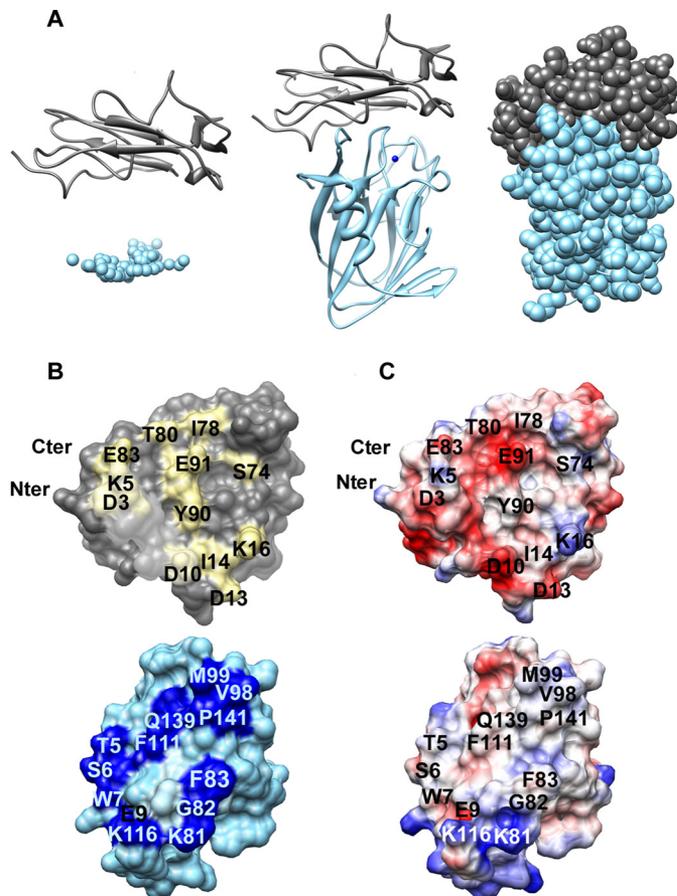


FIGURE 9. BIGGER molecular docking of the MSP1₁₉-Rc complex. *A*, left, best 100 models with the lowest energy values after alignment of MSP1₁₉ molecules with Rc geometry centers represented by spheres. Ribbon (center) and space-filling (right) representations for the best model are shown in the same orientation as on the left. MSP1₁₉ is represented in dark gray whereas Rc is in light blue. *B*, interface residues of the MSP1₁₉-Rc complex. MSP1₁₉ and Rc are independently rotated 90° to the top and to the bottom, respectively, with regard to their orientation in *A*. Contacting residues are depicted in light yellow for the MSP1₁₉ and dark blue for Rc. *C*, electrostatic potential surfaces of MSP1₁₉ (upper) and Rc (lower) with the same orientations as in *B*. The electrostatic potential surfaces were created with a color ramp for positive (blue) and negative (red) potentials at 300 mM ionic strength. The potentials were calculated in Chimera software (41).

To discard the theory that such a parasitemia inhibition could be ascribed to the well known apoptotic role of several cupredoxins (46, 47), control experiments were run with the holo- or apo-form of Pc. Holo-Pc was chosen as it does not bind to MSP1₁₉, as inferred from the herein presented NMR screening (see above), but it is structurally very similar to Rc: the two proteins belong to the type I blue copper-protein family, with almost identical folding and tetrahedral copper center. Upon addition of either holo- or apo-Pc (25 μM) under the same culture conditions, the *Plasmodium* growth first slightly decays to further reach a constant value until the end of the experiment. So the *Plasmodium* growth does not depend on protein concentration (the percentage of growth is maintained at approximately 70% even at 200 μM Pc) and is practically the same with either holo- or apo-Pc. Altogether these results indicate that holo-Rc inhibits parasitemia upon specific binding to MSP1₁₉.

DISCUSSION

Proteins from the cupredoxin family have demonstrated antimalarial activity and potential as therapeutic agents (26, 27). We have shown here that the cupredoxin rusticyanin interacts with MSP1₁₉ with micromolar K_d , forming a well defined complex. The interaction takes place on the same surface as that targeted by inhibitory antibodies, suggesting that a similar mechanism could take place. Its strength is dependent on the presence and, to a lesser extent, on the oxidation state of the metal center, which is consistent with the need for an intact interaction surface and charge conservation. Furthermore, we have observed the inhibition of *P. falciparum* parasitemia in infected cells where holo-Rc is present as a result of the interaction with MSP1₁₉. However, negligible effect on parasite growth could be observed for apo-Rc and Pc forms.

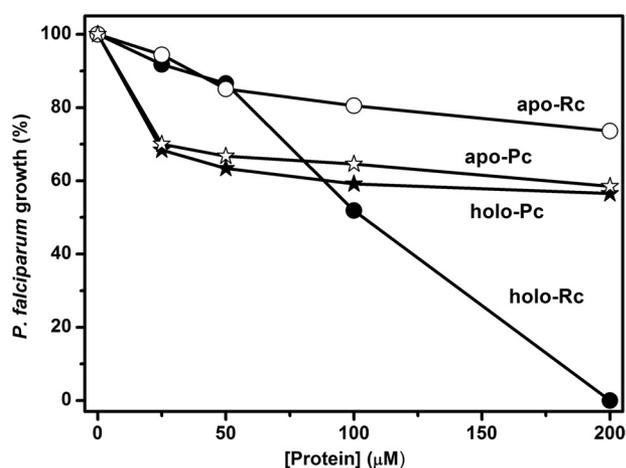


FIGURE 10. Inhibition of *P. falciparum* growth by Rc. Percentage of parasite growth was measured in the presence of either holo-Rc (filled circles), apo-Rc (open circles), holo-Pc (filled stars) or apo-Pc (open stars) at varying concentration (see "Experimental Procedures"). Parasite cultures were incubated with the Rc or Pc forms for a 96-h period, with a medium change after 48 h. Cultures in the absence of cupredoxin were used as a positive control.

MSP1₁₉ signals show slow exchange in the presence of the Fab fragments of the inhibitory mAb12.8 and mAb12.10, indicating the kinetic stability of complexes involving MSP1₁₉ with binding affinity constants in the micromolar to subnanomolar range (20). Rc binds specifically with a K_d of 2 μM to the interface between the two MSP1₁₉ EGF domains, overlapping the surface recognized by these inhibitory antibodies (20, 21), as indicated by our NMR-based mapping. A detailed comparison of MSP1₁₉ bound to either Rc or mAb12.10 and mAb12.8 reveals that the common interacting surfaces are located in the first EGF domain along with the first β -sheet and at the end of the second EGF domain. Interestingly, these regions of MSP1₁₉ form a shallow pocket (Fig. 1B) that may have a role in erythrocyte invasion (21). In addition, these residues involved in the formation of protein complexes with MSP1₁₉ are distributed in three segments: His-6 to Val-9, Lys-16 to Arg-22, and Tyr-89 to Cys-95 (numbering according to the *P. yoelii* MSP1₁₉ sequence; Fig. 1A), and they are strongly conserved in different species (21), supporting the idea that this is a functional site. The presence of additional perturbed residues identified in this study may be explained by the flat structure of MSP1₁₉ (5) because perturbations as a result of a protein-protein interaction may be transferred from one MSP1₁₉ face to the other.

Another point of interest arising from this study is the mode of interaction of MSP1₁₉ with Rc, compared with the interaction with mAbs. The study took as starting point a structural alignment of Rc with the Fab fragment of the non-inhibitory mAb G17.12 whose interaction with MSP1₁₉ has been solved through x-ray crystallography (15). The similarities with the β -barrel, as well as the loops connecting them, are clear (Fig. 2). However, the relative orientation between the mAb and MSP1₁₉ does not match that defined for the MSP1₁₉-Rc complex (Fig. 11A). In contrast, the models of mAb12.8-MSP1₁₉ and mAb12.10-MSP1₁₉ complexes built by Autore *et al.* (48) using NMR-based docking calculations match well with the Rc-MSP1₁₉ model reported here. In looking for structural sim-

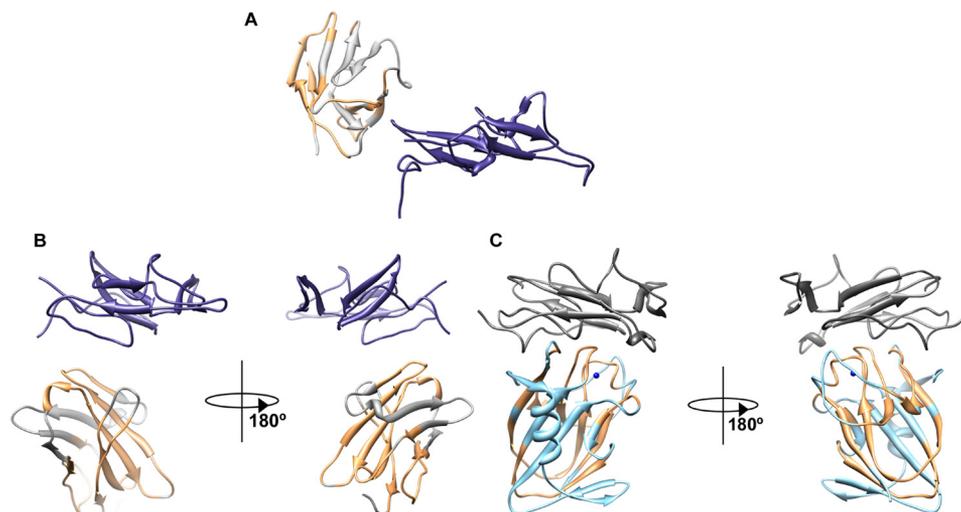


FIGURE 11. Comparison of complexes containing MSP1₁₉. A, MSP1₁₉-mAbG17.12 crystallographic structure (PDB ID code 1OB1; 15). Domain A1 of the noninhibitory mAbG17.12 is represented in light gray, whereas *P. falciparum* MSP1₁₉ is in purple. B, MSP1₁₉-mAb12.10 complex, whose PDB coordinates were kindly provided by Autore *et al.* (48). Domain A1 of the inhibitory mAb12.10 and *P. falciparum* MSP1₁₉ are colored as in A. C, MSP1₁₉-Rc complex studied in this work. *P. yoelii* MSP1₁₉ is depicted in dark gray and Rc in light blue. Copper center is represented by a blue sphere. Matching regions between mAbG17.12 or mAb12.10 and Rc after structural alignment using Dalilite are depicted in orange. The 180°-rotated views for both MSP1₁₉-mAb12.10 and MSP1₁₉-Rc complexes are shown.

MSP1₁₉-Rusticyanin Complex

ilarities between Rc and mAb12.10 with the DaliLite pairwise comparison server, the *Z*-score 2.8 (Fig. 11, *B* and *C*) suggested strong structural similarities, especially in the loops that interact with MSP1₁₉. In this paper, MSP1₁₉ complexes with mAb12.10 and Rc demonstrate (Fig. 11) a similar MSP1₁₉ orientation with respect to the other molecule (mAb12.10 or Rc) within each complex. The A1 region of mAb12.10 forms the main interaction with MSP1₁₉, similar to the structurally related region of Rc, especially at the level of β -strand connecting loops. As proposed by Autore *et al.* (48), a positively charged electrostatic spot on the antibody surface is required in the interaction and binding to MSP1₁₉. A similar positively charged area is located on the Rc surface, Lys-81 and Lys-116 in the complex interface, as inferred from our docking simulations (Fig. 9C). The Rc-MSP1₁₉ interface also involves hydrophobic contacts in the region surrounding the Rc copper center that presumably plays a key role in complex formation, as indicated by the NMR and ITC experiments. Reduced Rc binds MSP1₁₉ more efficiently than the oxidized form, suggesting that both electrostatic potential properties and protein folding depend on the metal redox state. This effect is even more drastic for apo-Rc, which is unable to interact with MSP1₁₉.

The practical significance of these observations can be found in the potential antimalarial role of the binding of Rc to MSP1₁₉ on the parasite surface, thereby blocking parasite growth and interfering directly with parasite invasion of red blood cells. In fact, the interaction might sterically interfere with the recognition between MSP1 and an erythrocyte surface molecule or with the proteolytic processing of MSP1, which occurs during invasion and can be inhibited by antibodies binding to MSP1₁₉. Such a role has been shown previously in the drug suramin (49). An alternative hypothesis, based on the fact that MSP1₁₉ is internalized with the malaria parasite during invasion and then trafficked to the food vacuole where it persists until the end of the next intraerythrocytic cycle (10), is that MSP1₁₉-bound Rc would be internalized and interfere with MSP1₁₉ functioning inside the cell, as shown for an antibody (11). Supporting this possibility, it has been reported that Rc is not only able to enter mammalian cells, but also to induce caspase-8-mediated apoptosis and/or to inhibit the cell cycle (47). Furthermore and unlike other cupredoxins, Rc is a copper protein with high acid stability and is biologically functional at a pH below 1.0 (50). The extreme acid stability of Rc may be important in understanding its function of blocking MSP1₁₉ because pH is \sim 4.5–5.5 inside the *Plasmodium* food vacuole. Altogether, Rc could be an excellent therapeutic tool for malaria treatment and expand our understanding of MSP1₁₉ function. In this way, as well, it could provide beneficial information for drug design.

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REFERENCES

1. Hobbs, C., and Duffy, P. (2011) Drugs for malaria: something old, something new, something borrowed. *Fl000 Biol. Rep.* **3**, 24
2. Wells, T. N., Alonso, P. L., and Gutteridge, W. E. (2009) New medicines to improve control and contribute to the eradication of malaria. *Nat. Rev. Drug Discov.* **8**, 879–891
3. Opar, A. (2011) Quarter-century quest for malaria vaccine shows signs of success. *Nat. Rev. Drug Discov.* **10**, 887–888
4. Holder, A. A. (2009) Malaria vaccines: where next? *PLoS Pathog.* **5**, e1000638
5. Holder, A. A. (2009) The carboxy-terminus of merozoite surface protein 1: structure, specific antibodies and immunity to malaria. *Parasitology* **136**, 1445–1456
6. Holder, A. A., and Blackman, M. J. (1994) What is the function of MSP-1 on the malaria merozoite? *Parasitol. Today* **10**, 182–184
7. Blackman, M. J., Heidrich, H. G., Donachie, S., McBride, J. S., and Holder, A. A. (1990) A single fragment of a malaria merozoite surface protein remains on the parasite during red cell invasion and is the target of invasion-inhibiting antibodies. *J. Exp. Med.* **172**, 379–382
8. Blackman, M. J., Ling, I. T., Nicholls, S. C., and Holder, A. A. (1991) Proteolytic processing of the *Plasmodium falciparum* merozoite surface protein-1 produces a membrane-bound fragment containing two epidermal growth factor-like domains. *Mol. Biochem. Parasitol.* **49**, 29–33
9. Blackman, M. J., Whittle, H., and Holder, A. A. (1991) Processing of the *Plasmodium falciparum* major merozoite surface protein-1: identification of a 33-kilodalton secondary processing product which is shed prior to erythrocyte invasion. *Mol. Biochem. Parasitol.* **49**, 35–44
10. Dluzewski, A. R., Ling, I. T., Hopkins, J. M., Grainger, M., Margos, G., Mitchell, G. H., Holder, A. A., and Bannister, L. H. (2008) Formation of the food vacuole in *Plasmodium falciparum*: a potential role for the 19 kDa fragment of merozoite surface protein 1 (MSP1₁₉). *PLoS ONE* **3**, e3085
11. Moss, D. K., Remarque, E. J., Faber, B. W., Cavanagh, D. R., Arnot, D. E., Thomas, A. W., and Holder, A. A. (2012) *Plasmodium falciparum* merozoite surface protein (MSP) 1₁₉-specific antibodies that interfere with parasite growth *in vitro* can inhibit MSP1 processing, merozoite invasion and intracellular parasite development. *Infect. Immun.* **80**, 1280–1287
12. Morgan, W. D., Birdshall, B., Frenkiel, T. A., Gradwell, M. G., Burghaus, P. A., Syed, S. E., Uthaipibull, C., Holder, A. A., and Feeney, J. (1999) Solution structure of an EGF module pair from the *Plasmodium falciparum* merozoite surface protein-1. *J. Mol. Biol.* **289**, 113–122
13. Garman, S. C., Simcoke, W. N., Stowers, A. W., and Garboczi, D. N. (2003) Structure of the C-terminal domains of merozoite surface protein-1 from *Plasmodium knowlesi* reveals a novel histidine binding site. *J. Biol. Chem.* **278**, 7264–7269
14. Babon, J. J., Morgan, W. D., Kelly, G., Eccleston, J. F., Feeney, J., and Holder, A. A. (2007) Structural studies on *Plasmodium vivax* merozoite surface protein-1. *Mol. Biochem. Parasitol.* **153**, 31–40
15. Pizarro, J. C., Chitarra, V., Verger, D., Holm, I., Pêtres, S., Darteville, S., Nato, F., Longacre, S., and Bentley, G. A. (2003) Crystal structure of a Fab complex formed with PfMSP1-19, the C-terminal fragment of merozoite surface protein 1 from *Plasmodium falciparum*: a malaria vaccine candidate. *J. Mol. Biol.* **328**, 1091–1103
16. Chitarra, V., Holm, I., Bentley, G. A., Pêtres, S., and Longacre, S. (1999) The crystal structure of C-terminal merozoite surface protein 1 at 1.8 Å resolution, a highly protective malaria vaccine candidate. *Mol. Cell* **3**, 457–464
17. O'Donnell, R. A., Saul, A., Cowman, A. F., and Crabb, B. S. (2000) Functional conservation of the malaria vaccine antigen MSP-119 across distantly related *Plasmodium* species. *Nat. Med.* **6**, 91–95
18. Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentini, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2007) ClustalW and ClustalX version 2.0. *Bioinformatics* **23**, 2947–2948
19. Hensmann, M., Li, C., Moss, C., Lindo, V., Greer, F., Watts, C., Ogun, S. A., Holder, A. A., and Langhorne, J. (2004) Disulphide bonds in merozoite surface protein 1 of the malaria parasite impede efficient antigen processing and affect the *in vivo* antibody response. *Eur. J. Immunol.* **34**, 639–648

20. Morgan, W. D., Lock, M. J., Frenkiel, T. A., Grainger, M., and Holder, A. A. (2004) Malaria parasite-inhibitory antibody epitopes on *Plasmodium falciparum* merozoite surface protein-1₁₉, mapped by TROSY NMR. *Mol. Biochem. Parasitol.* **138**, 29–36
21. Morgan, W. D., Frenkiel, T. A., Lock, M. J., Grainger, M., and Holder, A. A. (2005) Precise epitope mapping of malaria parasite inhibitory antibodies by TROSY NMR cross-saturation. *Biochemistry* **44**, 518–523
22. Ling, I. T., Ogun, S. A., and Holder, A. A. (1994) Immunization against malaria with a recombinant protein. *Parasite Immunol.* **16**, 63–67
23. Spencer Valero, L. M., Ogun, S. A., Fleck, S. L., Ling, I. T., Scott-Finnigan, T. J., Blackman, M. J., and Holder, A. A. (1998) Passive immunization with antibodies against three distinct epitopes on *Plasmodium yoelii* merozoite surface protein 1 suppresses parasitemia. *Infect. Immun.* **66**, 3925–3930
24. Benjamin, P. A., Ling, I. T., Clotey, G., Valero, L. M., Ogun, S. A., Fleck, S. L., Walliker, D., Morgan, W. D., Birdsall, B., Feeney, J., and Holder, A. A. (1999) Antigenic and sequence diversity at the C-terminus of the merozoite surface protein-1 from rodent malaria isolates, and the binding of protective monoclonal antibodies. *Mol. Biochem. Parasitol.* **104**, 147–156
25. Lazarou, M., Guevara Patiño, J. A., Jennings, R. M., McIntosh, R. S., Shi, J., Howell, S., Cullen, E., Jones, T., Adame-Gallegos, J. R., Chappel, J. A., McBride, J. S., Blackman, M. J., Holder, A. A., and Pleass, R. J. (2009) Inhibition of erythrocyte invasion and *Plasmodium falciparum* merozoite surface protein 1 processing by human immunoglobulin G1 (IgG1) and IgG3 antibodies. *Infect. Immun.* **77**, 5659–5667
26. Chaudhari, A., Fialho, A. M., Ratner, D., Gupta, P., Hong, C. S., Kahali, S., Yamada, T., Haldar, K., Murphy, S., Cho, W., Chauhan, V. S., Das Gupta, T. K., and Chakrabarty, A. M. (2006) Azurin, *Plasmodium falciparum* malaria and HIV/AIDS: inhibition of parasitic and viral growth by azurin. *Cell Cycle* **5**, 1642–1648,
27. Chakrabarty, A. M., Gupta, T. K. D., Yamada, T., Chaudhari, A., Fialho, A. M., and Hong, C. S. (November 30, 2006) Composition and methods for treating malaria with cupredoxin and cytochrome. U. S. Patent WO 2006/127477 A3
28. Hasegawa, H., and Holm, L. (2009) Advances and pitfalls of protein structural alignment. *Curr. Opin. Struct. Biol.* **19**, 341–348
29. Morgan, W. D., Kragt, A., and Feeney, J. (2000) Expression of deuterium-isotope-labelled protein in the yeast *Pichia pastoris* for NMR studies. *J. Biomol. NMR* **17**, 337–347
30. Jiménez, B., Piccioli, M., Moratal, J. M., and Donaire, A. (2003) Backbone dynamics of rusticyanin: the high hydrophobicity and rigidity of this blue copper protein is responsible for its thermodynamic properties. *Biochemistry* **42**, 10396–10405
31. Hall, J. F., Hasnain, S. S., and Ingledew, W. J. (1996) The structural gene for rusticyanin from *Thiobacillus ferrooxidans*: cloning and sequencing of the rusticyanin gene. *FEMS Microbiol. Lett.* **137**, 85–89
32. Díaz-Moreno, I., Díaz-Quintana, A., De la Rosa, M. A., and Ubbink, M. (2005) Structure of the complex between plastocyanin and cytochrome *f* from the cyanobacterium *Nostoc* sp. PCC 7119 as determined by paramagnetic NMR. *J. Biol. Chem.* **280**, 18908–18915
33. Crowley, P. B., Otting, G., Schlarb-Ridley, B. G., Canters, G. W., and Ubbink, M. (2001) Hydrophobic interactions in a cyanobacterial plastocyanin-cytochrome *f* complex. *J. Am. Chem. Soc.* **123**, 10444–10453
34. Lange, C., Cornvik, T., Díaz-Moreno, I., and Ubbink, M. (2005) The transient complex of poplar plastocyanin with cytochrome *f*: effects of ionic strength and pH. *Biochim. Biophys. Acta* **1707**, 179–188
35. van de Kamp, M., Hali, F. C., Rosato, N., Agro, A. F., and Canters, G. W. (1990) Purification and characterization of a nonreconstitutable azurin, obtained by heterologous expression of the *Pseudomonas aeruginosa* azu gene in *Escherichia coli*. *Biochim. Biophys. Acta* **1019**, 283–292
36. Goddard, T. D., and Kneller, D. G. (2006) SPARKY 3. University of California, San Francisco
37. Palma, P. N., Krippahl, L., Wampler, J. E., and Moura, J. J. (2000) BiGGER: A new (soft) docking algorithm for predicting protein interactions. *Proteins* **39**, 372–384
38. Crane, B. R., Di Bilio, A. J., Winkler, J. R., and Gray, H. B. (2001) Electron tunneling in single crystals of *Pseudomonas aeruginosa* azurins. *J. Am. Chem. Soc.* **123**, 11623–11631
39. Badsberg, U., Jørgensen, A. M., Gesmar, H., Led, J. J., Hammerstad, J. M., Jespersen, L. L., and Ulstrup, J. (1996) Solution structure of reduced plastocyanin from the blue-green alga *Anabaena variabilis*. *Biochemistry* **35**, 7021–7031
40. Zhao, D., and Shoham, M. (1998) Rusticyanin: extremes in acid stability and redox potential explained by the crystal structure. *Biophys. J.* **74**, 233
41. Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) UCSF Chimera: a visualization system for exploratory research and analysis. *J. Comput. Chem.* **25**, 1605–1612
42. Alcaraz, L. A., and Donaire, A. (2005) Rapid binding of copper(I) to folded aporusticyanin. *FEBS Lett.* **579**, 5223–5226
43. Zaballa, M. E., Abriata, L. A., Donaire, A., and Vila, A. J. (2012) Flexibility of the metal-binding region in apo-cupredoxins. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 9254–9259
44. Cruz-Gallardo, I., Díaz-Moreno, I., Díaz-Quintana, A., and De la Rosa, M. A. (2012) The cytochrome *f*-plastocyanin complex as a model to study transient interactions between redox proteins. *FEBS Lett.* **586**, 646–652
45. Díaz-Moreno, I., Díaz-Quintana, A., Molina-Heredia, F. P., Nieto, P. M., Hansson, O., De la Rosa, M. A., and Karlsson, B. G. (2005) NMR analysis of the transient complex between membrane photosystem I and soluble cytochrome *c₆*. *J. Biol. Chem.* **280**, 7925–7931
46. Yamada, T., Goto, M., Punj, V., Zaborina, O., Kimbara, K., Das Gupta, T. K., and Chakrabarty, A. M. (2002) The bacterial redox protein azurin induces apoptosis in J774 macrophages through complex formation and stabilization of the tumor suppressor protein p53. *Infect. Immun.* **70**, 7054–7062
47. Yamada, T., Hiraoka, Y., Das Gupta, T. K., and Chakrabarty, A. M. (2004) Rusticyanin, a bacterial electron transfer protein, causes G₁ arrest in J774 and apoptosis in human cancer cells. *Cell Cycle* **3**, 1182–1187
48. Autore, F., Melchiorre, S., Kleinjung, J., Morgan, W. D., and Fraternali, F. (2007) Interaction of malaria parasite-inhibitory antibodies with the merozoite surface protein MSP1₁₉ by computational docking. *Proteins* **66**, 513–527
49. Fleck, S. L., Birdsall, B., Babon, J., Dluzewski, A. R., Martin, S. R., Morgan, W. D., Angov, E., Kettleborough, C. A., Feeney, J., Blackman, M. J., and Holder, A. A. (2003) Suramin and suramin analogues inhibit merozoite surface protein-1 secondary processing and erythrocyte invasion by the malaria parasite *Plasmodium falciparum*. *J. Biol. Chem.* **278**, 47670–47677
50. Blake, R. C., 2nd, and Shute E. A. (1987) Respiratory enzymes of *Thiobacillus ferrooxidans*: a kinetic study of electron transfer between iron and rusticyanin in sulfate media. *J. Biol. Chem.* **262**, 14983–14989

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Isabel Cruz-Gallardo, Irene Díaz-Moreno, Antonio Díaz-Quintana, Antonio Donaire, Adrián Velázquez-Campoy, Rachel D. Curd, Kaveri Rangachari, Berry Birdsall, Andres Ramos, Anthony A. Holder and Miguel A. De la Rosa

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