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Methodology

Homology building as a means to define antigenic epitopes on dihydrofolate reductase (DHFR) from *Plasmodium falciparum*

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Abstract

Background: The aim of this study was to develop site-specific antibodies as a tool to capture *Plasmodium falciparum*-dihydrofolate reductase (Pf-DHFR) from blood samples from *P. falciparum* infected individuals in order to detect, in a sandwich ELISA, structural alterations due to point mutations in the gene coding for Pf-DHFR. Furthermore, we wanted to study the potential use of homology models in general and of Pf-DHFR in particular in predicting antigenic malarial surface epitopes.

Methods: A homology model of Pf-DHFR domain was employed to define an epitope for the development of site-specific antibodies against Pf-DHFR. The homology model suggested an exposed loop encompassing amino acid residues 64–100. A synthetic peptide of 37-mers whose sequence corresponded to the sequence of amino acid residues 64–100 of Pf-DHFR was synthesized and used to immunize mice for antibodies. Additionally, polyclonal antibodies recognizing a recombinant DHFR enzyme were produced in rabbits.

Results and conclusions: Serum from mice immunized with the 37-mer showed strong reactivity against both the immunizing peptide, recombinant DHFR and a preparation of crude antigen from *P. falciparum* infected red blood cells. Five monoclonal antibodies were obtained, one of which showed reactivity towards crude antigen prepared from *P. falciparum* infected red cells. Western blot analysis revealed that both the polyclonal and monoclonal antibodies recognized Pf-DHFR. Our study provides insight into the potential use of homology models in general and of Pf-DHFR in particular in predicting antigenic malarial surface epitopes.

Background

Antibodies raised against short peptide fragments of a given protein have been reported to be able to cross-react with the native protein [1]. The identification of peptide epitopes simulating the native protein has traditionally been based on amino acid sequences or sequence motifs exposed on the outer surface of the protein structure, thereby making these peptides potential candidates as antigen epitopes. Examples of algorithms for selecting and defining properties of exposed peptide sequences include plots of hydrophilicity, hydrophobicity, external flexibility and antigenic index. However, these algorithms provide only crude approximations of the native structures, and antibodies raised against the selected peptides are often lacking reactivity or show low degree of cross-reactivity with the native protein [2]. In recent years, the number of proteins for which three-dimensional structures have been determined by experimental and computational methods has increased dramatically [3]. The information could aid in a more precise identification of antigenic epitopes as opposed to defining epitopes based solely on the primary sequence of the protein.

The objective of the present study was to exploit the three-dimensional model of the *Plasmodium falciparum* dihydrofolate reductase (Pf-DHFR) domain to define peptide epitopes for the development of site-specific monoclonal antibodies against Pf-DHFR. The Pf-DHFR is a target of antifolatic drugs, i.e. pyrimethamine and cycloguanil. Resistance to pyrimethamine is linked to point mutations in the gene coding for Pf-DHFR [4-6].

The rationale for the development of site-specific antibodies as opposed to antibodies targeting the more general Pf-DHFR epitopes was a desire at later stage to develop secondary antibodies targeting specific epitopes on Pf-DHFR linked to drug resistance. Such antibodies would enable epidemiological studies of drug resistance in malaria. The feasibility of epitope mapping employing the structural model for Pf-DHFR was demonstrated by testing the reactivity of mouse polyclonal and monoclonal antibodies against native Pf-DHFR raised against a synthetic peptide identified as an exposed loop-peptide from the model structure. As a comparative experiment, rabbit polyclonal antibodies raised against the recombinant *P. falciparum* DHFR enzyme were used.

Materials and Methods

Computational analysis of Pf-DHFR

The homology model of Pf-DHFR was constructed based on the reported DHFR crystal structures of vertebrates (human and chicken), bacteria (*Escherichia coli* and *Lactobacillus casei*) and fungi (*Pneumocystis carinii*) as templates [7]. In these organisms, the DHFR and thymidylate synthase (TS) exist as separate monofunctional enzymes whereas the two enzymes in protozoa reside on the same polypeptide as a bifunctional DHFR-TS protein. Until recently, the only known three-dimensional structure of a bifunctional DHFR-TS protein was from *Leishmania major* [8]. Comparative analysis of the homology model of Pf-DHFR and the structures of mono-functional enzymes from other organisms revealed remarkable similarity with respect to its overall topology, except for the presence of two loop regions, a junctional peptide bridging the DHFR and TS domains, and the N-terminal extension in the case of *L. major* and *P. falciparum* DHFR-TS enzymes. Using SYBYL molecular modelling system, version 6.3 (Tripos Associates, St. Louis, MO, USA), the homology model of Pf-DHFR domain was exploited for the analysis of epitope candidates. Primary sequence analysis of Pf-DHFR was performed using the Protean software, (Lasergene, DNA Star software package, DNA Star Inc, Madison, Wl 53715, USA).

Immunization of mice with selected peptide and production of monoclonal antibodies

Ten mg of peptide (a 37-mer peptide selected as described in the results section with a purity of >95% as determined by HPLC) was synthesized by K.J. Ross-Petersen (Hørsholm, Denmark). The peptide was coupled to purified protein derivative of tuberculin (PPD) as a carrier at a ratio of 5:1 in 0.2% glutaraldehyde and the reaction was allowed to incubate overnight at 4°C. Five mice (CF1XBalbC) were immunized by intraperitoneal injection with 0.5 ml of the peptide solution (0.05 mg/ml peptide/PPD, 0.9% NaCI, 0.67 mg/ml $AI(OH)_3$, 0.05% merthiolate). After 2 weeks, the mice sera were tested for reactivity to the peptide by ELISA: Briefly, the ELISA plate was coated overnight at 4°C with 0.3 μg/ml peptide solution (in PBS). The plate was washed three times with washing buffer (0.37 M NaCI, 1% Triton X-100 in PBS, pH 7.4). Sera (1:10 dilution) in dilution buffer (washing buffer containing 1% BSA) were added to the wells and the reaction was allowed to incubate for 1 hour at room temperature. The plate was washed three times with washing buffer and secondary horseradish peroxidase rabbit anti-mouse-immunoglobulin-conjugated antibody (P260, DAKO, Glostrup, Denmark) with dilution buffer (1:1000) was added to each well. The plate was incubated for 1 hour at room temperature, followed by three times of washing with washing buffer. An o-phenylene-diamine (OPD) solution containing 1.5 mg/ml of 1,2-phenyldiamine dihydrochloride (DAKO, Glostrup, Denmark) dissolved in water and 0.015% H_2O_2 was added and the plate incubated at room temperature for 30 min. Adding 1 M H_2SO4 stopped the reaction and the optical density at 492 nm was measured by an ELISA reader.

Spleen cells from the highest responder mouse were fused with myeloma cells (X63Ag8.6.5.3) according to

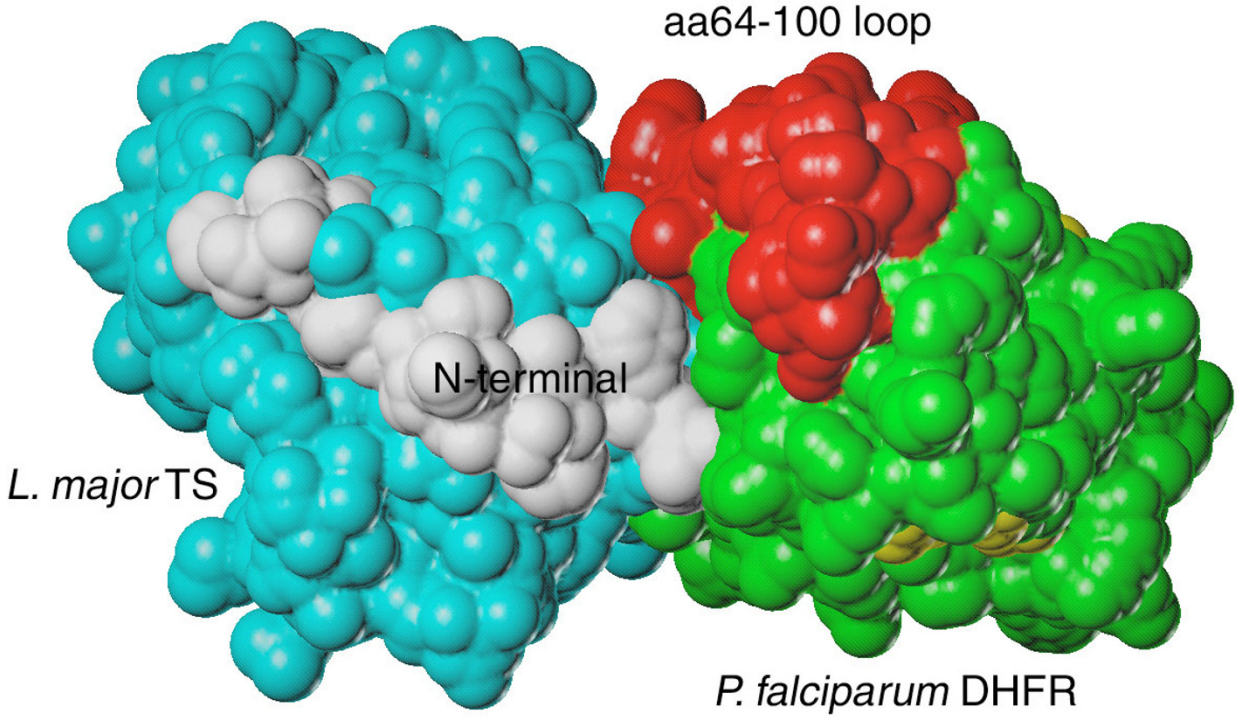

Figure 1
The molecular model of Pf-DHFR. The Pf-DHFR model (green) with the aa64-100 loop highlighted (red). The N-terminal part of the Pf-DHFR (white) was not included in the Pf-DHFR model and it is here shown together with the *Leishmania major*-TS structure (white and cyan, respectively) to illustrative that the aa64-100 loop does not interfere with these parts of the DHFR-TS complex. The Pf-DHFR substrate and cofactor are just visible at the lower right part of Pf-DHFR (orange). The surfaces are solvent-accessible surfaces generated with SYBYL.

procedures reviewed in [9]. The supernatant fractions from the fusions were tested by ELISA as described above, by incubating each supernatant in ELISA wells for 1 hour at room temperature (1:10 in dilution buffer). The well with the highest reactivity was selected and grown again and the procedure was repeated until a high reactivity from all wells with grown cells was obtained. The cell culture was now considered monoclonal allowing for mass-culture production of monoclonal antibodies and purification of antibodies.

Immunization of rabbits with recombinant DHFR and development of polyclonal antibodies

Recombinant DHFR (rDHFR) with a single point mutation at A16V was prepared by others, as described [10] and coupled to an immunogenic carrier-protein (S3, secreted proteins from cultures of mycobacteria (BCG)) in a molar ratio of 1:1. The complexed antigen was adsorbed onto $AI(OH)_3$ and emulsified with incomplete Freund's adjuvant. The final vaccine contained 25 μg carrier-protein/mg $AI(OH)_3$ and was given subcutaneously once, four times with two weeks intervals in the back of rabbits. The rabbit sera were tested by ELISA as described above for the mice, but the resulting polyclonal antibodies were not developed further into monoclonal antibodies.

Preparation of crude **P. falciparum** *protein extract*

The *P. falciparum* culture isolate 3D7 was grown to approximately 1.5×10^7 parasites/ml and the crude 3D7 protein extract enzyme was extracted according to [11]. The infected red blood cells were centrifuged at 2000 rpm for 10 min. The pellet was washed twice in 10 ml PBS and

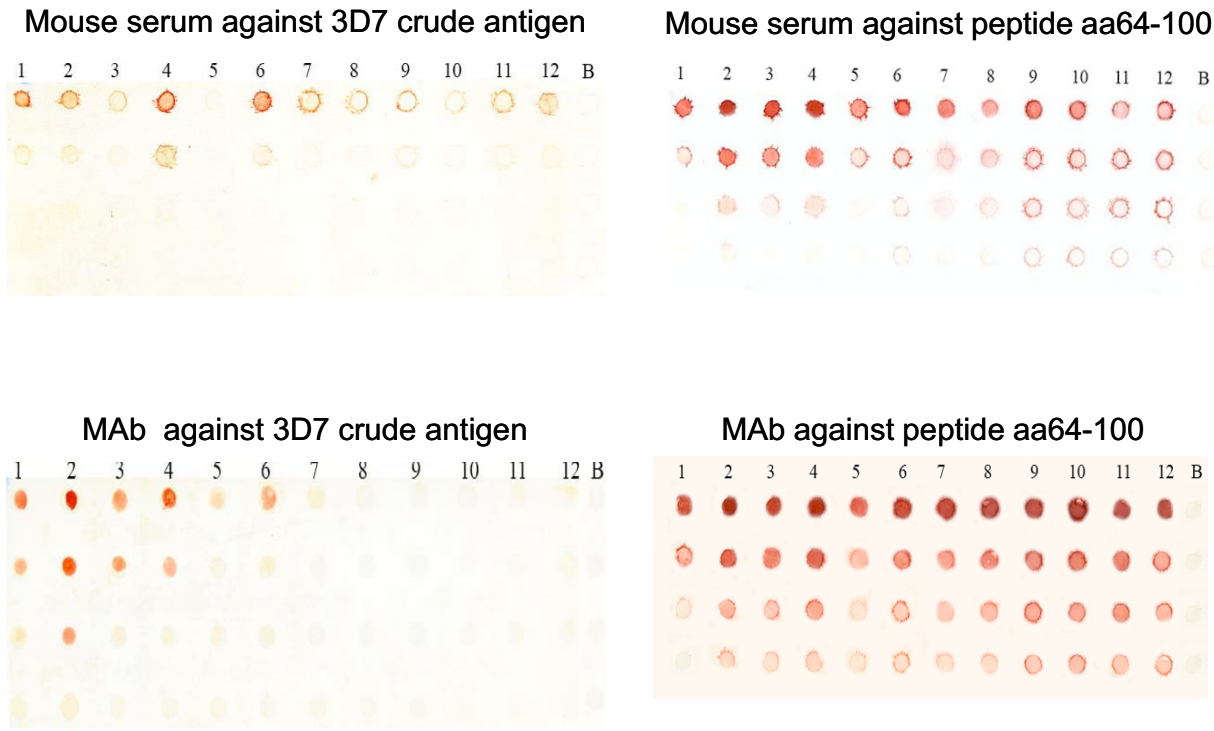

Figure 2
Dot blot analysis of mouse polyclonal and monoclonal antibodies raised against the aa64-100 peptide when tested against the loop peptide and crude *P. falciparum* 3D7 protein extract in the presence of various reagents interfering with protein structure. The reactivity to two-fold dilutions of crude 3D7 protein extract of polyclonal and monoclonal antibodies are shown. As a positive control the reactivity to the aa64-100 peptide solution (10 μg/ml, two-fold diluted) is shown. Crude 3D7 protein extract was pre-treated before dotting as indicated: 1: untreated, 2: boiled, 3: β-ME, 4: β-ME + boiled, 5: β-ME + IAA, 6: β-ME + IAA + boiled, 7: 0.1 % SDS, 8: 0.1 % SDS + boiled, 9: 0.1 % SDS + β-ME, 10: 0.1 % SDS + β-ME +boiled, 11: 0.1 % SDS + β-ME + IAA, 12: 0.1 % SDS + β-ME + IAA + boiled, B: Buffer control.

centrifuged at 2000 rpm for 10 min. A freshly made 0.015% saponin solution in PBS (20 × pellet volume) including one protease inhibitor cocktail tablet/10 ml saponin solution was added to the pellet (Roche, Cat. No. 1836170). The solution was incubated at 37°C for 30 min. and vortexed once within this period and at the end. The sample was subsequently kept on ice and centrifuged at 5000 rpm for 10 min. at 4°C. The pellet was washed twice in 10 ml of cold PBS. 3 ml of a 0.85% NaCI solution was added and the solution was sonicated four times for 15 sec with 30 sec rest in between. Finally, the solution was centrifuged at 15.000 rpm for 30 min. at 4°C and the supernatant was either used immediately or frozen at -20°C.

Dot blot analysis of mouse antiserum and of a monoclonal antibody tested against the loop-peptide and against an extract from infected red cells (crude 3D7 antigen)

The ability of mouse serum and monoclonal antibodies raised against the synthetic aa64-100 peptide to recognize the immunising peptide and crude 3D7 protein extract was tested by dot blot analysis: The peptide and the 3D7 protein extract were pre-treated in various ways (untreated, and combinations of boiling and incubation with SDS, β-ME (β-mecapto-ethanol), and IAA (imidoacetic acid)) and subsequently 2.5 μl was dotted onto nitrocellulose paper strips (Hybond, RPN2020E, Amersham) and allowed to dry. The strips were incubated in blocking buffer (0.25 M Tris-base, 2.5 M NaCI, 0.5% Tween-20 in H_2O, ph: 7.6) for 15 min and incubated with mouse serum or monoclonal antibody culture supernatant (1:5)

in washing buffer (0.25 M Tris-base, 2.5 M NaCI, 0.05% Tween-20 in water, pH: 7.6) over night at room temperature. The strips were washed twice in washing buffer for 2 × 10 min. Secondary goat anti mouse IgG conjugated to horse radish peroxidase (CHEMICON International, AP127P) was added (1:4000 in washing buffer) and incubated for 1 hour at room temperature. The strips were washed twice in washing buffer for 2 × 10 min. and then incubated in developing solution (3-amino-9 ethyl cabazol, N,N'-dimethyle-formamide and colour buffer (CH_3COOH and NaOH, pH 5,0) for 15 min. at room temperature protected from light. The strips were let to dry.

Western blot

SDS-PAGE and Western blot was prepared to estimate the size of the products recognized by the aa64-100 antibody and the polyclonal antibody raised in rabbits. SDS-PAGE was performed on gels consisting of 10 ml of separating gel (8%), made by adding 2 ml acrylamide solution (40% acylamide, 0.8% bis-acylamide), 2.5 ml separation buffer with 0.1% SDS (1.5 M Tris-HCI (pH: 8.8)) and 5.5 ml water with 100 μl 10% ammonium persulfate and 8 μl TEMED. 10 ml stacking gel (5%) was made by adding 1.25 ml 40% acrylamide solution, 2.5 ml stacking buffer with 0.1% SDS (1 M Tris-HCI (pH: 6.8)) and 6.25 ml H_2O with 100 μl 10% ammonium persulfate 8 μl TEMED. The samples were diluted 1:1 in sample buffer (125 mM Tris-HCI (pH: 6.8), 20% glycerol, 10% SDS, 0.01% bromophenol blue, 40 mM DTT 1.4 ml water). Electrophoresis was performed for 1.5 hours at 160 V. The gel was subsequently semi-dry blotted onto nitrocellulose paper in blotting buffer (25 mM Tris-base, 20% ethanol in H_2O, pH: 10.4) for 1 hour at 200 mA. The nitrocellulose membrane was incubated with blocking buffer (0.05 M Tris-base, 0.5 M NaCI, 0.5% Tween-20 and 5% skimmed milk in H_2O, pH: 7.6) at room temperature for 15–30 min. The monoclonal antibodies were diluted 1:5 in washing buffer (0.05 M Tris-base, 0.5 M NaCI, 0.05% Tween-20 in H_2O, pH: 7.6) and incubated overnight at room temperature. The membrane was washed twice in washing buffer and secondary antibody conjugated to alkaline phosphatase (P314, DAKO, Glostrup, Denmark) was added (1:1000 in washing buffer) and incubated for 1 hour at room temperature. The membrane was washed and 50 ml of a NBT/BCIP (NBT: nitro blue tetrazoleum, Sigma N-5515, BCIP: 5-bromo-4-chloro-3-indolyl-phosphate, Sigma B-0274) solution as recommended by the manufacturers was added to the membrane and incubated for 30 min. at room temperature and let to dry.

Results

The selection of a potential antigenic region

Using SYBYL molecular modelling system, the homology model of Pf-DHFR domain was exploited for the analysis of epitope candidates and a single potential antigenic region was identified remotely located in respect to the active site of the enzyme. The DHFR-TS and the chosen structure forming a loop spanning aa64-100 is shown in figure 1. Loops with certain length of amino acids are often highly flexible and positioned externally, hence are potentially antigenic. Although in the homology model the exact structure of the loop spanning aa64-100 has not been defined, the loop is predicted to locate externally and the TS domain does not conceal it. Furthermore, it does not interfere with the active sites of neither the DHFR nor the TS part of the bifunctional enzyme. According to the model aa64 (Tyr64) and aa100 (Asn100) are within Van Der Waals contact to each other and exhibit a distance between the C-alpha atoms of 6.0 Å. Thus, in order to attempt to induce the same conformation in the synthetic peptide, Tyr64 and Asn100 was replaced by Cys residues so that the synthetic peptide could form disulphide bonds and thereby presumed a loop structure similar to that predicted from the native Pf-DHFR protein. The validity of the antigenic epitope was analysed by primary sequence analysis of Pf-DHFR applying different algorithms (data not shown). According to the generated plots and the analyses of the stretch of amino acids spanning the residues 64–100, there were a few candidates selected that met the criteria predicted and that were highly flexible. However, the stretch of the epitope peptide would most likely have consisted of a fraction of the aa64-100 section if merely depending on these plots and the knowledge of the proximity of the aa64 and aa100 would not have been taken into account and considered as an important factor in the design of the synthetic peptides.

Dot blot analysis of immune mouse sera and monoclonal antibodies

The ability of the mouse polyclonal and monoclonal antibodies raised against the synthetic aa66-100 peptide to recognize the loop peptide and crude 3D7 protein is shown in figure 2. Of five monoclonal antibodies selected for strong reactivity, only one recognized the 3D7 antigen preparation (not shown), and this monoclonal antibody was selected for further analysis.

In order to find out under which conditions the crude 3D7 is recognized by the mono- and polyclonal antibodies (3D7 in its native form, denatured, reduced), the extract was tested in a variety of environments, see figure 2. It appears that boiling the 3D7 extract for 5 min. improved the reactivity of the monoclonal antibody, whereas treatment of the extract with 0,1% SDS inhibited the binding.

The polyclonal antibody raised against the synthetic peptide shows reactivity to the crude extract under all conditions except when the antigen was treated with β-ME +

IAA. The recognition of the untreated 3D7 crude antigen by the polyclonal antibodies indicates that the immune mouse serum contains antibodies against native Pf-DHFR from 3D7 crude extract.

Size estimation of the recognized product by Western blot

A Western blot analysis after separation of 3D7 proteins on an SDS-PAGE gel was performed in order to estimate the size of the product recognized by the monoclonal aa64-100 antibody selected for reactivity to 3D7 antigen and the polyclonal antibody to rDHFR raised in rabbits. As shown in figure 3, the Western blot revealed that both the aa64-100 monoclonal antibody and the polyclonal rDHFR antibody recognized one major product of approximately 70 kD.

Discussion

The objective of the present study was to analyse the feasibility of defining potential antigenic epitopes of a protein, *P. falciparum* DHFR (Pf-DHFR), by exploring a three-dimensional structure obtained by homology building from the known structure of DHFR from other species (vertebrates, bacteria, fungi). An epitope was selected based on its exposed loop-structure and potential flexibility and mice were immunized with a synthetic 37-mer peptide. Additionally, a polyclonal antibody towards recombinant DHFR enzyme (rDHFR) was raised in rabbits. Polyclonal antisera from the immunized mice showed strong reactivity with the peptide, with rDHFR, and with a preparation of crude antigen from *P. falciparum* infected red blood cells. Five monoclonal antibodies were developed with high reactivity to the peptide, however only one of these cross-reacted strongly with crude *P. falciparum* protein extract when tested by dot blot and was further analysed. The western blot showed that the aa64-100 antibody, as well as the polyclonal antibody towards rDHFR recognized one major product of approximately 70 kD, most likely to be Pf-DHFR. (including thymidylate synthetase, TS). The predicted molecular weight of Pf-DHFR-TS is 71.8 kD. The fact that the aa64-100 antibody reacted strongest after boiling of the crude extract and showed low reactivity to the untreated extract indicates that this monoclonal antibody has relatively low affinity to DHFR in its native state. However, the test (figure 2) of mouse serum immunized with the synthetic loop peptide aa64-100 leads us to the conclusion that it is possible to raise antibodies with high affinity to the DHFR in its native state.

Apart from the aa64-100 antibody, the other loop targeting monoclonal antibodies may have been directed against more extended conformations of the aa64-100 fragment. Despite the substitutions at aa64 and aa100 with cysteine, allowing the formation of a disulphide bond, only a small fraction of the peptide population may eventually form the intended loop structure. The aa64-100 fragment has a cysteine at aa78 and alternative disulphide bonds may have been formed of the aa64-100 peptide than the one intended. Furthermore, as a result of the high flexibility of loops in general, only a minor fraction of the formed peptide loops may actually resemble the native aa64-100 loop at any given time point, potentially leading to low immunization efficacy and/or low ELISA sensitivity towards the native protein. After completion of the biochemical studies the three-dimensional structure of the bifunctional Pf-DHFR-TS protein was determined by X-ray crystallography [12]. This provides a unique

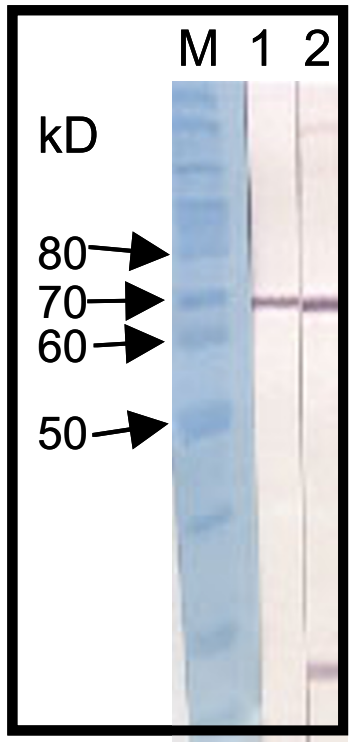

Figure 3
Western blots of crude *P. falciparum* 3D7 protein extract reacted with aa64-100-monoclonal antibody and rabbit polyclonal antibody. (M) Pre-stained protein marker, (1) aa64-100 (10 μg/ml) of the purified antibody; (2) 1:50 dilution of rabbit-antibody. In each lane approximately 6,25 μg total protein was added.

opportunity to see if the structural basis for the selection of the aa64-100 epitope was correct. In the experimental structure aa66-81 is forming an α-helix whereas aa86-95 cannot be located. In the model no secondary structure can be identified in the aa64-100 loop. Thus, although there are structural differences between the experimental structure and the model the similarities are also striking. In the experimental structure the distance between the Tyr64 and Asn100 C-alpha atoms is 7.5 Å compared with 6.0 Å in the model. Thus, the aa64-100 loop is anchored similarly and in both the experimental structure and the model this part of the Pf-DHFR is located on the surface and accordingly accessible for interactions with antibodies.

The three-dimensional structure analysis provided detailed knowledge of precise positions of surface exposed structures, enabling the development of site-directed antibodies that could not have been predicted by the primary structure analysis alone. This approach may therefore contribute significantly to the likelihood of selecting valuable peptide epitopes and whenever possible, epitope mapping by the primary sequence plots should be compared to the three-dimensional structures.

Authors' contributions

MA, AMR, ICB and CK designed and supervised the overall study. ITC and FSJ did the modelling, JEW, MC and YP contributed with various laboratory techniques, WS supplied the recombinant DHFR.

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