ITEM-THREE analysis of a monoclonal anti-malaria antibody reveals its assembled epitope on the pfMSP1\textsubscript{19} antigen

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Abstract
Rapid diagnostic tests are first line assays for diagnosing infectious diseases, such as malaria. To minimize false positive and false negative test results in population screening assays, high quality reagents and well characterized antigens and antibodies are needed. An important property of antigen – antibody binding is recognition specificity which best can be estimated by mapping an antibody’s epitope on the respective antigen. We have cloned a malarial antigen-containing fusion protein, MBP-pfMSP1\textsubscript{19}, in \textit{E. coli} which then was structurally and functionally characterized before and after high pressure-assisted enzymatic digestion. We then used our previously developed method, Intact Transition Epitope Mapping - Targeted High-Energy Rupture of Extracted Epitopes (ITEM-THREE), to map the area on the MBP-pfMSP1\textsubscript{19} antigen surface which is recognized by the anti-pfMSP1\textsubscript{19} antibody G17.12. We identified three epitope-carrying peptides: \textsuperscript{386}GRNISQHQC-VKKQCPQNSGCFRHLDE\textsuperscript{411}, \textsuperscript{386}GRNISQHQC-VKKQCPQNSGCFRHLDEREE\textsuperscript{414}, and \textsuperscript{415}CKCL-LNYKQE\textsuperscript{424} from the GluC-derived peptide mix-
Mortality among all the species (1,3). Parum is responsible for the highest morbidity and parasite lactate dehydrogenase (pLDH) (4). However, there have been reported malaria cases in 2018 worldwide, with 93% from Africa. Nineteen countries in sub-Saharan Africa together with India carry 85% of the global malaria burden (1). Impressively, improved malaria vector control, artemisinin-based combination therapy (ACT), and not least area-wide diagnostics resulted in the reduction of the worldwide malaria incidence rate by 37% within the period from 2000 to 2015 (2). Notwithstanding, there was an estimated 405,000 deaths reported globally in 2018 with 94% from Africa, of which 67% were children under 5 years of age (1). Malaria is caused by *Plasmodium* species. *Plasmodium falciparum* is responsible for the highest morbidity and mortality among all the species (1,3).

The clinical manifestation of acute malaria due to *Plasmodium falciparum* is normally associated with replication of merozoites, asexual blood-stage parasites, in circulating red blood cells (4). Clinical malaria diagnosis, though rather inexpensive, is difficult, because symptoms and signs of malaria are nonspecific and variable. Laboratory-based malaria diagnosis employs different methods, such as microscopy, PCR-, and ELISA-based methods. The antigens which are employed in immuno-analytical assays are histidine-rich protein 2 of *P. falciparum* (pfHRP2), Plasmodium aldolase, and parasite lactate dehydrogenase (pLDH) (4). However, there have been reported cases of cross reactivities with autoantigens, such as rheumatoid factor, leading to false positive test results ranging broadly from 3.3% to 83%. Also, of concern, rapid diagnostic tests (RDTs) for the diagnosis of malaria currently achieve sensitivities and specificities of around 90% (3-6). Therefore, the need for alternative or complementary malaria diagnostics still exists.

During infection, the merozoite’s surface proteins are responsible for initial interaction between the parasite and the host’s red blood cells. The merozoite surface protein 1 (MSP1) is a multi-domain protein (7). MSP1 is proteolytically cleaved at the end of schizogony into four polypeptides of characteristic sizes (MSP1\textsubscript{19}, MSP1\textsubscript{22}, MSP1\textsubscript{33}, and MSP1\textsubscript{38}) which are displayed as distinctive protein bands upon SDS-PAGE analysis (8). The MSP1\textsubscript{12} polypeptide experiences secondary proteolytic cleavages, generating MSP1\textsubscript{13} and MSP1\textsubscript{19}. All the MSP1\textsubscript{19} polypeptides are shed from the parasite surface with the exception of MSP1\textsubscript{19} which remains attached to the merozoite surface, and, upon the parasite’s entering of red blood cells, is presented on the cell surfaces (9,10). MSP1\textsubscript{19} as well as other MSP1 related antigens on their own or in combination with other malarial antigens are developed as vaccine candidates; some phase 2 clinical trials show promising efficacy (11-13). Since the MSP1\textsubscript{19} antigen possesses a highly conserved structure and amino acid sequence (14), it is also considered most suitable for developing a malaria screening assay. Together with a specific antibody which was to be applied as positive control, an improved immuno-analytical assay is envisaged for accurate malaria diagnosis. To ascertain assay precision, the antibody – antigen interaction of the positive control antibody needs to be studied in all possible molecular detail to reduce numbers of false positive and/or false negative test results when assaying patient sera.

In this study, precise characterization of the antigenic determinants on the MSP1\textsubscript{19} malaria antigen makes use of a novel mass spectrometry-based epitope mapping method. Mass spectrometers with ion-mobility separation capabilities allow separation of ions based on their *m/z* values, shapes and sizes (15,16) and this has found application for simple, robust, fast, and easy to apply antibody-antigen binding studies, termed ITEM-ONE (17,18). ITEM-ONE enables to identify epitopes of known antigens by precisely determining the mass of the extracted epitope peptide. Recently, ITEM-ONE has been advanced to ITEM-THREE, where mass spectrometric amino acid sequencing of unknown epitope peptides is performed to identify an antigenic determinant on an antigen surface (19). Specifically, ITEM-ONE and ITEM-THREE require mass spectrometric analysis of solution 1 (antigen peptide mixture), solution 2 (antibody) and solution 3 (formation of the antigen peptide-containing immune complex; mixtures of solutions 1 and 2), which can be electrospayed. The objective of this work is focused on charac-
terizing components of a mutual malaria RDT assay including a positive control antibody and to precisely characterize the interaction of this ‘malaria disease-specific’ monoclonal anti-pfMSP119 antibody with recombinant pfMSP1, which in this project is part of a fusion protein with maltose binding protein (MBP).

Results

Mass spectrometric and SDS-PAGE analyses of the recombinant MBP-pfMSP119 fusion protein

The amylose affinity-purified MBP-pfMSP119 fusion protein was cloned and expressed in E. coli as a starting material for mapping the epitope which is recognized by a monoclonal anti-pfMSP1 antibody. After purifying the overexpressed recombinantly produced fusion protein and upon reduction and alkylation, buffer exchange, and adjusting the pH to 6.8, the full-length MBP-pfMSP119 fusion protein was subjected to off-line nanoESI-MS analysis for checking structural integrity of the recombinantly produced starting material. The mass spectrum (Fig. S1A) showed multiply protonated ion series (26+ to 42+ ion signals) in the mass range between m/z 1100 and m/z 2100 which is typical for an unfolded protein. The experimental molecular mass of 54,311.25 ± 28.34 Da for the full-length MBP-pfMSP119 fusion protein which contains 12 fully alkylated cysteinyl residues was calculated from these m/z values and the respective charge states of the ion signals. This mass matched well with the calculated mass (Mr: 54,314.25) that was deduced from the chemically modified fusion protein’s amino acid sequence (Table S1). Each multiply charged ion signal showed a fine structure of satellite ion signals which could be attributed to under-alkylated protein species. An additional ion series in between the ion signals of the full-length MBP-pfMSP119 fusion protein monomer was assigned to a dimeric structure that may have been produced by air oxidation during work-up (20).

Fig. S1 and Table S1

In addition, in the high mass range between m/z 3000 and m/z 4600 the mass spectrum (Fig. S1B) showed multiply protonated ion series (12+ to 16+ ion signals), which is typical for a more compact protein structure. Each multiply charged ion signal showed a fine structure of satellite ion signals which could be attributed to reduced and unalkylated or partially alkylated protein species (Table S2).

Table S2

Additionally, the mass spectrum showed sharp molecular ion signals (charge states 11+ to 13+) in the high mass region (Fig. S1B) which led to the calculation of an experimental molecular mass of 42,799.28 ± 1.3 Da (Table S2). This experimentally determined mass agrees with the calculated mass for co-purified endogenous MBP from E. coli (Uniprot accession no. P0AEX9) which yields in Mr 42799.32 from the amino acid sequence of MBP when assuming that the amino acid sequence of this protein still contains the leader sequence and starts with acetylated Lys as amino acid 1 and extends to amino acid 391.

SDS-PAGE analysis was performed to confirm the presence of two or more protein components in the affinity-purified extract and indeed showed two stained protein bands with apparent molecular masses of 53 kDa and 42 kDa, respectively (Fig. S2, Lane 2), which is consistent with the nanoESI-MS results.

Fig. S2

Since reduction and alkylation of the MBP-pfMSP119 fusion protein turned out to be incomplete when applying standard reaction conditions, the protein extract was further characterized by MALDI-MS peptide mapping, after conducting high pressure-assisted tryptic digestion in-solution using the non-reduced and non-alkylated full-length protein. This rather harsh digestion procedure produced a peptide mixture with a fairly high peptide concentration of 0.1 μg/μL. The peptide ion signals derived from this peptide mixture after desalting were assigned to the MBP-pfMSP119 fusion protein, mainly its MBP part (Fig. S3, Table S3), and resulted in an overall amino acid sequence coverage of 65%.

Fig. S3 and Table S3

Interestingly, the peptide ion signal with m/z 1137.37 was the only one which was assigned to the pfMSP119 part of the fusion protein and corresponded to the partial amino acid sequence stretch between amino acid residues 398 and 407. Because there were no other peptide ion signals assigned to the pfMSP119 part of the fusion protein
Immuno-analytical characterization of the recombinant MBP-pfMSP1\(_{19}\) fusion protein

To further prepare for the epitope mapping experiments, antigen recognition of the MBP-pfMSP1\(_{19}\) fusion protein (accompanied by the endogenous MBP) by the chosen antibodies was checked first by conventional immuno-analytical means and was flanked by positive and negative control experiments. Western blot (WB) analysis of the protein extract with the anti-pfMSP1\(_{19}\) monoclonal antibody showed after decoration a single band (marked “a”) for the MBP-pfMSP1\(_{19}\) fusion protein, migrating at ca. 53 kDa apparent molecular mass (Fig. 1, Lane 1), which indicated strong and specific binding of the anti-pfMSP1\(_{19}\) monoclonal antibody to the - at least partially denatured - full-length MBP-pfMSP1\(_{19}\) fusion protein.

The WB with an anti-His-tag antibody also showed a single intense band (marked “a”) at the same location on the blot after decoration (Fig. 1, Lane 2), serving as positive control. As expected, the WB did not show any band after exposure of the MBP-pfMSP1\(_{19}\) fusion protein to rituximab (Fig. 1, Lane 3), which thereby served as negative control (rituximab binds CD20). For completion, the WB experiments included testing of binding to the MBP-pfMSP1\(_{19}\) fusion protein by an anti-MBP antibody which provided an intense band (marked “a”) at 53 kDa together with a weaker band (marked “b”) at 42 kDa apparent molecular mass after decoration (Fig. 1, Lane 4). This result showed recognition of both, the MBP-pfMSP1\(_{19}\) fusion protein and the endogenous MBP from \(E\). \(coli\).

The anti-MBP polyclonal antibody also stained a band (marked “c”) which migrated to approx. 33 kDa apparent molecular mass. This band may be a result of non-specific cross-reactivity of the polyclonal antibody to uncharacterized components in the sample. Presence of uncharacterized components was also seen by nanoESI-MS analysis of this preparation (see Fig. S1B). At last, no staining of any proteins was obtained when exposing the blotted proteins to only the secondary antibody (Fig. 1, Lane 5), thereby ascertaining no false positive staining.

Fig. 1

To test whether enzymatic cleavage of the full-length MBP-pfMSP1\(_{19}\) fusion protein produced any protein fragments which had preserved antigenicity, the peptide mixture which was obtained from high pressure-assisted tryptic digestion was subjected to WB analysis as well. Upon decoration with anti-pfMSP1\(_{19}\) monoclonal antibody, the Western blot showed a single band at ca. 11 kDa (marked “d”) for a tryptic fragment of the MBP-pfMSP1\(_{19}\) fusion protein (Fig. 1, Lane 6). Staining of this band is consistent with the assumption that some of the pfMSP1\(_{19}\) part had endured tryptic digestion unfragmented, whereas the MBP part was almost completely digested. Interestingly, the partial amino acid sequence 388-486 of the full-length MBP-pfMSP1\(_{19}\) fusion protein (Fig. S4) calculates for a molecular mass of 11.5 kDa and covers the pfMSP1\(_{19}\) part of the fusion protein in toto.

Fig. S4

Presence of an undigested pfMSP1\(_{19}\) part stands in agreement with the MALDI-MS peptide mapping data which showed peptide ion signals of nearly the complete MBP protein part of the fusion protein whereas peptide ion signals from the pfMSP1\(_{19}\) part were scarce (cf. Table S3). Similarly, the WB showed a single band (marked “b”) for endogenous MBP from \(E\). \(coli\) after high pressure-assisted tryptic digestion of the MBP-pfMSP1\(_{19}\) fusion protein and decoration with the anti-MBP polyclonal antibody (Fig. 1, Lane 9), which stands in agreement with the above-mentioned findings. At last, after high pressure-assisted tryptic digestion of the MBP-pfMSP1\(_{19}\) fusion protein, the WB showed no bands after exposure to neither the monoclonal anti-His-tag antibody nor to rituximab (Fig. 1, Lanes 7 and 8). These results were expected and can be explained, first by cutting off the His-tag from the protein by...
The fusion protein was represented by peptide linking the MBP part with the aa476-486. Further, the amino acid sequence (charge state 3+; aa 465-486) and 414), and the peptide mapping results of the tryptic digestion were not, because the yield of peptides, i.e. candidates for epitope peptides, from the pfMSP1p part of the MBP-pfMSP1 fusion protein was too poor. Therefore, the (partially) reduced and alkylated MBP-pfMSP19 fusion protein returned into focus and was subjected to enzymatic digestion with GluC. The resulting peptide mixture was characterized by nanoESI-MS analysis (Fig. 2 and Table S4) and showed multiply charged ion signals for both, the MBP part and the pfMSP19 part of the full-length MBP-pfMSP1 fusion protein. The sequence coverage for the MBP-pfMSP19 fusion protein was 96 % (Fig. S4). Hence, the GluC-derived peptide mixture was promising to function as solution 1 for ITEM-THREE experiments because the C-terminal partial amino acid sequence of the MBP-pfMSP19 fusion protein was found present from peptide ion signals, such as those with m/z 910.43 (charge state 3+; aa 465-486) and m/z 703.36 (2+; aa 476-486). Further, the amino acid sequence linking the MBP part with the pfMSP19 part of the fusion protein was represented by peptides with ion signals m/z 796.68 (4+; aa 386-411), m/z 1061.89 (3+; aa 386-411), m/z 900.23 (4+; aa 386-414), and m/z 1199.97 (3+; aa 386-414) (Fig. 2 and Table S4).

Fig. 2 and Table S4

Interestingly, the mass spectrum of the peptide mixture which was obtained from GluC digestion of the MBP-pfMSP19 fusion (Solution 1) also showed sharp molecular ion signals (charge states 11+ to 13+) in the high mass range (Fig. 2, insert). From the m/z values and the respective charge states of these ion signals was calculated the experimental molecular mass of 42,245.88 ± 3.2 Da. This mass matches to that of co-purified endogenous MBP from E. coli (Mr, 42,422.5; UniProt accession no. P0AEX9) which is acetylated at Lys1, three-fold carbamidomethylated and enzymatically truncated at the C-terminus, thus is assumed to encompass amino acids 1-384 (Table S5). This finding again stands in agreement with data from both, SDS-PAGE (Fig. S2) and WB analysis (Fig. 1). Despite the presence of this 42 kDa high molecular mass component in the GluC-derived peptide mixture, this solution 1 was deemed suitable for ITEM-THREE experiments, because it contained peptides with masses between 1 kDa and 4 kDa from the pfMSP19 part of the fusion protein whose ion signals were abundant and which potentially could function as epitope peptides.

Table S5

To continue with preparing for ITEM-THREE, the monoclonal antibodies were investigated by nanoESI-MS upon rebuffing them into ammonium acetate solutions (solutions 2). Each of the antibody-containing solutions with pH 6.8 provided good quality mass spectra. The mass spectrum of monoclonal anti-pfMSP19 antibody showed five baseline-resolved multiply protonated molecular ion signals (charge states 27+ to 23+) confirming high purity (Fig. S5A).

Fig. S5

The mass spectrum of monoclonal anti-His-tag antibody (Fig. S5B; positive control) similarly showed sharp and baseline resolved multiply charged molecular ion signals (charge states +22 to +26) and the mass spectrum of rituximab (Fig. S5C, negative control) showed also five sharp and baseline resolved multiply charged molecular ion signals (charge states +22 to +26). In addition, the mass spectrum of rituximab also revealed multiply protonated ion signals at around m/z 2000 which belonged to the light chain and indicated some deterioration of this antibody. Although, there was some deterioration of the rituximab antibody, all the antibodies (solution 2) were considered suitable for ITEM-THREE experiments, since the antibodies showed sharp and baseline resolved multiply charged molecular ion signals.
Immune complex formation in-solution (Solutions 3) and epitope mapping by ITEM-THREE

The ITEM-THREE experimental series started with generating immune complexes simply by mixing the antigen peptides-containing solution (solution 1) with one antibody-containing solution (solution 2). Upon forming immune complexes in solution, the epitopes were determined by nanoESI-MS analysis of the entire mixture (solution 3) without any further purification.

First, the epitope of monoclonal anti-\textit{p}MSP1\textsubscript{19} antibody was investigated by nano-electrospraying solution 3A which contained the immune complex which consisted of monoclonal anti-\textit{p}MSP1\textsubscript{19} antibody – MBP-\textit{p}MSP1\textsubscript{19} epitope peptide. Setting the quadrupole to block transmission of ions with \textit{m/z} values below 5500 ensured that no unbound peptide ions (UBPs) derived from the antigen by enzymatic digestion (GluC was used) reached the detector. As a consequence, there were no ions traversing the ion mobility separation chamber below 20 ms arrival time. Keeping the collision cell voltage difference in the TRAP cell low (4 V), ensured that only intact immune complex ions as well as free antibody ions (and multiply charged ions of undigested endogenous MBP) were able to traverse the mass spectrometer’s mass filters. These large ions reached the detector with arrival times between 20 ms and 27 ms (Fig. 3A). Important for the ITEM-THREE experiment was, that there were no peptide ion signals recorded within the low mass range (\textit{m/z} 500 and \textit{m/z} 1500) under these conditions (Fig. 3B).

Fig. 3

However, upon raising the collision cell voltage differences to 20 V in the TRAP cell, the immune complex ions (and multiply charged ions of undigested endogenous MBP and of free antibody) collided with argon gas atoms with higher energies which now caused dissociation of the immune complex and subsequently enabled recording of complex-released peptide ions (CoRPs) with arrival times between 10 ms and 15 ms (Fig. 3C). From recording the \textit{m/z} values of the CoRPs was deduced that an immune complex consisting of anti-\textit{p}MSP1\textsubscript{19} antibody – MBP-\textit{p}MSP1\textsubscript{19} epitope peptide had bound at least one of three epitope carrying peptides with ion signals at \textit{m/z} 678.33 (2+), \textit{m/z} 796.94 (4+) and \textit{m/z} 900.22 (4+), respectively (Fig. 3D). These ion signals were assigned to peptides from the \textit{p}MSP1\textsubscript{19} part of the fusion protein with partial amino acid sequences \textit{415\text{-}424}CKCLLNKYKQEC\textsuperscript{376}\textsuperscript{384}GRNISQHQCVKKQC-PQNSGCFRHLDE\textsuperscript{391}, \textsuperscript{386}GRNISQHQCVK-KQCPQNSGCFRHLDEERE\textsuperscript{414}, respectively. Recording more than one peptide whose amino acid sequences are different is consistent with the assumption of having dissociated an immune complex which is composed of an assembled, \textit{i.e.} a conformational epitope (19).

Second, to substantiate these ITEM-THREE results a positive control experiment was performed with the same solution 1 which had been generated by GluC digestion of reduced and alkylated MBP-\textit{p}MSP1\textsubscript{19} fusion protein. Mixing solution 1 with the anti-His-tag antibody-containing solution (solution 2B) generated an immune complex (solution 3B) consisting of anti-His-tag antibody – MBP-\textit{p}MSP1\textsubscript{19} epitope peptide. Upon electrospraying solution 3B and having had set the quadrupole to block transmission of all ions with \textit{m/z} values below 5500 allowed only the intact immune complex ions as well as the free antibody ions (and multiply charged ions of undigested endogenous MBP) to traverse the quadrupole ion filter and the ion mobility separation chamber with arrival times between 15 ms and 27 ms (Fig. 4A) when low collision cell voltage differences (4 V) in the TRAP cell were applied. There were no unbound peptide ion signals (UBPs) reaching the ion mobility separation chamber, and no peptides were released from the immune complex, as was seen by the absence of ion signals with arrival times below 15 ms within the mass range of \textit{m/z} 500 to \textit{m/z} 1500 (Fig. 4B).

Fig. 4

Increasing the collision cell voltage difference in the TRAP cell (20 V) caused collisions of the immune complex ions (and multiply charged ions of undigested endogenous MBP and free antibody) with argon gas atoms upon which occurred dissociation of the complex. Now, the complex-released peptide ions (CoRPs) were detected with arrival times between 10 ms and 15 ms (Fig. 4C) with ion signals at \textit{m/z} 703.32 (2+) and 910.43 (3+), respectively (Fig. 4D). These ion signals were assigned to peptides: \textit{476\text{-}486}\textsuperscript{496}\textsuperscript{486}CTKPDSYPLFDGIFCSHHHHH and \textit{465\text{-}476}\textsuperscript{476}\textsuperscript{486}GiFCShHHHHH, respectively. The anti-His-tag antibody had recognized from the peptide mixture exclusively two C-terminal His-tag epitope carrying peptides. Obviously, the anti-His-tag antibody had bound to a
consecutive (linear) epitope which was exposed to the antibody as part of either a shorter or a longer peptide.

Third, to complete our epitope mapping analyses, a negative control experiment was performed using rituximab (solution 2C) which was mixed with the GluC-derived MBP-p/MSP119 peptide solution (solution 1) to generate solution 3C. The ITEM-THREE experiment was conducted as before. When setting the quadrupole to block transmission of ions with m/z values below 5500 and keeping low collision cell voltage differences (4 V) in the TRAP cell, only the free antibody ions (and multiply charged ions of undigested endogenous MBP) traversed the quadrupole ion filter and the ion mobility separation chamber with arrival times between 15 ms and 27 ms (Fig. S6A) and there were no unbound peptide ions (UBPs) reaching the ion mobility separation chamber (below 15 ms). Accordingly, no peptide ion signals within the mass range of m/z 500 to m/z 1500 (Fig. S6B) were recorded.

Fig. S6

Application of higher collision cell voltage differences (20 V) in the TRAP cell caused collisions of the antibody ions (and multiply charged ions of undigested endogenous MBP) with argon gas atoms but could not dissociate any MBP-p/MSP119-derived peptides and consequently the mass spectra showed no ion signals with arrival times between 10 ms and 15 ms (Fig. S6D). Because rituximab did not recognize any peptides from the MBP-p/MSP119 fusion protein there were again no ion signals in the mass spectra (Fig. S6D) which (i) proved that the negative control was working successfully and (ii) confirmed that the above described epitope mapping experiments produced reliable results for both, the monoclonal anti-p/MSP119 antibody and the anti-His-tag antibody. Since length of peptides produced by enzymatic digestion can be controlled to some extent, depending on cleavage site locations in the antigen’s amino acid sequence, a survey of peptides derived from digestions of the antigen with different enzymes is suggested before starting an ITEM-THREE experiment. In our case, we had tested trypsin and AspN in addition to GluC. Focusing on the MSP119 region of MBP-p/MSP119 (amino acid residues 388 to 480) we found that in-gel trypsin digestion generated rather short peptides (lengths varied from 5-mers to 11-mers) from the suspected epitope range (cf. Fig. S7 and Table S6) whereas AspN produced a very long peptide (a 46-mer) of this protein region (cf. Fig. S8 and Table S7), similar to high pressure tryptic digestion of non-reduced and non-alkylated MBP-p/MSP119 (see above). GluC-derived peptides were considered optimal in length.

Fig. S7 and Table S6
Fig. S8 and Table S7

Comparison of ITEM-THREE results with epitope data from X-ray structure analysis

While the anti-His-tag antibody epitope is a consecutive epitope, the anti-p/MSP119 antibody epitope is called assembled since it comprises amino acid residues from different regions of the antigen’s amino acid sequence which are located closely spaced together on the same partial surface of the antigen, as is suggested from the X-ray crystallography data of the immune complex (22). The crystal structure of the complex led to the identification of the “assembled” (discontinuous / conformational) epitope by selecting those residues as being part of the antibody interacting surface which are placed on the MSP119 surface and with a distance of ≤ 4 Å to surface-exposed amino acid residues on the antibody paratope. Twelve surface amino acid residues 8VKKQ11, 13PQ14, 24ERE29, 28C, and 38GD39 of MSP119 within the assembled epitope were suggested to be important for binding (Fig. 5) as they fulfilled the distance criteria. Of note, ten of these twelve amino acid residues on the MSP119 surface are placed on the two neighboring GluC-derived peptides of the MBP-p/MSP119 fusion protein, 386GRNISQHQC-VKKQCPQNSGCGRHLDEREENSE24 and 415CKCL-LNYKQE24, and these two were among the three peptides which were identified as CoRPs by ITEM-THREE.

Fig. 5

In conclusion, the determined CoRPs from ITEM-THREE experiments comprise a partial surface of 2721.90 Å² of the total surface area of MSP119 which is 6543.02 Å². For comparison, the summed-up partial surface which spans over the “assembled epitope”, i.e. extends from amino acid residues 8 to 39 on the MSP119 surface is 2325.07 Å², to which the twelve binding residues contribute 1210.48 Å². Whether or not the GluC-derived CoRPs function as mimotope peptides or whether
or not the flexibility of the CoRPs allowed them to be assembled in a way by which a surface structure was generated such that the important amino acid residues for binding were placed to fit into the paratope of the antibody and thereby enabled direct contacts remains to be investigated. Obviously, the CoRPs that were identified by ITEM-THREE, were the ones which had formed strong binding forces with the anti-p/MSP1<sub>19</sub> antibody in solution. Upon dissociation of the immune complex in the gas phase the released peptide ion masses were unequivocally and accurately determined, thereby identifying them as the epitope peptides.

**Discussion**

Although, structure-based methods, such as X-ray crystallography have been used to study antibody-antigen interactions in great detail (23,24), large sample consumption, challenges with crystallization, and lack of speed of analysis remain major concerns (25,26). On the other hand, despite the simplicity of ITEM-THREE it enabled identification of the “assembled epitope” to the anti-p/MSP1<sub>19</sub> monoclonal antibody with very little sample consumption and by relatively easy and speedy analysis with few in-solution handling steps (19). Nevertheless, results with comparable preciseness as compared to those from 3D structure analysis were reached (17).

Success of an ITEM-THREE experiment depends critically on the antibody of interest being able to bind to partial peptides derived from its antigen with sufficient affinity to capture peptide - antibody complexes for mass spectrometric analysis. Hence, as the vast majority of protein epitopes are assumed to be conformational in nature (27), relatively short partial peptides derived from the respective epitopes may generally bind weakly, if at all, to the investigated antibody. Nevertheless, the importance of sequential epitopes has been emphasized (28). Defining either of them, requests good understanding of their minimal sizes. A non-mass spectrometry example shall be mentioned to illustrate this important point. A minimal length prediction of the human ACE2 alpha 1 helix suggested as the requested partial structure sufficient for binding to the receptor binding domain (RBD) from the SARS-CoV2 spike protein a peptide which spanned from amino acid residue 27 to 38 (termed SPB2; 12 amino acids in length) because analyzing an X-ray structure of the protein - protein complex in combination with molecular dynamics calculations suggested that it possessed all the important core residues which were needed for stable interactions (29). Yet, when investigating binding properties of the synthetic 12-mer peptide by Bio-Layer Interferometry it turned out that this peptide did not bind. By contrast, a longer peptide which encompassed amino acid residues 21 to 41 (termed SBP1; 23 amino acids in length) proved strong binding by experimental investigation. This longer peptide possessed in addition to the core amino acid residues flanking amino acid residues on either side of the core whose importance for binding was initially overlooked. In our ITEM-THREE experiment the lengths of the GluC-derived peptides were found ideal, as by binding it turned out that they encompassed all the amino acid residues which were needed for forming stable immune complexes.

The fact that the recombinantly produced and affinity-purified MBP-p/MSP1<sub>19</sub> fusion protein was accompanied by another high mass protein component asked for extra attention to be paid. Thorough analysis of the starting material by SDS-PAGE, WB, and mass spectrometry accumulated the side product’s characteristics like proteolysis resistance (21), presence of leader peptide, and therefore starting with acetylated Lys1 (19,30-33). All evidence pointed to endogenous MBP from *E. coli*, which not interfered with ITEM-THREE results, since neither by enzymatic cleavage nor by collision induced fragmentation could endogenous MBP have produced irritating ion signals which accidentally might have been assigned as MSP1<sub>19</sub>-derived CoRPs.

Of accentuated interest was the finding that the natively folded MSP1<sub>19</sub> “assembled epitope” was maintained during WB analysis, at least to such an extent that decoration with the monoclonal anti-MSP1<sub>19</sub> antibody was successful. Similar cases have been described showing that “assembled epitopes” were occasionally displayed on SDS-PAGE-denatured and blotted proteins (34).

The by ITEM-THREE determined CoRPs might be regarded as mimotope peptides which mimicked the (partial) structure of an epitope on an antigen surface, but resulted in an immune complex which can be considered similar to the one which was elicited by the epitope of the full-size antigen (35). While differentiating between these cases of binding possibilities was not in the focus of this study, ITEM-THREE enabled to identify the binding region which by X-ray analysis had been assigned as being part of an “assembled
epitope”. By contrast, the anti-His-tag antibody which was used as a positive control had been shown previously to form immune complexes with peptides that contained a 6-times histidinyl residue tag which were enzymatically cleaved off from recombinant proteins (19). The ability of the anti-His-tag antibody to bind unequivocally to only peptides containing the 6-times histidinyl residue tag, independent of the presence of further flanking amino acid residues, demonstrates its specificity and hence provides a means of quality assurance for ITEM-THREE experiments. Similarly, rituximab was used for negative control experiments to ascertain whether non-specific binding was observable. The results of the respective positive and negative control experiments agree with those from previous ITEM-THREE studies (19).

Obviously, malaria-specific antibody titer determination enables assessment of recent exposure to malaria infection (36) and, together with neutralization assays (37,38), to estimate the risk of future infection for an individual. To further enhance protection against malaria, we suggest to determine antibody titers in individuals to monitor vaccination success for a given person, i.e. to estimate the personal “immune status” which should become an important means for disease prevention and treatment (39,40) also with other infectious diseases. Having determined the structural and functional integrity of the test antigen, the individual “protection status” might in future be assayed by using the MBP-\( p/\text{MSP1}_{19} \) fusion protein or even by a mimotope peptide therefrom. Mimotope peptides have been studied as potential immunotherapeutic agents (41-43) and as diagnostics means (44-46). Moreover, the here investigated anti-\( p/\text{MSP1}_{19} \) monoclonal antibody is a candidate to serve as a positive control for a future malaria “protection status” screening assay platform, to help in ruling out potential false positive and/or negative results because of assay failure (3-6).

Furthermore, “key amino acid residues” with respect to antigen – antibody binding within epitope peptides can now be studied to determine which mutations in the identified (mimotope) peptide could be tolerated without harming stable immune complex formation. This information is of importance when mutations on the antigen have to be taken into consideration and can be retrieved by determining the binding strength similarity or differences of the “wild-type” peptide with respect to the “mutated” mimotope peptide. First approaches in this direction have been undertaken and led to the development of ITEM-TWO (47). ITEM-TWO is a newly developed mass spectrometry-based method which can determine the role of each amino acid at any position in any peptide with respect to its energetic contribution to the binding strength by which the peptide is bound to an antibody’s paratope, lending a novel malaria screening assay a higher level of fidelity.

**Experimental procedures**

**Cloning of an expression vector encoding the MBP-\( p/\text{MSP1}_{19} \) fusion protein and overexpression in \( E. \ coli \)**

Standard recombinant DNA technology (48) was used to construct the prokaryotic expression vector for the C-terminal fragment \( p/\text{MSP1}_{19} \) of the major merozoite surface protein from *Plasmodium falciparum*. The initial coding sequence was obtained by commercial gene synthesis (Eurogentec, Köln, Germany), codon-optimized for expression in *E. coli* (49). The gene sequence was C-terminally extended to encode a histidine-tag (His-tag, six consecutive His residues) and was equipped with Hpal or Sall restriction sites at the ends. The Hpal/Sall DNA fragment was cloned into the prokaryotic expression vector pMal-c2x (New England Biolabs Inc., Hitchin, UK) and cut with XmnI/Sall restriction enzymes. The final expression vector encodes for a fusion protein with *E. coli* maltose binding protein (MBP) on the N-terminus and the His-tagged \( p/\text{MSP1}_{19} \) at the C-terminus. After cloning in *E. coli* strain XL1blue (Stratagene, Agilent, Santa Clara, USA), the expression vector was transformed into the *E. coli* strain K12 TB1, genotype [\( F^{+} \) ara \( \Delta(\text{lac-proAB}) [\Phi 80 dlac \Delta(\text{lacZ})M15] rpsL(\text{Str}^{\text{r}}) \text{ thi hsdR} \)] (New England Biolabs Inc.) for recombinant protein production. Details of overexpression are described in the Supporting Information.

**Ultrasound assisted \( E. \ coli \) cell lysis (crude protein extract) and protein concentration determination**

Upon re-suspending one bacterial pellet (~20 mg) in 200 µL TNA buffer (0.02 M TRIS-HCl, 0.1 M NaCl, pH 8.0) of induced cells which had overexpressed the MBP-\( p/\text{MSP1}_{19} \) fusion protein and transferring the cell suspension (100 µg/µL) into a Covaris micro tube (130 µL), cells were lysed in the M220 ultra-sonicator (Covaris Inc., Woburn, USA) as is described in the Supporting Information. The protein concentration of the crude protein extract solution was determined using the
Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, USA), see (18) and Supporting Information. MBP-pfMSP1\textsubscript{19} was affinity-purified through the MBP part of the fusion protein using an amylose resin suspension (E8022S; New England Biolabs Inc.) in batch mode over night at 4° C (50). Details are described in the Supporting Information.

Reduction and alkylation of MBP-pfMSP1\textsubscript{19} fusion protein (reduced and alkylated antigen solution) and off-line NanoESI-MS analysis
Reduction and alkylation of the MBP-pfMSP1\textsubscript{19} fusion protein was performed as previously described (18); see Supporting Information for details. Then, off-line nanoESI-MS measurement of the reduced and alkylated MBP-pfMSP1\textsubscript{19} fusion protein was performed as described (18); see detail in the Supporting Information.

High pressure-assisted in-solution trypsic digestion of MBP-pfMSP1\textsubscript{19} fusion protein (antigen peptide solution), desalting of antigen peptide solution, peptide concentration determination, and MALDI-MS peptide mapping of desalted antigen peptide solution
A volume of 20 µL of MBP-pfMSP1\textsubscript{19} solution (antigen solution; protein dissolved in 200 mM ammonium acetate buffer, pH 6.8) was transferred into an Eppendorf tube (0.5 mL) and 10 µL of trypsin solution (Promega Corporation, Madison, USA; 20 µg dissolved in 2 mL 50 mM ammonium bicarbonate solution, pH 8.4) was added and vortexed. The digestion solution (30 µL) was transferred into a barocycler tube (50 µL) and closed with a tightly fitting pestle. High pressure in-solution trypsic digestion of MBP-pfMSP1\textsubscript{19} fusion protein was performed without reduction / alkylation using the Barocycler 2320 EXT (Pressure BioScience, South Easton, USA). The following instrument parameters were applied: pressure, 20 kpsi; cycles, 60; on time, 50 sec; off time, 10 sec; temperature, 50 °C; digestion time, 1 h. The resulting antigen peptide solution (~30 µL, antigen peptide solution) was transferred into an Eppendorf tube (0.5 mL) and was either stored at -20 °C or was used immediately for further analysis. The tryptic peptide mixture from the antigen peptide solution was stepwise desalted using C\textsubscript{18} ZipTips (Merck Millipore Ltd, Tullagreen, Carrigtwohill, Ireland) following published protocols (51). The peptide concentrations of peptide solutions were determined using the Qubit 2.0 Fluorometer as described in the Supporting Information (18). MALDI-MS peptide mapping analysis was performed as described in the Supporting Information (52,53).

SDS-PAGE analysis of MBP-pfMSP1\textsubscript{19} fusion protein before and after trypsic digestion
SDS-PAGE analysis was performed as described in the Supporting Information (54,55). Gels were shrink-wrapped, moistened with water and 150 µL sodium azide and stored at 4 °C (56).

Antibody sources
The monoclonal anti-p/MSP1\textsubscript{19} antibody (G17.12; isotype, IgG2a; 1 µg/µL) was obtained from Creative Biolabs (New York, USA). Monoclonal mouse anti-His-tag antibody (MCAl396; batch, 0309; 1 µg/µL) was obtained from Bio Rad (Munich, Germany). Rituximab (batch, H0013; protein concentration, 10 µg/µL) was obtained from Roche Ltd. (Welwyn Garden City, UK). Polyclonal rabbit anti-maltose binding protein antibody (anti-MBP; Cat #, NBPI-97370, 0.5 mg) was obtained from Novus Biologicals (Abingdon, UK). Biotin-SP-conjugated AffiniPure polyclonal goat anti-human antibody (IgG, H+L) was obtained from DiaNovad (Hamburg, Germany). Polyclonal IRDye 800CW conjugated goat anti-mouse antibody and polyclonal IRDye 800CW conjugated donkey anti-rabbit antibody were from LI-COR (Nebraska, USA).

Western Blot analysis of MBP-pfMSP1\textsubscript{19} fusion protein before and after trypsic digestion
Upon SDS-PAGE separation of the MBP-pfMSP1\textsubscript{19} fusion protein before (antigen solution) and after trypsic high pressure assisted digestion (antigen peptide solution), separated proteins were subjected to Western blotting (57), see details in the Supporting Information.

In-gel trypsin digestion of MBP-pfMSP1\textsubscript{19} fusion protein
In-gel trypsin digestion (53) was performed as described in the Supporting Information.

In-solution AspN digestion of MBP-pfMSP1\textsubscript{19} fusion protein
In-solution AspN digestion (55) was performed as described in the Supporting Information.
**GluC digestion of MBP-pfMSP1\textsubscript{19} fusion protein after reduction and alkylation (Solution 1)**

To reduced and alkylated MBP-pfMSP1\textsubscript{19} fusion protein solution (reduced and alkylated antigen solution, 50 μL, 0.66 μg/μL) dissolved in 200 mM ammonium acetate buffer, pH 6.8 was added 1.3 μL of 500 ng/μL GluC solution (Roche, Mannheim, Germany; dissolved in water) to yield an enzyme : substrate ratio of 1 : 50 (w/w). Digestion was performed overnight at room temperature. The resulting peptide solution (51 μL) was either stored at -20 °C or was used immediately for further analysis without any purification. Peptide concentration determination was performed as described in the Supporting Information.

**Preparation of nanoESI-MS compatible antibody solutions (Solutions 2)**

Preparation of nanoESI-MS compatible antibody solutions (solutions 2) was performed as described (19). Monoclonal anti-pfMSP1\textsubscript{19} antibody solution was rebuffered into 200 mM ammonium acetate buffer, pH 6.8 (solution 2A) by loading 20 μL of the antibody stock solution (~20 μg) into a centrifugal filter (ca. 30 μL). To the retentate on the filter (ca. 30 μL) was added 200 mM ammonium acetate buffer, pH 6.8, to reach again a total volume of 500 μL. After discarding the filtrate, the solution was centrifuged again. This centrifugation / re-filling procedure was repeated eight times. All the filtrates were discarded. After the last spinning, the filter unit was inverted into a new vial and centrifuged at 4500 rpm for 5 min to collect the retentate (ca. 30 μL). This procedure was applied for buffer exchange for monoclonal anti-His-tag antibody and for rituximab as well. Antibody solutions (solutions 2) were either directly used for preparation of antibody-epitope peptide complexes (19), after their concentrations had been determined using the above described procedure, or they were stored at 4 °C.

**Preparation of immune complex-containing and nanoESI-MS compatible solutions (Solutions 3)**

Immune complex-containing and nanoESI-MS compatible solutions were prepared as described (19). The anti-pfMSP1\textsubscript{19} antibody – pfMSP1\textsubscript{19} peptide solution (solution 3A) was prepared by adding 6 μL of 0.52 μg/μL pfMSP1\textsubscript{19} peptide solution (solution 1) to 10 μL of 0.38 μg/μL anti-pfMSP1\textsubscript{19} antibody solution (solution 2A). Similarly, the anti-His-tag antibody – pfMSP1\textsubscript{19} peptide solution (solution 3B) was prepared by adding 1 μL of 0.52 μL pfMSP1\textsubscript{19} peptide solution (solution 1) to 8 μL of 0.3 μg/μL anti-His-tag antibody solution (solution 2B). Also, the rituximab – pfMSP1\textsubscript{19} peptide solution (solution 3C) was prepared by adding 4.5 μL of 0.52 μL pfMSP1\textsubscript{19} peptide solution (solution 1) to 7.5 μL of 0.5 μg/μL rituximab solution (solution 2C). All the immune complex-containing solutions (solutions 3A, 3B, 3C) were left to stand at room temperature for at least 1 h prior to analysis.

**Off-line NanoESI-IMS-MS/MS instrument settings and spray needle preparation**

NanoESI-IMS-MS/MS measurements were carried out in positive ion mode on a quadrupole ion-mobility separation TOF mass spectrometer (Synapt G2-S, Waters MS-Technologies, UK) as described (18,19,47,58). The TOF analyzer was calibrated externally from m/z 200 – 8000 with 1 mg/mL sodium iodide solution, dissolved in an isopropanol / water mixture (50:50, v/v). The following instrumental settings were used: source temperature, 50 °C; capillary voltage, 1.60–2.25 kV; source offset, 80–110 V; sample cone voltage, 90–110 V; TRAP cell gas flow, 6.0 mL/min; cone gas flow, 100 L/h. Automatic gas controls were used as follows: TRAP cell gas flow, 2.0 mL/min; helium cell gas flow, 180 L/h; IMS cell gas flow, 102 mL/min. The following parameters were optimized and used for IMS measurements for each experiment to obtain adequate ion mobility separation: wave velocity, 650 m/s; wave height, 40 V; start wave height, 30–35 V; end wave height, 20–25 V. Pusher width and pusher cycle times were both set to automatic. Scan duration of 1.0 s and inter scan delay of 0.015 s were set for both IMS and MS measurements. Reflection grid, flight tube and reflectron voltages were 1.46 kV, 10.00 kV and 3.78 kV, respectively, and detector sensitivity was set to normal. Pressure settings within the various parts of the mass spectrometer were as follows: TRAP cell, ~3.53 × 10\textsuperscript{−2} mbar; Helium cell, ~1.35 × 10\textsuperscript{−2} mbar; IMS cell, ~3.35 × 10\textsuperscript{−2} mbar; TRANSFER cell, ~3.87 × 10\textsuperscript{−2} mbar; ToF analyzer, ~1.40 × 10\textsuperscript{−6} mbar. Spray needles were prepared in-house from borosilicate glass tubes of 1 mm outer and 0.5 mm inner diameters with a P-1000 Flaming/Brown\textsuperscript{TM} Micropipette Puller System (Sutter Instruments, Novato, CA).
USA) followed by gold coating, applying the Sputter Coater SCD 005 (BAL-TEC Inc., Balzers, Liechtenstein) as described (47,59).

**ITEM-THREE measurements**

ITEM-THREE experiments were performed as described (19). Approximately 3 μL, each, of solutions 3A, 3B, and 3C were loaded into separate spray needles using 20 μL microloader pipette tips (Eppendorf AG, Hamburg, Germany). After mounting the capillaries onto the sprayer, immune complex containing solutions were electrosprayed without any purification. The quadrupole mass analyzer in the mass spectrometer was first manually tuned to filter off ions below m/z 5500 (18,19). The collision cell voltage differences in the TRAP cell were raised in a stepwise manner (5 – 20 V / step), which was optimized for each experiment to ensure adequate dissociation of the epitope peptide - antibody complex with minimal antibody fragmentation. The dissociated complex constituents then entered the ion mobility chamber. Both ion mobility raw data (arrival time of ions) and mass spectral raw data from the ToF analyzer were collected and stored using MassLynx software 4.1 (Waters MS-Technologies, Manchester, UK). All mass spectrometry data have been deposited in the PRIDE database (60).

**Visualization of 3D protein structures**

The coordinates of the 3D structure data files of the MSP1₁₉ antigen plus the Fab component of monoclonal G17.12 (anti-pfMSP1₁₉) antibody complex (PDB ID: 1OB1, (22)) were downloaded from the RCSB Protein Data Bank (www.rcsb.org). A new pdb-file which contained only the atom coordinates of the MSP1₁₉ antigen was generated by editing the 1OB1 pdb-file using a word processing program and opening the pdb-file as a text file. Then, all the lines that contained the coordinates of the atoms from the G17.12 Fab components were deleted. Visualization of the protein structures was done using the UCSF Chimera (http://www.cgl.ucsf.edu/chimera/) molecular visualization software (61).

**Data availability**

The mass spectrometry data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (60) with the dataset identifier PXD019717.

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**Conflict of interest**

The authors declare that they have no conflicts of interest with the contents of this article.

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Figures

**Figure 1. Western blot of MBP-pf/MSP1<sub>19</sub> before and after high pressure assisted tryptic digestion.** Lane M: Molecular mass marker. Lanes 1-5: MBP-pf/MSP1<sub>19</sub>, 1.3 μg in each lane, before digest. Lanes 6-10: MBP-pf/MSP1<sub>19</sub>, 1.6 μg in each lane, after tryptic digest. Lanes 1 and 6: Decoration with anti-pf/MSP1<sub>19</sub> antibody. Lane 2: Decoration with anti-His-tag antibody. Lane 7: Exposure to anti-His-tag antibody. Lanes 3 and 8: Exposure to rituximab. Lanes 4 and 9: Decoration with anti-MBP antibody. Lanes 5 and 10: Exposure to only secondary antibodies. Band “a”: MBP-pf/MSP1<sub>19</sub> fusion protein. Band “b”: Endogenous MBP from *E. coli*. Band “c”: Non-specific cross-reactivity of the polyclonal anti-MBP antibody to uncharacterized components in the sample. Band “d”: pf/MSP1<sub>19</sub> fragment. Blot images have been cropped above 110 kDa and below 3 kDa, respectively.

**Figure 2. NanoESI mass spectrum of ions from MBP-pf/MSP1<sub>19</sub> upon GluC digestion (solution 1).** Selected m/z values are given, and charge states are indicated in parentheses (cf. Table S4). Multiply charged ion signals (top right insert) are from truncated endogenous MBP from *E. coli* which is chemically modified and enzymatically truncated (cf. Table S5). Sequence coverage was 96%.
Figure 3. ITEM-THREE with MBP-pfMSP\textsubscript{19} peptides from GluC digest as epitope peptide containing mixture and anti-pfMSP\textsubscript{19} antibody. Ion mobility arrival time plots of A, and C (solution 3A) - ITEM-THREE experiment. Dashed lines mark the regions for mass spectra selections. B, and D, nanoESI mass spectra (low m/z range) of ions from selected arrival time ranges. Selected m/z values are given, and charge states are indicated in parentheses.

Figure 4. ITEM-THREE with MBP-pfMSP\textsubscript{19} peptides from GluC digest as epitope peptide containing mixture and His-tag antibody. Ion mobility arrival time plots of A, and C (solution 3B) - positive control experiment. Dashed lines mark the regions for mass spectra selections. B, and D, nanoESI mass spectra (low m/z range) of ions from selected arrival time ranges. Selected m/z values are given, and charge states are indicated in parentheses.
Figure 5. Three-dimensional structure representations of the pfMSP1<sub>19</sub> antigen. The atoms of the amino acid residues of the epitopes on the antigen surface (Connolly surfaces using van der Waals radii) are colored whereas the antigen surface is indicated in white. The backbone of the amino acid sequence is shown as ribbon (cartoon view). The side chain atoms of the epitope-contributing amino acids are depicted as stick models. A: ITEM-THREE results. Green: amino acid residues from peptide <sub>386</sub>GRNISQHQCCVKKQCPQN-SGCFRHLDEREE<sub>414</sub>. Blue: amino acid residues from peptide <sub>415</sub>CCKLLNYKQE<sub>424</sub>. The red circle surrounds amino acid residues that overlap with X-ray results. B: X-ray results. Green: amino acid residues of the epitope identified by Pizarro et al 2003 (22); yellow: Cysteiny1 residues forming disulfide bonds. The red circle surrounds amino acid residues that overlap with ITEM-THREE results.
ITEM-THREE analysis of a monoclonal anti-malaria antibody reveals its assembled epitope on the pfMSP119 antigen

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