The malaria parasite *Plasmodium falciparum* degrades host cell hemoglobin inside an acidic food vacuole during the blood stage of the infectious cycle. A number of aspartic proteinases called plasmepsins (PMs) have been identified to play important roles in this degradation process and therefore generated significant interest as new antimalarial targets. Several x-ray structures of PMII have been described previously, but thus far, structure-guided drug design has been hampered by the fact that only inhibitors comprising a staine moiety or derivatives thereof have been published. Our drug discovery efforts to find innovative, cheap, and easily synthesized inhibitors against aspartic proteinases yielded some highly potent non-peptidomimetic achiral inhibitors. A highly resolved (1.6 Å) x-ray structure of PMII is presented, featuring a potent achiral inhibitor in an unprecedented orientation, contacting the catalytic aspartates indirectly via the “catalytic” water. Major side chain rearrangements in the active site occur, which open up a new pocket and allow a new binding mode of the inhibitor. Moreover, a second inhibitor molecule could be located unambiguously in the active site of PMII. These newly obtained structural insights will further guide our attempts to improve compound properties eventually leading to the identification of molecules suitable as antimalarial drugs.

Malaria is a major public health issue in many areas of the world, with *Plasmodium falciparum* being the causative agent of the most severe and deadliest form of this disease. Each year, 500 million new infections resulting in up to 2 million deaths and enormous economic damage (1) are attributed to this parasite. Drug resistance in *P. falciparum* has been aggravating the problem in many parts of the world during the last two decades, and new antimalarial agents addressing new targets are desperately needed.

The protozoan parasite resides in erythrocytes of infected individuals during the asexual part of its life cycle. Recent studies indicated that hemoglobin degradation in a parasitic acidic organelle represents a major metabolic pathway and is crucial for survival of the parasite. Multiple proteinases appear to be actively involved in hemoglobin degradation (2–5). In particular, three members of a family of *P. falciparum* aspartic proteinases (PMI, PMII, and PMIV) have been localized in the food vacuole (4, 5) and shown to be able to degrade hemoglobin *in vitro*. Another sequence-related proteinase with a new catalytic apparatus called PMIII or histo-aspartic proteinase (6) is also involved in hemoglobin catabolism *in vitro*. A number of research groups have reported attempts to find potent inhibitors of plasmepsins (7–12). Many of the identified molecules are peptidomimetic in nature, a compound class often associated with relatively low bioavailability and, importantly for use in developing countries, unfeasible due to significant cost of goods. We have discovered and subsequently optimized a new class of potent PMII inhibitors that could potentially overcome both these problems (8). X-ray structures of PMII and PMIV, respectively, were available (11, 13–15) and could be used for structure-based drug design efforts. All attempts to model our achiral inhibitors into the active site of currently available structures of PMII or PMIV led to unsatisfactory results and were unable to explain the structure-activity relationship. As a result, studies of highly resolved structural information of relevant protein-inhibitor complexes were initiated.

Presented here is a highly resolved structure of PMII inhibited by a potent non-peptidomimetic achiral inhibitor. This PMII-inhibitor complex reveals new features not yet observed in structures of aspartic proteinases, and it can serve as a starting point for structure-guided molecular design of novel compound classes with further optimized properties.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—DNA encoding the last 48 residues of the pro-part and the whole mature sequence of *P. falciparum* plasmepsin II was cloned into the T7-dependent vector pET3a for expression (clone was obtained from C. Berry, Cardiff School of Biosciences, Cardiff University, Cardiff, Wales, UK). Protein was expressed in *Escherichia coli* BL21 (DE3) as inclusion bodies, solubilized, and refolded as described by Hill et al. (16), with minor modifications to the protocol. The resulting soluble refolded protein was purified using a ligand affinity column, followed by size exclusion chromatography into 20 mM sodium/potassium phosphate, 100 mM NaCl, pH 6.5, and concentrated using an Amicon Ultra centrifugal concentrator for crystallization.

**Crystallization and Data Collection**—Crystals were obtained applying the hanging drop method using protein versus reservoir drop ratios of 2:1 and 3:1, respectively. Crystals were obtained with 25–30% polyethylene glycol 4000 at phosphate buffer (pH 5.0–6.0) after 2 weeks. Data were collected at the PX beamline at the Swiss Light Source, Paul Scherrer Institute (Villigen, Switzerland). The structure was determined by molecular replacement using a structure of inhibited PMII (PDB code 1LF3). Data processing, structure solution, and refinement were performed with the program suite CCP4 (17). For manual rebuilding of the structure, the program Moloc (18) was used. A summary of data processing and refinement is given in Table I.

*The abbreviations used are: PM, plasmepsin; PDB, Protein Data Bank; HIV, human immunodeficiency virus.*
interactions, allowed the flap to twist and lift its tip by 6 Å and the flap, as well as diminished attractive hydrophobic
sence of hydrogen bonds between the tightly bound inhibitor as the catalytically important aspartates. The complete ab-

| X-ray data processing and refinement |      |
|-------------------------------------|------|
| Space group                         | P 21 2 |
| Cell constants (Å)                  | 67.9, 157.1, 38.9 |
| Resolution range (last shell)       | 157 to 1.55 (1.64 to 1.55) |
| No. of observed reflections         | 247,290 |
| Unique observations                 | 60,622 |
| Multiplicity (last shell)           | 5.5 (3.7) |
| Completeness (last shell)           | 94% (84.7%) |
| R<sub>merge</sub> (last shell)       | 11.8% (25.8%) |
| No. of atoms                        | 3,117 |
| Used reflections (test set)         | 53,885 (2,885) |
| Deviation bond length               | 0.012 |
| Deviation bond angles               | 1.447 |
| R<sub>free</sub> (last shell)        | 0.196 (31.5) |
| Free R<sub>free</sub> (last shell)   | 0.228 (36.5) |

Surface Calculations—The program XSAE (an unpublished program obtained from Dr. C. Broger, Hoffmann-La Roche, Basel, Switzerland) was used to calculate surface interactions between enzyme and inhibitors. An algorithm as described in Ref. 19 was applied in there. A probe radius of 1.4 Å was used for calculations.

Assay for Plasminogen II—Proteolytic activity of plasminogen II was determined in fluorescence resonance energy transfer-based assays with a substrate purchased from Bachem (M-2120). Approximately 1 nM enzyme was incubated with 1 μM substrate at 37 °C in 50 mM sodium acetate, pH 5, 12.5% (w/v) glycerol, 0.1% (w/v) bovine serum albumin, and 10% Me<sub>2</sub>SO. Enzyme activity was derived from the turn-over rate of the substrate and monitored by the increase of the fluorescent signal over time. Fluorescence was determined with a FluoroStar Galaxy from BMG using excitation and emission filters of 355 and 520 nm, respectively. Test compounds were dissolved and diluted in 100% Me<sub>2</sub>SO. Inhibitory activity of the compounds was expressed as IC<sub>50</sub>, which represents the concentration of compound that inhibits 50% of the maximal (uninhibited) enzyme activity. IC<sub>50</sub> values determined for published inhibitors corresponded well with the reported data.

RESULTS

The x-ray crystal structure of mature wild type PMII inhibited by the potent (34 nM) non-peptidomimetic and achiral inhibitor 1 (Fig. 1) was solved at a resolution of 1.6 Å. All 329 amino acids of PMII were visible and could be built into electron density. The enzyme crystallized in space group P2<sub>1</sub> 2<sub>1</sub> 2<sub>1</sub> comprising one enzyme molecule in the asymmetric unit, an arrangement not seen in any of the structures of PMII published so far. A Ramachandran plot revealed that with the exception of two residues (Leu<sup>191</sup> and Asn<sup>238</sup>), all amino acids were found in “most favored” or “allowed” regions.

General Description of Structure—The overall architecture of the single enzyme molecule in the asymmetric unit compares well with structures inhibited by statin derivatives. A superposition with a structure of PMII inhibited by the prototypic peptidomimetic compound pepstatin A (PDB code 1SME) resulted in a root mean square deviation of 0.55 Å for 288 of 329 C<sub>α</sub> atoms (Fig. 2). A significant difference in protein conformation was found in the region of a long β hairpin called “flap” (Lys<sup>22</sup>-Phe<sup>67</sup>) that was covering parts of the active site as well as the catalytically important aspartates. The complete absence of hydrogen bonds between the tightly bound inhibitor 1 and the flap, as well as diminished attractive hydrophobic interactions, allowed the flap to twist and lift its tip by 6 Å (Val<sup>26</sup> to 9 Å (Ser<sup>79</sup>) compared with the structure of PDB code 1SME (Fig. 3). The highly conserved Tyr<sup>77</sup> that was part of this flap was lifted up 4.6 Å (C<sub>α</sub>) and rotated ~120° around χ1, thereby losing a hydrogen bond contact with the indole NH of the highly conserved Trp<sup>31</sup>. The interaction between these two side chains was typically observed in structures of aspartic proteinases from eukaryotic sources that were inhibited by peptidomimetic inhibitors (e.g. pepstatin, PDB code 1SME).

Compared with known structures of monomeric aspartic proteinases, the side chain of Trp<sup>31</sup> was rotated ~120° around χ1 and 180° around χ2, opening up a large, mostly hydrophobic pocket toward the core of the protein (Figs. 2 and 4). Such a concerted rearrangement was never observed in structures with peptidomimetic inhibitors. Similar movements have been described for structures of renin (20) that were available from the PDB but have been withdrawn recently. Amino acid side chains in the active site that were not part of the flap superimpose well in our structure and the structure of PDB code 1SME, respectively, with the already mentioned exception of Trp<sup>31</sup>. Unexpectedly, the active site of each enzyme molecule hosted not just one but two inhibitor molecules (Fig. 5). One molecule was tightly bound and deeply buried in the reshaped active site of the proteinase and was most likely solely responsible for the high inhibitory potency. The second inhibitor experienced significantly less protein contact. It is noteworthy that this molecule is also in contact with amino acids Val<sup>199</sup>-Leu<sup>242</sup> of a loop residing in a neighboring PMII proteinase (Fig. 5). Superposition of our new structure with a PMII-pepstatin A complex (PDB code 1SME) revealed that besides the newly created pocket, the more tightly bound “inner” inhibitor molecule also occupied subsites S1’, parts of S1 and S3, and the S5 pocket (21) (Fig. 6). The second “outer” inhibitor molecule occupied the S2, S4, and the S6 pocket as well as space filled by the backbone of the peptidomimetic inhibitor and the tip of the flap in pepstatin A-inhibited PMII (1SME).

Specific Enzyme Inhibitor Interactions—As briefly men-

![Fig. 1. Topology of inhibitor 1 is depicted.](image)
strongly reduced motion. The tertiary amide moiety was rotated by $75^\circ$ compared with the phenyl ring and, as such, was in an almost unstrained conformation. There was no direct hydrogen bond between enzyme and the amide carbonyl oxygen. However, there was a water molecule in ideal distance and geometry to form a H-bond, although without any further H-bond contact to the enzyme. The tertiary amide nitrogen was linked on one side to the central piperidine moiety and on the other side to the 4-$\alpha$-substituted biphenyl-methylene unit that occupied parts of the S1 and S3 pockets (Fig. 6). The proximal phenyl ring was in contact with side chains of Ile$^{32}$ and Ile$^{123}$, whereas the distal aromatic moiety touches side chains of Met$^{15}$, Ile$^{32}$, and Phe$^{120}$. Side chains of Ile$^{14}$, Met$^{15}$, and Ser$^{118}$ in collaboration with parts of the second inhibitor molecule tightly embraced the terminal 4'-methyl-acetate substituent, thereby diminishing its freedom of rotation. This was clearly reflected by well-defined electron density for the methyl-acetate group, with the exception of the ester methyl carbon.

The amide-linked piperidine moiety of 1 was located "above" the two catalytic aspartates. This was in contrast to the situation described in Ref. 20, in which a protonated secondary amine of the piperidine ring forms two strong hydrogen bond contacts, one with each of the two catalytic aspartates. Such interactions were impossible for the N-substituted piperidine in 1 due to steric reasons. To overcome this lack of required space, the piperidine ring was lifted with the nitrogen moved
up by 2.5 Å and C4 by 1 Å, respectively, compared with the structure described in Ref. 20. The tertiary amino group contacted the two aspartates indirectly via a very strong hydrogen bond to the catalytic water, which was not expelled from its position between the two carboxylates of the catalytic aspartates (Fig. 4). The water molecule in our structure superimposed very well with the nitrogen atom of the unsubstituted piperidine described in Ref. 20. (Structure PDB code 1PR8 was temporarily available from PDB but has been withdrawn recently for unknown reasons.)

The 2-methylene-imidazole unit linked to the piperidine nitrogen occupied the S1' pocket in an unstrained conformation, and its N1 participated in a hydrogen bond to the outer oxygen of Asp214 (Fig. 7). The second imidazole nitrogen was involved in a water-mediated contact to the alcoholic ester oxygen of the second inhibitor molecule.

The second, or outer, inhibitor molecule ran in the opposite direction compared with the inner molecule, with piperidine rings touching terminal aromats of the biphenyl moiety in the opposite inhibitor (Fig. 5). The outer inhibitor mainly occupied the remaining space in the active site, with the biphenyl moiety located in the S2 pocket and compensating for the relocated Val74 at the tip of the flap that had been lifted. The methylene tether to the amide bond packed against the biphenyl unit of the inner inhibitor, thereby establishing attractive hydrophobic interactions. The amide carbonyl oxygen accepted a hydro-

Fig. 4. PMII is represented as Cα backbone (green) with the catalytic aspartates (white; only partially visible) forming strong hydrogen bonds (red dashed lines) with the catalytic water (red ball). Inhibitor 1 (yellow) is located in the active site of the enzyme (blue surface) filling a mostly hydrophobic cavity with its n-pentyl chain. The piperidine moiety is engaged in a H-bond with the catalytic water, whereas the imidazole ring is involved in a H-bond contact with the outer oxygen of Asp214 (see also Fig. 7).

Fig. 5. PMII is displayed in Cα representation (green). Two inhibitor molecules in its active site are represented in yellow and blue, respectively. A neighboring PMII molecule is shown in red, with the Lys238-Pro243 loop represented with all atoms except hydrogens.
gen bond from the backbone NH of Ser218, whereas the 4-pentyl-phenyl residue was mimicking part of the natural substrate backbone as well as partially filling the S6 pocket. The piperidine ring was located next to the relocated flap, with its nitrogen engaged in water-mediated contacts to the phenolic OH of Tyr77 and the hydroxy group of Thr114, respectively (Fig. 7).

Using the software program XSAE (an unpublished program obtained from Dr. C. Broger, Hoffmann-La Roche), we calculated surface interactions for PMII hosting two inhibitor molecules and a neighboring enzyme molecule, respectively, to qualitatively judge individual interactions between inhibitors and enzyme(s). Note that different inhibitor conformations lead to slightly different total surfaces for the two inhibitors. Of a total surface of 979 Å² of the buried inner inhibitor, the large majority (757 Å²) were covered by PMII, whereas only 501 of 956 Å² of the outer inhibitor were in contact with this enzyme molecule. Some 125 Å² of the outer inhibitor were shielded from solvent by a symmetry-related PMII molecule (Fig. 5). The two inhibitors covered 151 Å² of each other’s surface. The symmetry-related PMII molecule did not touch the inner inhibitor at all.

**DISCUSSION**

Plasmepsins play an important role in hemoglobin degradation in *Plasmodium* parasites because they are supposed to perform initial cleavages before numerous other proteinases.
get involved in the degradation process. Recent knock-out studies have shown that none of the four proteinases (PMI–PMIV) is absolutely crucial for survival of the parasite (5, 22). Therefore, effective drugs must inhibit multiple plasmapepsins to increase antimalarial effects and, at the same time, decrease the likelihood of resistance development. Designing compounds active against multiple enzymes with reasonable bioavailability and devoid of unwanted side effects is a difficult endeavor. It is therefore of great help if structural details of all target enzymes are known in atomic resolution. A number of x-ray structures of PMII and PMIV have been published (11, 13–15), but they were considered to be irrelevant for our purposes because we identified inhibitors with new topologies (8) that could not be reliably modeled into the active site of existing PMII structures. We therefore embarked on a project aimed at solving structures of relevant enzymes inhibited by our new compounds.

We could obtain an x-ray structure of PMII complexed to a potent achiral inhibitor at 1.6 Å resolution, currently the highest resolved structure of this enzyme. Unexpectedly, we could locate not just one but two inhibitors with clearly defined electron density in the active site of one enzyme molecule. To our knowledge, this had not been observed thus far in structures of monomeric aspartic proteinases. However, two inhibitor molecules per active site could be localized in x-ray structures of homodimeric HIV-1 protease inhibited by a dithioketal derivative of haloperidol (23). By coincidence, in analogy to our compound 1, these inhibitors also comprised a N-substituted piperidine moiety with an additional substituent in position 4. Their binding modes in wild type and a Q7K mutant from HIV-1 were very different from each other, but in both cases, a halide anion was located near the protonated tertiary amino group. In neither of these two structures was the piperidine nitrogen found to be within hydrogen bond distance to either one of the catalytic aspartates. Inhibitory activity of these haloperidol derivatives was strongly dependent on the presence of halide anions in the assay solution, whereas in our system, no halide anions were required for inhibition of PMII.

The finding of not just one but two clearly defined inhibitor molecules in the active site came rather unexpectedly. To estimate whether the second inhibitor was simply a “crystallization artifact” or must be considered part of the measured enzyme inhibition, we calculated enzyme-inhibitor surface interactions. Whereas the inner molecule was deeply buried, and more than 77% of its mostly hydrophobic surface was occluded from access to water, the outer molecule had only a total of 65% of its surface buried, including 13% covered by a neighboring enzyme molecule (Fig. 5). Assuming that nanomolar concentrated PMII in solution was not favoring such close interactions with a second enzyme molecule, as observed in the crystal structure, one would conclude that only 52% of the surface of the outer molecule was involved in enzyme-inhibitor interactions. This led to a difference of covered (mostly hydrophobic) surface for the two inhibitors of ~240 Å² that was estimated (24) to lead to a difference in binding energy of more than 10 kcal/mol affinity. On this basis, it seemed to be fair to conclude that the inner inhibitor was exclusively responsible for the determined inhibiting effect. In addition, there were no observations from inhibition measurements indicating a biphasic kinetic within the investigated range of inhibitor concentrations. This second inhibitor in the active site of each enzyme was judged to be a crystal artifact necessary solely to complete and stabilize the crystal lattice.

It was tempting to speculate whether this newly found pocket already exists in uninhibited PMII, or whether the inhibitor has to enforce a “door-opening” of the pocket by pushing Tyr27 and Trp41 to their new conformation. A strong hint came from the x-ray structure of proPM II (25), which had a wide open unoccupied active site and was inhibited only by its propeptide and not by any further molecule. In the asymmetric unit there were four enzyme molecules, each showing Trp41 side chain in the open conformation similar to the one found in our structure, whereas the whole tip of the flap including Tyr27 could not be located in any of the four enzyme molecules. Because there was no obvious external influence/molecule enforcing this open conformation, there must be an inherent driving force to populate this open state in proPMII, leading to the conclusion that no large energy barrier had to be surmounted in order to “unlock” and shift the Trp41 side chain in this new position. In the case of non-peptidomimetic renin inhibitors (20), authors have already come to the conclusion that the two observed side chain conformations of Trp20 in renin are likely to be separated only by a small energy barrier.

The highly flexible n-pentyl chain of 1 fitted very tightly into this newly opened hydrophobic pocket of PMII in an unstrained conformation. The rather high affinity of 1 indicated that attractive interactions gained by burying 190 Å² of hydrophobic surface in a hydrophobic environment compensated for the entropic loss of significantly reducing the rotational degrees of freedom of this alkyl chain. Additionally, the benzoyl unit was involved in two favorable edge on face contacts with Trp41 and Phe111, respectively, allowing the 3,5-hydrogens of its benzene ring to interact with π-electrons of the electron-rich amino acid side chains. This observation indicated that substitution of carbons by heteroatoms or introduction of substituents at these positions might yield less favorable interactions.

One of the major problems in our modeling efforts was the correct location of the putatively protonated piperidine nitrogen. Identical interactions as described for some renin inhibitors comprising secondary amine piperidine derivatives (20) could be excluded for steric reasons. Positioning of the putatively charged piperidine nitrogen close to the catalytic aspartates was assumed due to favorable electrostatic attractions, but correct locating of the hydrophobic parts of 1 was anything but obvious. Directing hydrogen bond interactions between the secondary amine of the piperidine and catalytic aspartates, as observed in renin x-ray structures, was not possible for 1. The binding mode chosen by nature was striking. Instead of a protonated secondary amine directly interacting with both catalytic aspartates via hydrogen bonds, the N-substituted piperidine ring was simply lifted up, allowing one strong hydrogen bond between the ring nitrogen and the catalytic water that remained between the catalytically important aspartates. This is the first report of a highly potent inhibitor interacting with the catalytic aspartates by means of a hydrogen bond to the water sitting between their side chains. This water molecule gets activated upon substrate binding and initiates amide bond breaking by attacking the carbonyl carbon of the cleavable peptide bond.

In a recently published x-ray structure of endothiapepsin (26), a Ser-Thr dipeptide with significantly reduced occupancy could be located in the S1’ and S2’ subpockets, with its N-terminal nitrogen contacting this catalytic water. However, neither the origin nor the sequence of the di-peptide could be unambiguously determined. It was speculated that it could be a cleavage product of an autoproteolytic degradation process at the N terminus of the protein because crystal growth had lasted for 12 years. In a second example, Fraser et al. (27) mentioned a low affinity inhibitor of penicillopepsin (110 μM) comprising a phosphonate moiety that contacted the catalytic aspartates indirectly via a water molecule sitting between their side chains. During preparation of this manuscript, a short publication appeared (28) mentioning a low micromolar β-site
amyloid precursor-cleaving enzyme inhibitor performing a water-mediated contact to the catalytic aspartates via N-H of an amide bond. It remains to be seen whether a derivative of 1 comprising an appropriate replacement of the piperidine nitrogen would be able to expel the catalytic water from its position, thereby profiting from the entropy gain leading to further improved affinity.

The 2-imidazole-methylene group of 1 that was involved in H-bond contacts in both inhibitors located in the active site might have played a key role in the growth of a regular crystal lattice. N1 of the inner inhibitor is involved in a H-bond contact to Asp214, whereas N3 participates in a water-mediated H-bonding of crystals.

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