The staphylococcal inhibitory protein SPIN binds to human myeloperoxidase with picomolar affinity but only dampens halide oxidation

Urban Leitgeb, Paul G. Furthmüller, Stefan Hofbauer, Jose A. Brito, Christian Obinger, and Vera Pfanzagl

From the 1University of Natural Resources and Life Sciences, Vienna, Department of Chemistry, Institute of Biochemistry, Vienna, Austria; 2Universidade Nova de Lisboa, Instituto de Tecnologia Química e Biomolecular António Xavier, Oeiras, Portugal

Edited by Ruma Banerjee

The heme enzyme myeloperoxidase (MPO) is one of the key players in the neutrophil-mediated killing of invading pathogens as part of the innate immune system. MPO generates antimicrobial oxidants, which indiscriminately and effectively kill phagocytosed pathogens. *Staphylococcus aureus*, however, is able to escape this fate, in part by secreting a small protein called SPIN (Staphylococcal Peroxidase Inhibitor), which specifically targets and inhibits MPO in a structurally complex manner. Here, we present the first crystal structures of the complex of SPIN-*aureus* and a truncated version (SPIN-truncated) with mature dimeric leukocyte MPO. We unravel the contributions of the two domains to the kinetics and thermodynamics of SPIN-*aureus* binding to MPO by using a broad array of complementary biochemical and biophysical methods. The C-terminal “recognition” domain is shown to mediate specific binding to MPO, while interaction of the N-terminal “inhibitory” domain is guided mainly by hydrophobic effects and thus is less sequence dependent. We found that inhibition of MPO is achieved by reducing substrate migration, but SPIN-*aureus* cannot completely block MPO activity. Its’ effectiveness is inversely related to substrate size, with no discernible dependence on other factors. Thus, SPIN-*aureus* is an extremely high-affinity inhibitor and highly efficient for substrates larger than halogens. As aberrant MPO activity is implicated in a number of chronic inflammatory diseases, SPIN-*aureus* is the first promising protein inhibitor for specific inhibition of human MPO.

Phagocytosis by neutrophils and subsequent killing of invading pathogens is a highly effective line of defense of the innate immune system (1–3). Upon activation, neutrophils phagocytose bacteria and release antimicrobial proteins and peptides from subcellular granules into the nascent phagosomal compartment. The heme enzyme myeloperoxidase (MPO) is the most abundant antimicrobial protein and contributes significantly to oxidative killing of pathogens through hydrogen peroxide (H₂O₂) mediated formation of highly reactive oxidants. Its most important products are hypochlorous (HOCl) and hypotiocyanous (HOSCN) acid (4–6) but it can oxidize a range of other physiologically relevant molecules such as bromide, nitrite, phenols, or sulphide (6–12). However, MPO activity is a two-sided sword as the generated oxidants will indiscriminately attack surrounding biomolecules (13, 14). Thus, MPO is both a key enzyme in host defense and a causative agent in inflammatory diseases, if it is active outside the phagosome. It has therefore attracted considerable attention for the development of therapeutically useful MPO inhibitors (15–18).

Interestingly, a few pathogens have developed strategies to escape MPO-mediated killing. The most successful human pathogen in combating neutrophil-mediated killing is *Staphylococcus aureus* (*S. aureus*), a bacterium that has evolved strategies against every aspect of host defense (19). In 2017, de Jong *et al.* were able to identify a small 8 kDa protein that has evolved to specifically bind and inhibit human MPO (20). This protein, called SPIN—Staphylococcal Peroxidase Inhibitor—is unique to the *Staphylococcus* genus and is the first known protein-based specific inhibitor of MPO (17, 18, 21).

De Jong *et al.* (2017) first solved the structure of the complex of recombinant monomeric heme-free MPO (rMPO) and SPIN from *S. aureus* (SPIN-*aureus*). The X-ray structure shows that SPIN is composed of two distinct domains. The 6.3 kDa C-terminal alpha-helical domain binds close to the active site. The N-terminal peptide domain is inserted into the substrate channel. Studies using truncations and mutations identified the C-terminal domain as the binding domain responsible for recognition of MPO, while the N-terminal domain and a conserved HDD-motif are required for inhibition. De Jong *et al.* described the mode of inhibition as a “molecular plug” that prevents substrates from entering the active site (22).

Based on the crystal structure, it can be assumed that the two domains bind consecutively. Importantly, the N-terminal domain changes from an unstructured loop in solution to the β-hairpin domain in the course of complex formation with MPO (22). This resembles the “folding-upon-binding” mechanism proposed for several intrinsically disordered proteins (23–25). Surprisingly, the contribution of the N-terminal peptide to the overall affinity is reported to be rather small (22).
SPIN—S. aureus’ inhibitory shield against myeloperoxidase

Although it amounts to half of the SPIN-MPO surface area and contains 7 out of 17 bond-forming residues (21). This contradicts the general observation that protein–protein interactions are typically governed by favorable enthalpic contributions and protein complexes with higher affinities exhibit large interface areas, a higher number of hydrogen bonds, and high geometric complementarity (26, 27). Clearly, binding and inhibition of MPO by SPIN are closely linked. Therefore, understanding the inhibitory capacity of SPIN—an aspect both vital for S. aureus and interesting for MPO inhibitor design—requires understanding not only of the kinetics and thermodynamic driving forces of SPIN binding and complex formation but also of the effect on the activity of MPO including ligand binding and the individual reaction steps of catalyzed redox reactions.

In this article, we present the first crystal structures of the complex of SPIN-aureus and a truncated version (SPIN-truncated) with mature dimeric leukocyte MPO showing full heme occupancy (Fig. 1). SPIN-truncated contains neither the N-terminal domain nor the HDD motif. We could unravel the contribution of the two domains to the kinetics and thermodynamics of SPIN binding to MPO. We demonstrate a strong impact of the N-terminal domain to the overall affinity, which is in contrast to the published data. We can show that although the SPIN N-terminal domain reaches the active site to a certain extent, there is no interaction with the heme cofactor. Moreover, we analyzed the inhibition kinetics and can show that this “molecular plug” (as reported in (22)) is not tight enough for the smaller biological substrates of MPO, such as hydrogen peroxide or halides (6), whose Van der Waals volumes are not significantly larger than that of water http://www.chemaxon.com. Finally, we discuss the structure–function mechanism of MPO inhibition by SPIN with respect to the individual (redox) reaction steps of MPO (28).

Results

The crystal structure of native human leukocyte MPO in complex with SPIN-aureus suggests a split binding interface

As described previously, de Jong et al. solved the crystal structure of recombinant monomeric MPO (rMPO) in complex with SPIN-aureus. As rMPO differs in several aspects from the native protein, we decided to obtain a cocrystal structure of native human MPO with SPIN-aureus (Fig. 1). While rMPO is a single chain monomer with low heme incorporation (29), native human MPO is a ~150 kDa dimer with each subunit consisting of a heavy and a light chain formed by posttranslational modifications, which are linked by disulfide bridges (30, 31). The heme cofactor is autocatalytically modified and covalently linked to the protein via two ester and one sulfonium linkage. Additionally, native MPO is heavily glycosylated at seven N-glycosylation sites (32), with one site in close proximity to the binding site of SPIN-aureus.

We obtained single crystals of the complex of human native MPO with SPIN-aureus that diffracted to a resolution of 2.18 Å and solved the structure to Rwork/Rfree of 0.20/0.24 (Protein Data Bank [PDB] code: 7QZR). The MPO–SPIN-aureus complex crystalized in space group P432121 with one monomer per asymmetric unit. Overall the crystal structure of the MPO–SPIN-aureus complex is similar to the previously solved complex with rMPO (Fig. S1). Superimposition of the structures gave an rmsd of 0.27. The C-terminal globular “reognition” domain binds close to the active site access channel while the N-terminal “inhibitory” domain forms a β-hairpin motive inserted in the active site (Figs. 2 and S1). The C-terminal domain contributes 680 Å² (45%) and the N-terminal domain contributes 880 Å² (55%) to the overall interface area (1500 Å²). We did not find any interaction with the resolved sugar moieties at Asn355, suggesting that SPIN-aureus binding is likely not influenced by the divergent glycosylation patterns found in MPO (33). Gln37, the SPIN-aureus residue closest to the heme cofactor, is 3.2 Å away from O1D of the heme propionate, a distance where a weak interaction is possible. Importantly, no direct ligation of the heme iron is observed. This is reflected by
the unaltered UV-visible and electronic CD spectra obtained in solution (Fig. S2).

We next analyzed the protein–protein interfaces with regard to (i) electrostatic surface potential (Fig. 2A), (ii) hydrophobicity (Fig. 2B), and (iii) specific interactions between side chains of SPIN-\textit{aureus} and side chain and main chain atoms of MPO (Fig. 2, C and D). Analysis of the electrostatic surface potential shows that the MPO substrate access channel is highly negatively charged (Fig. 2A). However, no complementarily charged interface can be found in the N-terminal domain of SPIN-\textit{aureus}. In both subunits, only two residues of the N-terminal domain (Tyr35 and Gln37) form hydrogen bonds with residues of MPO, which is in stark contrast to the seven interactions reported by Ploscariu et al. in 2018 (21). The reported salt bridge of the nonconserved Lys33 could form. Lys33 is, however, not well resolved, which suggests a high degree of flexibility and contradicts a stable salt bridge. His43 is the only residue of the conserved HDD-motif—reportedly essential for inhibition—that directly interacts with MPO through a salt bridge with Asp380. The interactions of Asp44 and Asp45 reported for rMPO-SPIN-\textit{aureus} are not present in our structure. In total, we found three salt bridges and only four hydrogen bonds with a distance lower than 3.3 Å in both subunits and two with distances up to 3.5 Å. These are

![Figure 2. The interface between mature dimeric human MPO and SPIN-\textit{aureus} (PDB 7QZR).](image)

\textit{A}, electrostatic surface potential (calculated by ABPS) of MPO (left, SPIN-\textit{aureus} depicted as \textit{white loops}) showing the negative potential of the active side (red), which is not matched by SPIN-\textit{aureus} (right). \textit{B}, hydrophobicity of the active site of MPO (left) and of the N terminus of SPIN-\textit{aureus} (right) colored according to the Eisenberg hydrophobicity scale. The remaining surface is colored cyan (MPO) or \textit{white} (SPIN-\textit{aureus}). \textit{C}, hydrogen bonds and salt bridges between side chains of SPIN-\textit{aureus} and side chains (left) or the backbone (right) of MPO. The central panel depicts interactions between residues of SPIN-\textit{aureus} with side chain residues of MPO in \textit{magenta} and with main chain residues of MPO in \textit{orange}. \textit{D}, interface sequence (boxed residues) of SPIN-\textit{aureus} with secondary structure elements and the SPIN domains illustrated below. The color code of the N-terminal residues reflects their hydrophobicity. Interacting residues are highlighted with “\textit{blue circled} H” for hydrogen bonds or “\textit{orange circled} S” for salt bridges. MPO, myeloperoxidase; PDB, Protein Data Bank.
mainly in the interface between the C-terminal domain and MPO (Fig. 2, C and D), suggesting that in this domain, polar interactions are the dominant driving force behind protein binding. A “hot spot” seems to be the stretch of residues 47 to 55 with four out of nine residues forming polar interactions with MPO.

In addition to shape complementarity and polar interactions, interface hydrophobicity is a key driving force for protein binding. The N-terminal domain of SPIN-aureus is unstructured in solution but upon binding adopts a distinct β-sheet structure with a hydrophobic patch (residues 39–42) that has a complementary interface with MPO (Fig. 1B). Calculation of the solvation free energy gain (ΔiG) of the overall crystal structure and of the two domain interfaces of SPIN-aureus individually (Tables 1 and S1) using PISA demonstrates that the N-terminal SPIN domain–MPO interface has a higher ΔiG (ΔiG = -8 kcal/mol) than the C-terminal domain (ΔiG = -4 kcal/mol). This suggests that hydrophobicity plays an important role in folding and binding of the N-terminal domain.

Next, we solved the crystal structure of human MPO in complex with a truncated version of SPIN-aureus. SPIN-truncated contains neither the N-terminal domain nor the HDD motif, which are both essential for inhibition. The cocystal structure of the truncated molecules with the C-terminal domain of SPIN-aureus (chain E) were between 0.25 and 0.30. Overall, the crystal structure suggests that SPIN-truncated is a good model to understand the initial recognition and binding of the C-terminal domain to MPO (Fig. S1).

### Solvation of the interface area of the N-terminal domain of SPIN-aureus with MPO and of the substrate-binding sites

In addition to formation of electrostatic protein–protein interactions, binding of the N-terminal domain includes displacement of solvent water in the substrate channel. Interestingly, we found that large areas of the MPO access channel, corresponding to the less hydrophobic regions in the interface between MPO and SPIN-aureus, contained crystal waters (8 within 5 Å of the N-terminal domain). This includes the positions immediately surrounding the heme cofactor (Fig. 3, yellow boxes). To assess accessibility to the heme cavity in the MPO-SPIN-aureus complex, we calculated potential access routes using Caver3.0 (https://www.caver.cz/) (34). Two main potential access routes with divergent ends exist (Fig. 3 green box, blue and yellow-orange, Fig. S3). The calculated bottleneck radii were small with 1.1 to 0.9 Å (Table S2), in line with the proposed plug mechanism. SPIN-aureus residues framing the bottleneck were Asn38 in the most likely tunnel (blue tunnel, Fig. 3) or the hydrophobic patch residues 39 to 42 and His43 (yellow-orange tunnels, Fig. 3).

We directly compared the crystal waters in the active site and access channel in the MPO–SPIN-aureus complex with the MPO-SPIN-truncated cocystal structure and literature data (30). Importantly, one has to bear in mind that the MPO metal center in all crystals likely represents the ferrous form due to the inevitable photoreduction of the heme iron (III) to iron (II) during data collection (35, 36), which influences solvent organization in the first and second coordination sphere of the iron. Five distinct water positions are described (Fig. 4C). These are w1, at the position where H2O2 and iron ligands would bind, w2 & w5, which are at the postulated halogen binding sites, and two conserved waters at w3 & w4. We did not observe the water positions to be as strictly conserved (Fig. 4) as suggested. We observed waters at positions w1 (red spheres) in only two subunits of the MPO-SPIN-truncated structure. However, this position is the most affected by the iron oxidation state. The general area of the bromide binding site w2 (raspberry, highlighted with orange cycle) is occupied in two subunits but without a clearly

### Table 1

PISA interface analysis of the crystal structure of native MPO–SPIN-aureus complex (PDB: 7QZR), the recombinant MPO-SPIN-aureus complex (PDB: SUZU), and the MPO-SPIN-truncated complex (PDB: 7Z53)

| Protein/domain/subunit | Interface area [Å²] | ΔG [kcal/mol] | ΔG p-value | H-bonds (>3.3Å) | Salt bridges |
|------------------------|---------------------|---------------|-------------|-----------------|--------------|
| SPIN-aureus (average)   | 1434 ± 22           | 12.2 ± 0.3    | 0.131 ± 0.03| 6 (4)           | 3            |
| Subunit 1 (chains AB & E) | 1442               | -12.5         | 0.106       | 6 (5)           | 3            |
| Subunit 2 (chains CD & F) | 1428               | -11.9         | 0.157       | 4 (4)           | 2            |
| C-terminal domain E     | 1500 ± 21           | -9.4          | 0.283       | 4 (4)           | 2            |
| N-terminal domain E     | 824                 | -8.5          | 0.137       | 2 (1)           | 1            |
| Recombinant MPO (Suzu)  | 1541.9              | -10.7         | 0.280       | 18 (5)          | 8            |
| SPIN-truncated (average) | 653 ± 24           | -4.1 ± 0.5    | 0.35 ± 0.04 | 6 (4)           | 3            |
| Subunit 1 (chains AB & E) | 611                | -4.1          | 0.345       | 4 (3)           | 2            |
| Subunit 2 (chains CD & F) | 661                | -4.1          | 0.356       | 4 (3)           | 2            |
| Subunit 3 (chains GH & K) | 675               | -4.8          | 0.317       | 3 (2)           | 2            |
| Subunit 4 (chains II & L) | 652               | -3.8          | 0.42        | 3 (2)           | 2            |
| Subunit 5 (chains MN & Q) | 634               | -4.8          | 0.329       | 4 (4)           | 2            |
| Subunit 6 (chains OP & R) | 638               | -3.5          | 0.316       | 4 (3)           | 2            |
| Subunit 7 (chains ST & W) | 676               | -4.1          | 0.343       | 4 (3)           | 2            |
| Subunit 8 (chains UV & X) | 676                | -3.9          | 0.363       | 4 (4)           | 2            |
**SPIN—S. aureus’ inhibitory shield against myeloperoxidase**

conserved position and the position of w5 (brown spheres, circled in gray) is not occupied. For these, the effect of photoreduction can only be speculated on. However, the electron density for Glu408 shows that it is only partially linked in all subunits. If Glu408 is not linked, it appears to be very flexible and the carboxyl group would occupy the space of w5 (31). Therefore, we suggest that the previously described heterogeneity observed in the Glu408 ester linkage is the main reason we do not observe waters at this position. More stably coordinated waters (green, wheat, and blue spheres, Fig. 4B) were observed around the propionate groups and along the most likely access channel (Fig. 3, blue channel). Three of these coordinated waters need to be actively replaced by SPIN-aureus residues Gln37 and Asn38 (highlighted with blue circles in Fig. 4). Finally, the crystal waters in the access channel are not clearly conserved and we observed between 1 and 11 water molecules that would overlap with the N-terminal residues of SPIN-aureus.

**SPIN-aureus binds MPO with picomolar affinity, which is predominantly governed by an extremely low dissociation rate mediated by the N-terminal domain**

The structural data suggest that the two domains of SPIN-aureus interact and bind to MPO through different mechanisms. To better understand and dissect the kinetic and thermodynamic driving forces behind complex formation, we analyzed the kinetic and thermodynamic properties of SPIN-aureus with SPIN-truncated during binding to MPO. Interestingly, de Jong et al. 2018 reported that SPIN-truncated binds MPO with the same high affinity ($K_D \sim 10$ nM) as SPIN-aureus (22). However, the N-terminal domain contributes more than half of the overall interface area, forms a salt-bridge (His43), and is tightly packed within the active site pocket. Both hydrophobic interactions and hydrogen bonds are known to increase protein stability (37), which might be reflected in an increased thermostability. MPO is known to exhibit a high thermostability ($T_m$) of 86.7 °C at pH 7.4 (38). While SPIN-truncated did not have a stabilizing effect, we found that the MPO–SPIN-aureus complex is significantly stabilized compared to MPO alone in both physiological (pH 7.4: $T_m$ shift of $\sim 1.5$ °C) and acidic pH conditions (pH 5: $T_m$ shift $\sim 5$ °C) (Table S3 and Fig. 5A). The increase in thermostability of the MPO–SPIN-aureus complex compared to the truncated version implies a strong interaction, which is in contrast to the minimal effect on binding observed by de Jong et al (22).

We therefore tested the binding of SPIN-aureus and SPIN-truncated by surface plasmon resonance (SPR) using biotinylated native leukocyte MPO immobilized in a two-layer capture strategy via hybridization of a DNA-streptavidin conjugate to the sensor surface. Importantly, this setup allows reversible immobilization of the biotinylated protein. We found that SPIN-truncated binding to native MPO was comparable to the literature data ($K_D = 21 \pm 8$ nM compared to 29 nM, Table 3). Binding of SPIN-truncated to MPO was
monophasic (Fig. 5B) and analysis of the sensograms using a 1:1 binding model allowed calculation of the secondary binding constants (Table 3). Conversely, binding of SPIN-aureus was biphasic (Fig. 5B) with low to almost no observable dissociation. In accordance with the apparent two domain structure of SPIN-aureus, we used a two-state binding model, assuming an initial binding event followed by a conformational change with first order kinetics. For the initial binding event (presumably the C-terminal domain of SPIN-aureus), we obtained values similar to SPIN-truncated and the reported literature values of SPIN-aureus (Table 3), with \( k_{on1} \sim 6 \times 10^6 \text{ M}^{-1}\text{s}^{-1} \) and \( k_{off1} \sim 5 \times 10^{-3} \text{ s}^{-1} \). The association rate constant for the second binding phase (presumably folding and binding of the N-terminal domain) is extremely slow (\( k_{on2} \).)

Figure 3. Water pockets and channels present after SPIN-aureus binding in the MPO substrate channel (PDB 7QZR). Sliced representation of the MPO–SPIN-aureus cocrystal structure in full (center) or of the substrate channel and active site pocket from a frontal, right, and left hand perspective (yellow boxes). The water molecules in the active site are shown as blue spheres. MPO is shown as surface and SPIN-aureus is shown as cartoon putty. The access channel of MPO and the N terminus of SPIN-aureus are colored according to hydrophobicity (cyan = low hydrophobicity in MPO, white = low hydrophobicity in SPIN-aureus). Potential access channels (green boxes) to the heme cofactor (white stick representation) were calculated by Caver3.0 and are shown in the frontal and left hand view as blue or yellow-orange spheres. MPO, myeloperoxidase; PDB, Protein Data Bank.

Figure 4. Three partially conserved crystal waters are replaced by SPIN-aureus. Crystal waters in the active site of MPO in the presence (A) or absence (B) of the N-terminal domain of SPIN-aureus compared to the structure of MPO alone (PDB code: 1cxp, C). Shown are overlays of all subunits (two in the MPO-SPIN-aureus cocrystal (A) and eight in the MPO-SPIN-truncated cocrystal (B)). The waters are colored according to position, red shows waters in the vicinity of the heme iron, green shows waters surrounding the propionate groups, and blue corresponds to waters along the blue access channel shown in Figure 2. Conserved water positions replaced by residues of SPIN-aureus are highlighted with blue circles in the SPIN-truncated cocrystal structure (B). The label w1 represents the water at the position where \( \text{H}_2\text{O}_2 \) and ligands of the heme iron would bind, w2 & w5 represent the waters at the postulated halogen-binding sites, and w3 & w4 are two highly conserved water positions coordinated by the heme propionate groups. The heme cofactor is shown as black outline; the N-terminal residues (Q37 & N38) of SPIN-aureus are shown as white sticks. MPO, myeloperoxidase; PDB, Protein Data Bank.
**Figure 5. Kinetics and thermodynamics of binding of SPIN-aureus and SPIN-truncated to human leukocyte MPO.**

A, DSC thermograms of MPO in complex with SPIN-aureus (top), MPO alone (center), and MPO in complex with SPIN-truncated (bottom) in 50 mM phosphate buffer at pH 7.4. The corresponding fits using a non-two state transition model are depicted in red, the individual transitions in yellow, gray, or violet. The highest T_m values of each complex are highlighted with black lines (full = MPO-SPIN-aureus complex, dashed = MPO-SPIN-truncated complex). B, SPIN-aureus (top) and SPIN-truncated (bottom) binding to human MPO monitored by surface plasmon resonance spectroscopy. A 2-fold dilution series (32–2 nM) of recombinant SPIN was injected over biotinylated MPO noncovalently immobilized on a Biotin CAPture chip. The reference-subtracted sensograms (black traces) were fitted to a two-state binding model (SPIN-aureus, yellow traces) or a 1:1 binding model (SPIN-truncated, violet lines). Representative sensogram series are shown; all experiments were performed in triplicates. C, analysis of SPIN-aureus (top) or SPIN-truncated (bottom) binding to MPO by isothermal titration calorimetry. Shown are profiles of the heat difference obtained after titration of SPIN-aureus or SPIN-truncated (150 μM) to MPO (10 μM). Insets show the corresponding integrated injection heats, corrected for the heat of dilution (circles). The line shows the best least-squares fit to the one-site binding model. All experiments were performed in triplicates. MPO, myeloperoxidase.

Time dependency of MPO inhibition is biphasic

SPR analysis suggests a large time difference between initial binding of MPO by the C-terminal domain of SPIN-aureus ($k_{on} \sim 10^6 \text{ M}^{-1}\text{s}^{-1}$) and inhibition upon binding of the N-terminal domain ($k_{on} = 0.008 \text{ M}^{-1}\text{s}^{-1}$). To test whether and how this two-phase binding affects the inhibition of the enzyme, we measured the inhibition of the bromination activity of MPO preincubated with SPIN-aureus for defined time intervals. Figure 6 clearly shows that MPO inhibition is biphasic with an initially fast loss in activity followed by a slower second inhibition phase. Two half maximal inhibition times $IT_{50}$ from separate hyperbolic fits of the two phases (Fig. 6, B and C) were determined. MPO inhibition in the early phase (incubation times 0–5 min) leads to ~80% total inhibition with an $IT_{50}$ of 2.4 s, while inhibition up to 99.5% is only achieved after 2 h of incubation ($IT_{50} = 24$ min).

Inhibition efficiency of SPIN-aureus depends on the molecule size of substrate or ligand

The crystal structure of the complex, as well as in solution data from UV-visible and electronic CD spectroscopies...
SPIN—S. aureus’ inhibitory shield against myeloperoxidase

Table 3

| Protein          | Analyte          | $k_{on} \text{ [M}^{-1} \text{s}^{-1}]$ | $k_{off} \text{ [s}^{-1}]$ | $k_{on} \text{ [M}^{-1} \text{s}^{-1}]$ | $k_{off} \text{ [s}^{-1}]$ | $K_D \text{ [nM]}$ |
|------------------|------------------|-------------------------------------|---------------------------|-------------------------------------|---------------------------|------------------|
| SPIN-aureus      | native MPO       | 5.9 ± 2.4 × 10^4                    | 5.0 ± 1.0 × 10^-3         | 0.008 ± 0.001                      | 3.5 ± 2.4 × 10^-6        | 0.005 ± 0.006    |
| SPIN-aureus (22) | native MPO*      | 5.4 ± 10^6                         | 5.0 ± 10^-3              | 9.3                                 |                           |                  |
| SPIN-aureus (20) | native MPO*      | 20.1 ± 2.2 × 10^4                  | 3.2 ± 0.1 × 10^-3        | 15.9 ± 2.1                          |                           |                  |
| SPIN-aureus (22) | rec. MPO*        | 4.5 × 10^5                         | 5.2 × 10^-3              | 4.5 ± 1.4                           |                           | 11.8             |
| SPIN-truncated   | native MPO       | 1.1 ± 0.6 × 10^5                   | 1.6 ± 0.2 × 10^-2        | 21 ± 8                              |                           |                  |
| SPIN-truncated (22) | native MPO*     | 6.2 ± 10^5                         | 1.9 × 10^-2              | 29.8                                |                           |                  |
| SPIN-truncated (22) | rec. MPO*       | 4.3 × 10^5                         | 1.5 × 10^-2              | 35.1                                |                           |                  |

Abbreviations: BSA, bovine serum albumin.
The average and SD was calculated from three independent injection series, reported literature values with native or recombinant MPO immobilized by amine coupling.

(Fig. S2), clearly show that there are no significant direct interactions between the heme iron and residues of the N-terminal domain of SPIN-aureus. As outlined previously, we have found a large number of water molecules still present in the MPO substrate channel and heme pocket even when SPIN-aureus is bound. We also observed residual activity even after 2 h of incubation. In principle, the main biological substrates of MPO, that is, H_2O_2, chloride, bromide, and thiocyanate (SCN^−), as well as its reaction products, that is, (pseudo) hypohalous acids, are all relatively small, with Van der Waals radii close to that of water. This also applies to typical heme ligands, which might allow access of these molecules to the heme cavity even in the presence of SPIN-aureus. We therefore decided to test the impact of the molecular properties (i.e., charge, size, and geometry) of substrates and ligands on the accessibility to the heme iron. We analyzed the reaction of native MPO and MPO-SPIN-aureus (equimolar concentration, one-hour pre-incubation) with hydrogen peroxide and the heme ligands cyanide (HCN), nitrite (NO_2^−), and sulfide (HS^-/H_2S) by using conventional stopped-flow spectroscopy.

Hydrogen peroxide mediates the two-electron oxidation of ferric MPO to the redox intermediate Compound I. This rapid reaction represents the initial step of both the peroxidase and halogenation cycle of MPO and can easily be followed by the decrease in Soret absorbance at 430 nm (Fig. 7B) (28, 32). At pH 7.0, the apparent bimolecular rate constant of Compound I formation was determined to be 1.0 × 10^7 M^-1 s^-1 and 7.1 × 10^5 M^-1 s^-1 for native MPO and MPO-SPIN-aureus,

Figure 6. Inhibition of the bromination activity of human MPO by SPIN-aureus is time dependent. A, impact of the incubation time of MPO with SPIN-aureus on the residual bromination activity. The bromination activity was determined photometrically using the MCD assay, that is, following the decrease in absorbance at 290 nm after addition of 100 μM MPO-SPIN-aureus complex to 5 mM bromide and 150 μM H_2O_2 in 50 mM phosphate-citrate buffer pH 5. As a control bromination of MPO without SPIN and after incubation with SPIN-truncated were measured (i.e., 100% activity). B, determination of the half maximum inhibition time (IT_50) of the first phase (light brown cycles, incubation time up to 5 min). Black line: hyperbolic fit. C, determination of the half maximum inhibition time (IT_50) of the second phase (light brown cycles, prolonged incubation times from 5 min to 120 min). Black line: hyperbolic fit. All experiments were performed in triplicates and represented as mean (SD). MCD, monochlorodimedone; MPO, myeloperoxidase.
respectively (Fig. 7B). The presence of SPIN-aureus had no impact on the spectral transitions accompanying the oxidation of ferric MPO to Compound I (Fig. S4).

Binding of the low-spin ligands cyanide, nitrite, and sulfide can be followed by a red shift of the Soret maximum of high-spin MPO or MPO–SPIN-aureus to the Soret maximum of the respective low-spin complexes (28, 32). The obtained kinetic data clearly demonstrate that the limited accessibility due to the presence of SPIN-aureus is more pronounced with bigger ligands following the hierarchy sulfide < cyanide (<H₂O₂) < nitrite (Fig. 7A). The calculated association (k_on) and dissociation rates (k_off) for MPO and MPO–SPIN-aureus are summarized in Table 4. In all ligands, both k_on and the k_off are reduced to the same extent in the presence of SPIN-aureus. The degree of reduction is strictly dependent on the ligand size (native MPO: 100%): ~30% (sulfide) > 20% (cyanide) > 7% (H₂O₂) > 0.2% (nitrite). As a consequence, binding of SPIN-aureus had only a small impact on the respective K_D values (Table 4).

It has to be mentioned that, compared to cyanide or nitrite, sulfide binding to MPO is more complex because it acts as both ligand and reductant. It is a two-step mechanism that includes binding to native MPO followed by the formation of the ferrous protein (39). The overall spectral transitions accompanying this two-step reaction are similar with MPO and MPO–SPIN-aureus complex upon reaction with 2 mM hydrogen sulfide. Spectra are colored according to time after mixing (initial: black, up to 0.015 s: cyan, up to 200 s: gray, last spectrum: red). D, schematic representation of the reaction of the heme cofactor with hydrogen sulfide and time traces of the reaction of MPO and MPO–SPIN-aureus complex at 467 nm. MPO, myeloperoxidase.

**Figure 7. Compound I formation and ligand binding of MPO and MPO–SPIN-aureus.** A, molecular parameters of the tested ligands and substrates (hydrogen sulfide, cyanide, hydrogen peroxide, and nitrite) and the change in rate constants k_on, k_off, and k_HOOH relative to free MPO. B, compound I formation of MPO (green, single exponential fit in black) or MPO–SPIN-aureus (yellow, single exponential fit in red) upon addition of 10 μM followed at 430 nm and pH 7. C, spectral transitions of MPO and MPO–SPIN-aureus complex upon reaction with 2 mM hydrogen sulfide. D, schematic representation of the reaction of the heme cofactor with hydrogen sulfide and time traces of the reaction of MPO and MPO–SPIN-aureus complex at 467 nm. MPO, myeloperoxidase.
Finally, we determined the steady-state kinetic parameters for H$_2$O$_2$-dependent bromination and chlorination of monochlorodimedone (MCD) to test if the halogen binding site is perturbed by SPIN–S. aureus (Table 5). We found that while $k_{\text{cat}}/K_M$ for H$_2$O$_2$ and bromide were both reduced by a factor of $\sim$100, this was due to a reduction in the turnover number (MPO: $k_{\text{cat}} \sim 27$ s$^{-1}$, MPO–SPIN–S. aureus: $k_{\text{cat}} \sim 0.7$ s$^{-1}$). The same was observed for chloride (MPO: $k_{\text{cat}} \sim 26$ s$^{-1}$, MPO–SPIN–S. aureus: $k_{\text{cat}} \sim 0.5$ s$^{-1}$). As chloride has a smaller ionic radius than bromide, the inhibition by SPIN–S. aureus should be less efficient. Unfortunately, due to the low residual activity of the complex and the corresponding high uncertainty of the kinetic measurements, we can only speculate on this. Importantly, the presence of SPIN–S. aureus had no significant impact on the $K_D$ values. This supports the hypothesis that the binding sites of both hydrogen peroxide and bromide or chloride are not perturbed (Table 5).

Discussion

* S. aureus* is uniquely inventive in its fight to evade the human innate immune system. Among others, it has evolved the fascinating small protein SPIN–S. aureus that specifically binds and inhibits human MPO. This work aimed at understanding the MPO–SPIN–S. aureus complex in detail in order to elucidate the molecular mechanisms of how SPIN–S. aureus inhibits the generation of antimicrobial oxidants by MPO.

Despite its small size, SPIN–S. aureus has two distinct domains, with the N-terminal domain changing from an unstructured state to a $\beta$-sheet structure during complex formation with MPO. Conformational changes due to protein–protein interactions as well as “folding-upon-binding” mechanisms known for disordered protein regions or intrinsically disordered proteins are often seen in biology but mostly found at surface areas. The N-terminal peptide of SPIN–S. aureus, however, adopts a $\beta$-sheet structure that reaches $\sim$18 Å into the access channel of MPO, a fact not to be neglected in terms of folding. Folding of the N-terminal domain of SPIN–S. aureus is only induced after molecular recognition of MPO by the globular C-terminal domain that binds close to the substrate access channel. Thus, SPIN–S. aureus binding to MPO follows a distinct sequence of events, that is, (i) binding of the C-terminal recognition domain, followed by (ii) folding, and (iii) insertion of the N-terminal inhibitory domain (or vice versa). By comparing SPIN–S. aureus with SPIN–truncated as a model for the C-terminal domain, we were able to dissect the thermodynamics and kinetics of these reaction steps and demonstrate that the published $K_D$ for both native and recombinant MPO of 10 nM to 20 nM (which already placed the MPO–SPIN–S. aureus complex among high affinity protein complexes) is too high. This is because it only reflected the binding of the C-terminal globular domain and fully neglected the contribution of the N-terminal domain (Table 3). In this work, we estimate the $K_D$ value of the MPO–SPIN–S. aureus complex to be $\sim$10$^{-12}$ M or likely is even lower. This places the MPO–SPIN–S. aureus complex close to the highest affinity protein–protein heterocomplexes. For comparison, the streptavidin–biotin complex has a $K_D$ of 10$^{-15}$ M (40). In this case, it is due to a large conformational change and structural adaptation of the N-terminal domain of streptavidin and the concomitant low dissociation rate. Unfortunately, it still remains technically challenging to determine such high protein–protein affinities accurately as it is at or beyond the detection limit of most methods.

Binding of the small globular C-terminal domain to MPO is predominantly enthalpically driven ($\Delta H \sim -17$ kcal/mol) and results in a nanomolar affinity. The overall picomolar affinity is only achieved upon the comparably slow binding of the N-terminal domain ($k_{\text{on}} \sim 0.008$ M$^{-1}$s$^{-1}$) and governed by the practically unmeasurable dissociation rate ($k_{\text{off}} = 10^{-6}$ s$^{-1}$ or

### Table 4

Association and dissociation rate constants and $K_D$ values determined from pre–steady-state kinetics of ligand binding to MPO alone or in complex with SPIN–S. aureus

| Substrate/Ligand                  | $k_{\text{on}}$ [M$^{-1}$s$^{-1}$] | $k_{\text{off}}$ [s$^{-1}$] | $K_D$ [M]   | $k_{\text{on}}$ [M$^{-1}$s$^{-1}$] | $k_{\text{off}}$ [s$^{-1}$] | $K_D$ [M]   |
|----------------------------------|-----------------------------------|-----------------------------|-------------|-----------------------------------|-----------------------------|-------------|
| Cyanide                          | 2.4 × 10$^7$                      | 2.26                        | 0.95        | 5.2 × 10$^3$                      | 0.22                        | 0.43        |
| Hydrogen sulfide                 | n.d.                              | n.d.                        | 8.5         | 2.88                              | 33.9                        |
| Nitrite                          | 4.71                               | 0.011                       | 2.3 × 10$^3$| 1.62                              | 4.3 × 10$^{-3}$            | 2.6 × 10$^3$|
| Hydrogen peroxide                | $k_{\text{HOOH}}$ [M$^{-1}$s$^{-1}$] | 54.57                       | 18.33       | $k_{\text{HOOH}}$ [M$^{-1}$s$^{-1}$] | 0.15                        | 24.16       |

### Table 5

Kinetic parameter (mean ± SD) of MPO bromination and chlorination of MCD without or in complex with SPIN–S. aureus

| Kinetic constant | MPO | MPO–SPIN–S. aureus complex | MPO | MPO–SPIN–S. aureus complex |
|------------------|-----|---------------------------|-----|---------------------------|
| $V_{\text{max}}$ [µM Ms$^{-1}$] | 2.76 ± 0.85 | 0.07 ± 0.03 | 2.64 ± 0.43 | 0.05 ± 0.01 |
| $K_{\text{MCD}}$ [H$_2$O$_2$] [µM] | 26.67 ± 12.31 | 24.74 ± 6.28 | 64.04 ± 13.62 | 133.70 ± 15.52 |
| $K_{\text{MCD}}$ [Br/Cl] [µM] | 1.32 ± 0.78 × 10$^3$ | 2.44 ± 0.13 × 10$^5$ | 6.31 ± 1.32 × 10$^4$ | 12.87 ± 10.7 × 10$^4$ |
| $k_{\text{cat}}$ [s$^{-1}$] | 27.63 ± 8.60 | 0.69 ± 0.05 | 26.39 ± 4.31 | 0.50 ± 0.10 |
| $k_{\text{cat}}/k_{\text{cat}}$ [M$^{-1}$s$^{-1}$] | 1.04 ± 0.30 × 10$^6$ | 2.79 ± 0.96 × 10$^8$ | 4.14 ± 2.09 × 10$^5$ | 3.70 ± 3.94 × 10$^5$ |
| $k_{\text{cat}}/k_{\text{cat}}$ [M$^{-1}$s$^{-1}$] | 2.10 ± 1.20 × 10$^4$ | 2.83 ± 0.59 × 10$^5$ | 3.90 ± 0.1 × 10$^5$ | 6.4 ± 6.1 |
SPIN—S. aureus’ inhibitory shield against myeloperoxidase

even lower). Contrary to the previous published structures with monomeric recombinant MPO, we did not find significant electrostatic interactions between the N-terminal peptide and the heme enzyme. Instead, we identified the hydrophobic GLVL patch preceding the HDD motif that aligns along an equally hydrophobic area in the MPO access channel (Fig. 2). Desolvation of the access channel, which imposes an enthalpic penalty on binding, appears to be limited, as several water-containing cavities remain (Fig. 3). Of all well-coordinated crystal waters present in the X-ray structure of MPO-SPIN-truncated, only three are replaced by residues Gln37 and Asn38 of the N-terminal domain (Fig. 4). Interestingly, we observe an endotherm overlying the binding exotherm in ITC titration experiments only with SPIN-aureus (Fig. 5). Therefore, desolvation of the active site, which imposes an enthalpic penalty (ΔH > 0) is not compensated by the newly formed electrostatic interactions of the N terminus and HDD motif, as is the case during binding of the C-terminal domain. As binding of the N terminus is enthalpically disfavored, it must be entropically driven. The overall entropy change of this binding step includes both the entropy contributions of the proteins, that is, the cost of forming the MPO–SPIN complex and the conformational entropy cost upon folding of the N-terminal domain, together with solvent entropy changes due to desolvation of the interfaces. As the contribution of the proteins will be unfavorable, the increase in solvent entropy due to release of waters from the active site and from the N-terminal domain into the bulk solvent must be the driving force. An example for entropically driven binding is the class I MHC, where this effect was attributed to the hydrophobic nature of the peptide binding groove (41). Interestingly, a recent study on MHC suggested that interface waters in nonpolar cavities may have a higher entropy than bulk solvent (42). We also observe a number of water molecules in three cavities in the interface between the MPO active site and the N-terminal hydrophobic GLVL patch (Fig. 4), which may contribute in a similar manner.

The crystal structure, as well as in-solution spectroscopic data and the unaltered affinity for all tested ligands, show that SPIN-aureus does not directly interact with the heme iron. Additionally, the relatively small change in K_M determined for bromide oxidation suggest that the substrate-binding sites for H_2O_2 and halogens are not perturbed by SPIN-aureus. Thus, it is reasonable to assume that the peculiar redox properties of human MPO (28) and the reduction potentials of its catalytically relevant couples Fe(III)/Fe(II), Compound I/Fe(III), Compound I/Compound II, as well as Compound II/Fe(III) (43) are similar to those of S. aureus MPO. As a result, we can use the reduction potentials of these couples to estimate the electron transfer rates of the S. aureus MPO turnover. However, we found a strong correlation between the rates of electron transfer and the rates of protein turnover, which suggests that the electron transfer rates are limited by the rate of protein turnover. This is not consistent with the assumption that the electron transfer rates are limited by the rate of protein turnover.

Experimental procedures

Highly purified dimeric leukocyte MPO of a purity index (A_280/A_350) of at least 0.85 was purchased as lyophilized powder from Planta Natural Products (http://www.planta.at/) and the concentration was determined spectrophotometrically using ε_280 = 91,000 M⁻¹ cm⁻¹ per heme (47).

Cloning, expression, and purification of SPIN-aureus and SPIN-truncated

The codon optimized sequences for SPIN-aureus and SPIN-truncated were cloned in frame with an His-SUMO expression tag (backbone pSUMO) using the NEBuilder HiFi DNA Assembly Master Mix (NEB # E2621S); PCR amplification of all fragments was done using Q5 High-Fidelity 2x Master Mix (NEB # M0492L) and primers 3’-TGAGGCTTCACCCGCAA CAGATGGAGGTAAGGTTATTTCCGAGAAC-5’ and 3’-GGTCTGGGAAAATACCTTTACCTCCAATCTGTGGCGG TGAACCCTCA-5’ (SPIN-aureus), 3’-ACCGCGAACACATT GGAGGTGCCAATTTCGAGCAGC-5’ and 3’-GTTCT GGAAAATAACCTTTACCCATCTGTTGCCGGTGAGCC TCA-5’ (SPIN-truncated), and 3’-TGATGACTCGAGCAC CACCACCCAC-5’ and 3’-GTTCTGGGAATAAACT TTACCTCCAATCTGTTGCCGGTGAGCCCTCA-5’ (pSUMO backbone). For protein expression, all plasmids were transformed in Escherichia coli BL21 C41 and cultivated in LB
medium with kanamycin (final concentration 100 μg mL⁻¹) in shaker flasks (180 rpm, 37 °C) until OD₆₀₀ of ~0.6 to 0.8, followed by reduction of the temperature to 16 °C and induction using IPTG (final concentration 0.5 mM) and growth was continued at 16 °C and 180 rpm overnight.

Protein purification was performed by His-affinity purification, SUMO protease cleavage, and subsequent size-exclusion chromatography (SEC). For protein purification, cells were harvested by centrifugation (4 °C, 2700g, 20 min), resuspended in lysis buffer (binding buffer with 0.5% Triton X-100), and lysed by pulsed ultrasonication (1 s interval, 95% power, two times 3 min) on ice. The lysate was cleared by centrifugation (40 min, 38,720 × g power, two times 3 min) on ice. The lysate was cleared by centrifugation (40 min, 38,720 × g) and stored at −80 °C. For purification, the filtrate was loaded on a His-trap affinity column (5 ml, GE-Healthcare) pre-equilibrated with binding buffer (50 mM phosphate buffer, pH 7.4, 500 mM NaCl, 20 mM imidazole) on an ÄKTA or Bio-Rad system. The loaded column was washed with binding buffer and the protein was eluted by gradient elution with elution buffer (binding buffer with 500 mM imidazole). The fractions were concentrated by centrifugation (4500 × g, 4 °C) using a centrifugal filter unit (Amicon Ultra-15, Merck Millipore Ltd Tullagreen, Carrigtwohill Co Cork Ireland, 10 kDa cut-off). For cleavage, SUMO-protease was added at a molar ratio of 1:100 and incubated overnight at 4 °C. SEC was done using a HiLoad 26/600 Superdex 75 pg, GE Healthcare column equilibrated with 50 mM phosphate buffer pH 7.4. The collected fractions were pooled and concentrated to 1 to 2 mM and stored at −80 °C. Quality and monodispersity of the samples was verified by HPLC SEC (Superdex 75 26/200 GL (GE Healthcare)) coupled to multiangle light scattering and performed on a LC20 prominence HPLC system equipped with a refractive index detector RIF-10A, a photodiode array detector SPD-M20A (Shimadzu), and a multiangle light scattering Heleos Dawn+ with QELY detector (Wyatt Technology). The column was equipped with running buffer (PBS with 200 mM NaCl (pH 7.4)). Experiments were performed at a flow rate of 0.75 ml min⁻¹ and 25 °C and resulting data were analyzed using the ASTRA 6 software (Wyatt Technology). All samples were filtered through an Ultrafree-MC filter with a pore size of 0.1 μm (Merck Millipore) and 80 μg of protein was loaded per run.

SPR spectroscopy

SPR experiments were performed with the BiacoreT200 instrument (GE Healthcare). Human native MPO was biotinylated using the EZ-Link Sulfo-NHS-LC-Biotinylation kit (Thermo Fischer Scientific) and was immobilized on a CAP sensor chip (Cytiva) using the Biotin CAPture kit according to the manufacturer’s protocol (Cytiva). Biotinylated MPO in 50 mM phosphate buffer pH 7.4 was immobilized at a concentration of 2.5 ng/μl and a flow rate of 30 μl/min to a density of 500 resonance unit (RU) on flow cell 2. Flow cell 1 served as a reference surface. Single cycle kinetic experiments were performed using increasing concentrations of SPIN variants (between 2 and 64 nM) in running buffer (PBS, 0.05% Tween, 0.1% bovine serum albumin). Association times were 600 s, dissociation times were 600 s, and the flow rate was set to 30 μl/min. To determine the equilibrium dissociation constant K_D, equilibrium response units the data were either fitted to a 1:1 binding model (SPIN-truncated) or a two-state transition model (SPIN-aureus), which assumes a rebinding event. The R_max values were set to constant.

Differential scanning calorimetry

Differential scanning calorimetry (DSC) experiments were performed with a MicroCal PEAQ-DSC Automated (Malvern Panalytical Ltd) equipped with an autosampler for 96-well plates and controlled by the MicroCal PEAQ-DSC software (Malvern Panalytical Ltd) (cell volume: 130 μl). Samples were measured over a temperature range of 20 to 90 °C with a heating scan rate of 90 °C h⁻¹, cooled to 20 °C, and rescanned with the same settings. Two micromolar MPO alone or with 8 μM SPIN-aureus or SPIN-truncated were preincubated in buffer for 1 h at room temperature (RT). Buffer used were phosphate citrate buffer pH 5 or phosphate buffer pH 7.4, the rescan was used for baseline correction. Data analysis was performed with the MicroCal PEAQ-DSC software using a non–two-state equilibrium unfolding model.

ITC

ITC experiments were performed with a Microcal PEAQ-ITC Automated (Malvern Panalytical Ltd) equipped with an autosampler for 96-well plates and controlled by the MicroCal PEAQ-ITC software. All samples were diluted in 50 mM phosphate buffer pH 7.4 or pH 5, 10 μM MPO was in the cell, and 150 μM SPIN variants were used as titrant with 19 injections of 3 μl. Data analysis was performed using the Microcal PEAQ-ITC analysis software.

Steady state and pre–steady state kinetics

For all steady and transient state experiments with the MPO–SPIN complex, the samples were preincubated with equimolar or 2-fold excess of SPIN-aureus for at least 1 h at RT. The apparent steady-state kinetic parameters of MPO with and without inhibition by SPIN-aureus with H₂O₂ and bromide or chloride were determined by monitoring the decrease in absorbance of MCD at 290 nm (E₂₉₀ nm = 19.9 mM⁻¹ cm⁻¹) upon reaction of MCD with NaBr using a stirred cuvette and a scanning photometer (Cary 60 spectrophotometer, Agilent Technology). A reaction consisted of 1 ml of 50 mM phosphate–citrate buffer, pH 5.0, and 100 μM MCD, H₂O₂ concentrations were varied between 50 and 200 μM, bromide concentrations between 1 and 25 mM, and chloride between 50 and 200 mM. The reaction was initiated with 100 nM MPO or MPO–SPIN-aureus complex; all reactions were carried out in triplicates at
SPIN—S. aureus’ inhibitory shield against myeloperoxidase

25 °C. The initial rate of reaction ($v_o$) was obtained from the slope between 20 and 40 s; the kinetic parameters were calculated according to the following:

$$v_o = \frac{k_{cat}[AB]}{E_0 K_A[B] + K_B[A] + [AB]}$$

which describes a ping pong bi-bi steady-state kinetic mechanism with $E_0$ representing the enzyme concentration, $K_A$ and $K_B$ are the Michaelis constants for bromide (A) and $H_2O_2$ (B), and $k_{cat}$ is the turnover number at saturating concentration. Time dependency of the inhibition of MPO was determined by measuring the MCD activity after defined incubation at RT (time points 5 min to 2 h) or by addition of SPIN-aureus to MPO after initiation of the reaction (time points 10 s to 1 min) and determining the change in MCD oxidation in 3 s increments at specific time points.

All transient state experiments were conducted with a stopped-flow apparatus (SX-18MV or pi-star equipped with diode array detector or a monochromator) from Applied Photophysics. All measurements were performed at 25 °C. For single wavelength measurements, a minimum of three repeats were performed for each ligand or substrate concentration. Typically, 2 μM MPO or MPO–SPIN-aureus complex (5 mM phosphate buffer, pH 7.0) was mixed with at least a 5-fold excess of ligand or substrate in 100 mM buffer (phosphate buffer pH 7 or 7.4 or phosphate-citrate buffer pH 5). The reaction was monitored at distinct wavelength and pH (cyanide: 455 nm, pH 7, nitrite: 455 nm, pH 5, $H_2O_2$: 430 nm, pH 7).

Crystallization and structure refinement

Native human MPO (10 mg ml⁻¹) was incubated for 1 h or overnight with stoichiometric concentrations of SPIN-aureus or SPIN-truncated in 50 mM phosphate buffer pH 7. Crystallization experiments were performed using SWISSCI MRC 3-well crystallization plates (Molecular Dimensions) adopting the vapor diffusion method. Crystallization drops

Table 6

| Data collection and refinement statistics for SPIN structures |
|---------------------|---------------------|---------------------|---------------------|
|                     | 7QZR                | –                   | 7Z53                | –                   |
| PDB entry           | autoPROC/STARANISO  | autoPROC/STARANISO  | autoPROC/STARANISO  | autoPROC/STARANISO  |
| Synchrotron         | ESRF (Grenoble – France) | ESRF (Grenoble – France) |
| Wavelength (Å)      | ID23–2              | 0.873               | ID23–2              | 0.873               |
| Resolution range (Å) | 19.81–2.18 (2.33–2.18) | 19.81–2.28 (2.32–2.28) | 166.56–2.28 (2.44–2.28) | 166.56–2.41 (2.46–2.41) |
| Space group         | P 4₁ 2₁ 2           | P 4₁ 2₁ 2           | P 2₁ 2           | P 2₁ 2           |
| Cell a, b, c (Å)    | 112.09, 112.09, 249.95 | 90, 90, 90         | 257.02, 157.20, 166.56 | 90, 90, 90         |
| Data collection     | 5,25,797 (18,209) | 5,51,475 (19,365) | 1,497,194 (63,791) | 15,93,333 (81,424) |
| Multiplicity        | 68,945 (34,988) | 72,785 (35,51) | 2,42,698 (98,994) | 2,57,787 (12,769) |
| Completeness spherical (%) | 82.4 (23.5) | 99.9 (99.9) | 79.3 (17.9) | 99.9 (100.0) |
| Completeness ellipsoidal (%) | 92.9 (48.4) | 93.1 (58.8) | 4.1 (1.5) | 4.0 (1.2) |
| Rmerge (%)          | 6.2 (1.6) | 5.9 (1.1) | 36.2 (16.9) | 38.2 (21.1) |
| CC1/2 (%)           | 98.8 (51.2) | 98.8 (38.9) | 96.4 (49.4) | 96.4 (38.3) |

Refinement

| Rmerge (%)¹ | 20.38 (27.12) | 20.28 (27.12) |
| Rfree (%)² | 23.74 (31.63) | 23.74 (31.63) |
| Number of non-H atoms | 10,267 | 10,267 |
| Protein | 760 | 760 |
| Ligands | 526 | 526 |
| Waters | 0.09 | 0.09 |
| RMSD bonds (Å) | 0.98 | 0.98 |
| Ramachandran plot | Most favored (%) | 97.22 | 97.22 |
| Outliers (%) | 0.24 | 0.24 |
| Rotamer outliers (%) | 0.89 | 0.89 |
| Clash score⁶ | 1.23 | 1.23 |
| MolProbity score⁶ | 0.99 | 0.99 |
| B-factors (Å²) | Protein | 32.98 | 32.98 |
| Ligands/ions | 39.03 | 39.03 |
| Waters | 29.80 | 29.80 |

¹ Information in parenthesis refers to the last resolution shell.
² Rmerge = Σ<|h|I(h)−<|h|I(h)>|Σ<|h|I(h)> <h>ₙ, where I(h) is the n__th observation of reflection h and <h>ₙ.
³ C₁/2 = ΣΣ|Fobs|²/Fcalc|²|ΣΣ|Fobs|²/Fcalc|², where Fobs and Fcalc are the observed and calculated structure factors for reflection h, respectively.
⁴ Rmerge = Σ|Fobs|²−|Fcalc|²|ΣΣ|Fobs|²/Fcalc|², where Fobs and Fcalc are the observed and calculated structure factors for reflection h, respectively.
⁵ Rfree was calculated the same way as R_merget but using only 5% of the reflections which were selected randomly and omitted from refinement.
⁶ RMSD, root mean square deviation.
were set using a Mosquito LCP (TTP Labtech, Melbourne Science Park). The MPO–SPIN-aureus complex crystallized in 8% (w/v) PEG 20000, 0.1 M BICINE pH 9, 0.5% (V/V) Dioxane. The SPIN-truncated complex crystallized in 8.5% (w/v) PEG 20000, 0.1 M BICINE pH 9, 2.0% (V/V) Dioxane. The reservoir was filled with 40 μl crystallization solution. Single drops were set up with a ratio of 100:150, 150:150, and 200:150 protein:nl crystallization. Crystallization plates were sealed and stored at 22 °C. For cryo-protection, the crystallization conditions were supplemented with 25% (V/V) flash-vitrified in liquid nitrogen. Datasets were collected at beamline ID23-2 (48) and at ESRF (European Radiation Synchrotron Facility, Grenoble, France). Data for MPO–SPIN-aureus and MPO-SPIN-truncated cocrystals (PDB ID: 7QZR and 7Z53) were indexed and integrated with XDS (49), the space group was determined with POINTLESS (50) and scaled with AIMLESS (51), all within the autoPROC data processing pipeline (52). Rfree flags for all datasets were created at this stage corresponding to 5% of the measured reflections for each dataset. STARANISO (53) was used for anisotropic cut-off of the merged intensity data. The phase problem for the MPO–SPIN-aureus structure was solved by molecular replacement using Phaser-MR (54) taking PDB structure 5UZU of recombinant MPO in complex with SPIN-aureus. The phase problem for the MPO–SPIN-truncated cocrystals was solved by molecular replacement using 7QZR as a search model and MORDA (55), followed by automated model building with ARP/wARP (56, 57). Data collection and processing statistics are listed in Table 6. Matthews coefficient calculations (58) indicated the presence of one tetramer per asymmetric unit in the MPO–SPIN-aureus cocrystals and four tetramers in the MPO-SPIN-truncated cocrystal. The models were further improved by iterative model building using maximum likelihood refinement with phenix.refine (59) (with flags set for individual b-factor refinement, TLS, and occupancy refinement), manual model building by SSM superpositioning (60) and COOT (61). Models were further optimized by automated model building using PBD-redo (62). Final refinement rounds were performed with BUSTER (63, 64) (correcting for wavelength and form factor, with flags set TLS refinement and occupancy refinement). Grade (65) was used to generate optimized restraints for the heme b cofactor and refinement was carried out with hydrogen atoms (at zero occupancy) added to the model using aB_hydrogenate within the BUSTER-TNT package. The final models were validated with Molprobity (66). Figures were prepared with PYMOL (http://www.pymol.org/).

Chemicalize was used for prediction of Van der Waals volume (November 2021) shown in Figure 5, https://chemicalize.com developed by ChemAxon (http://www.chemaxon.com), UCSF Chimera (67) and PISA (68) were used for prediction of hydrogen bonds, salt bridges, and analysis of the interfaces. In UCSF Chimera hydrogen bonds parameters were set to 0.6 Å distance and 45° angle deviation.

Data availability

The data for the described crystal structures is available under the PDB accession codes 7QZR and 7Z53, all other data is contained within the article.

Supporting information—This article contains supporting information.

Acknowledgments—The study was funded by the FWF Project P33997.

Author contributions—V. P. conceptualization; J. A. B. formal analysis; U. L. investigation; P. G. F. resources; V. P. writing—original draft; P. G. F., S. H., and C. O. writing—review & editing; V. P. visualization; V. P. supervision; C. O. and V. P. funding acquisition.

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

Abbreviations—The abbreviations used are: DSC, differential scanning calorimetry; ITC, isothermal titration calorimetry; MCD, monochlorodimedone; MPO, myeloperoxidase; PDB, Protein Data Bank; SEC, size-exclusion chromatography; SPR, surface plasmon resonance.

References

1. Nauseef, W. M. (2007) How human neutrophils kill and degrade microbes: an integrated view. ImmunoL Rev. 219, 88–102. https://doi.org/10.1111/j.1600-065X.2007.00550.x
2. Nauseef, W. M., and Borregaard, N. (2014) Neutrophils at work. Nat. Immunol. 15, 602–611. https://doi.org/10.1038/ni.2921
3. Klebanoff, S. I. (2005) Myeloperoxidase: friend and foe. J. Leukoc. Biol. 77, 598–625. https://doi.org/10.1189/jlb.1204697
4. Kettle, A. J., and Winterbourn, C. C. (1997) Myeloperoxidase: a key regulator of neutrophil oxidant production. Redox Rep. 3, 3–15. https://doi.org/10.1056/13510002.1997.11747085
5. van Dalen, C. J., Whitehouse, M. W., Winterbourn, C. C., and Kettle, A. J. (1997) Thiocyanate and chloride as competing substrates for myeloperoxidase. Biochem. J. 327, 487–492. https://doi.org/10.1042/bj3270487
6. Winterbourn, C. C., Kettle, A. J., and Hampton, M. B. (2016) Reactive oxygen species and neutrophil function. Annu. Rev. Biochem. 85, 765–792. https://doi.org/10.1146/annurev-biochem-060815-014442
7. Senthilmohan, R., and Kettle, A. J. (2006) Bromination and chlorination reactions of myeloperoxidase at physiological concentrations of bromide and chloride. Arch. Biochem. Biophys. 445, 235–244. https://doi.org/10.1016/j.abb.2005.07.005
8. Burner, U., Furtmüller, P. G., Kettle, A. I., Koppenol, W. H., and Obinger, C. (2000) Mechanism of reaction of myeloperoxidase with nitrite. J. Biol. Chem. 275, 20597–20601
9. Garai, D., Rios-Gonzalez, B. B., Furtmüller, P. G., Fukuto, J. M., Xian, M., Lopez-Garriga, J., et al. (2017) Mechanisms of myeloperoxidase catalyzed oxidation of H2S by H2O2 or O2 to produce potent protein Cys-poly-sulphide-inducing species. Free Radic. Biol. Med. 113, 551–563. https://doi.org/10.1016/j.freeradbiomed.2017.10.384
10. Eiserich, J. P., Hristova, M., Cross, C. E., Jones, A. D., Freeman, B. A., Halliwell, B., et al. (1998) Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. Nature 391, 393–397. https://doi.org/10.1038/34923
11. Van der Veen, B. S., de Winther, M. P., and Heeringa, P. (2009) Myeloperoxidase: molecular mechanisms of action and their relevance to human health and disease. Antioxid. Redox Signal. 11, 2899–2937. https://doi.org/10.1089/ars.2009.2538
21. Ploscariu, N. T., de Jong, N. W. M., van Kessel, K. P. M., van Strijp, J. A. (2015) Structure of the low-spin form of mature human myeloperoxidase by X-ray crystallography. *Arch. Biochem. Biophys.* 645, 1–11.

22. de Jong, N. W. M., Ploscariu, N. T., Ramyar, K. X., Garcia, B. L., Herrera, A. I., Prakash, O., et al. (2018) A structurally dynamic N-terminal region drives function of the staphylococcal peroxidase inhibitor (SPIN). *J. Biol. Chem.* 293, 2260–2271.

23. Dunker, A. K., Lawson, J. D., Brown, C. J., Williams, R. M., Romero, P., Oh, J. S., et al. (2001) Intrinsically disordered protein. *J. Mol. Graph. Model.* 19, 26–59. https://doi.org/10.1016/s0939-3263(00)00138-8.

24. Receiveur-Brechet, V., Bourhis, J. M., Uversky, V. N., Canard, B., and Longhi, S. (2006) Assessing protein disorder and induced folding. *Proteins* 62, 24–45. https://doi.org/10.1002/prot.20750.

25. Bourhis, J. M., Johansson, K., Receiveur-Brechet, V., Oldfield, C. J., Dunkor, K. A., Canard, B., et al. (2004) The C-terminal domain of measles virus nucleoprotein belongs to the class of intrinsically disordered proteins that fold upon binding to their physiological partner. *Virus Res.* 99, 157–167. https://doi.org/10.1016/j.virusres.2003.11.007.

26. Janin, J. (1995) Principles of protein-protein recognition from structure to thermodynamics. *Biochimie* 77, 497–505. https://doi.org/10.1016/0300-9849(95)81166-1.

27. Erijman, A., Rosenthal, E., and Shifman, J. M. (2014) How structure defines affinity in protein-protein interactions. *PLoS One* 9, e910085. https://doi.org/10.1371/journal.pone.0110085.

28. Arnhold, J., Monzani, E., Furtmuller, P. G., Zederbauer, M., Casella, L., and Obinger, C. (2006) Kinetics and thermodynamics of halide and nitrite oxidation by mammalian heme peroxidases. *Eur. J. Inorg. Chem.* 2006, 3801–3811. https://doi.org/10.1002/ejic.200600436.

29. Grishkovskaya, I., Paumann-Page, M., Tscheließnig, R., Stamperl, I., Hofbauer, S., Soudi, M., et al. (2017) Structure of human promyeloperoxidase (proMPO) and the role of the propeptide in processing and maturation. *J. Biol. Chem.* 292, 8244–8261.

30. Fiedler, T. J., Davey, C. A., and Fenna, R. E. (2000) X-ray crystal structure and characterization of halide-binding sites of human myeloperoxidase at 1.8 A resolution. *J. Biol. Chem.* 275, 11964–11971.

31. Carpena, X., Vidossich, P., Schroeetter, K., Calisto, B. M., Banerjee, S., Stamperl, J., et al. (2009) Essential role of proximal histidine-asparagine interaction in mammalian peroxidases. *J. Biol. Chem.* 284, 25929–25937.

32. Furtmuller, P. G., Zederbauer, M., Jantschko, W., Helm, J., Bogner, M., Jakopitsch, C., et al. (2006) Active site structure and catalytic mechanisms of human peroxidases. *Arch. Biochem. Biophys.* 445, 199–213. https://doi.org/10.1016/j.abb.2005.09.017.

33. Van Antwerpen, P., Slomianny, M.-C., Boudjeltia, K. Z., Delporte, C., Faid, V., Calay, D., et al. (2010) Glycosylation pattern of mature dimeric leukocyte and recombinant monomeric myeloperoxidase: glycosylation is required for optimal enzymatic activity. *J. Biol. Chem.* 285, 16351–16359.

34. Chovancova, E., Pavelka, A., Benes, P., Strnad, O., Brezovsky, J., Kozlikova, K., et al. (2012) Caver 3.0: a tool for the analysis of transport pathways in dynamic protein structures. *PLoS Comput. Biol.* 8, e1002708. https://doi.org/10.1371/journal.pcbi.1002708.

35. Pfanzagl, V., Beale, J. H., Michlits, H., Schmidt, D., Gabler, T., Obinger, C., et al. (2020) X-ray-induced photoreduction of heme metal centers rapidly induces active-site perturbations in a protein-independent manner. *J. Biol. Chem.* 295, 13488–13501.

36. Ishigami, I., Russi, S., Cohen, A., Yeh, S.-R., and Rousseau, D. L. (2022) Temperature-dependent structural transition following X-ray-induced metal center reduction in oxidized cytochrome c oxidase. *J. Biol. Chem.* 298, 10179.

37. Pace, C. N., Fu, H., Fryar, K. L., Landua, J., Trevino, S. R., Shirley, B. A., et al. (2011) Contribution of hydrophobic interactions to protein stability. *J. Mol. Biol.* 408, 514–528. https://doi.org/10.1016/j.jmb.2011.02.053.

38. Banerjee, S., Stamperl, J., Furtmuller, P. G., and Obinger, C. (2011) Conformational and thermal stability of mature dimeric human myeloperoxidase and a recombinant monomeric form from CHO cells. *Biochim. Biophys. Acta* 1814, 375–387. https://doi.org/10.1016/j.bbapap.2010.09.015.

39. Palinkas, Z., Furtmuller, P. G., Nagy, A., Jakopitsch, C., Pirker, K. F., Magierowski, M., et al. (2015) Interactions of hydrogen sulfide with myeloperoxidase. *Br. J. Pharmacol.* 172, 1516–1532. https://doi.org/10.1111/bph.12769.

40. Green, N. M. (1963) Avidin. 3. The nature of the biotin-binding site. *Biochem. J.* 89, 599–609. https://doi.org/10.1042/bj089059.

41. Binz, A. K., Rodriguez, R. C., Biddison, W. E., and Baker, B. M. (2003) Thermodynamic and kinetic analysis of a peptide-class I MHC interaction highlights the noncovalent nature and conformational dynamics of the class I heterotrimer. *Biochemistry* 42, 4954–4961. https://doi.org/10.1021/bi03077m.

42. Petrone, G., Bella, M., Zederbauer, M., Furtmüller, P. G., Sola, M., and Obinger, C. (2006) Redox thermodynamics of the Fe(III)/Fe(II) couple of human myeloperoxidase in its high-spin and low-spin forms. *Biochemistry* 45, 12750–12755. https://doi.org/10.1021/bi061647k.

43. Furtmüller, P. G., Arnhold, J., Jantschko, W., Pichler, H., and Obinger, C. (2003) Redox properties of the couples compound I/compound II and compound II/native enzyme of human myeloperoxidase. *Biochem. Biophys. Res Commun* 301, 551–557. https://doi.org/10.1016/s0006-291x(02)03075-9.

44. Arnhold, J., Furtmüller, P. G., and Obinger, C. (2003) Redox properties of myeloperoxidase. *Redox Rep* 8, 179–186. https://doi.org/10.1117/15.1310003235025664.
**SPIN—S. aureus’ inhibitory shield against myeloperoxidase**

46. Galijasevic, S. (2019) The development of myeloperoxidase inhibitors. *Bioorg. Med. Chem. Lett.* 29, 1–7. https://doi.org/10.1016/j.bmcl.2018.11.031

47. Furtmuller, P. G., Burner, U., and Obinger, C. (1998) Reaction of myeloperoxidase compound I with chloride, bromide, iodide, and thiocyanate. *Biochemistry* 37, 17923–17930. https://doi.org/10.1021/bi9818772

48. Flot, D., Mairs, T., Giraud, T., Guijarro, M., Lesourd, M., Rey, V., et al. (2010) The ID23-2 structural biology microfocus beamline at the ESRF. *J. Synchrotron Radiat.* 17, 107–118. https://doi.org/10.1107/S0909049509041168

49. Kabash, W. (2010) Xds. *Acta Crystallogr. D* 66, 125–132. https://doi.org/10.1107/S090744491003982X

50. Evans, P. R. (2011) An introduction to data reduction: space-group determination, scaling and intensity statistics. *Acta Crystallogr. D* 67, 282–292. https://doi.org/10.1107/S0907444911003982X

51. Evans, P. R., and Murshudov, G. N. (2013) How good are my data and what is the resolution? *Acta Crystallogr. D* 69, 1204–1214. https://doi.org/10.1107/S0907444913000461

52. Vonrhein, C., Flensburg, C., Keller, P., Sharff, A., Paciorek, W., et al. (2011) Data processing and analysis with the autoPROC toolbox. *Acta Crystallogr. D* 67, 293–302. https://doi.org/10.1107/S0907444911007773

53. Vonrhein, C., Tickle, I. J., Flensburg, C., Keller, P., Paciorek, W., Sharff, A., et al. (2018) Advances in automated data analysis and processing within autoPROC, combined with improved characterisation, mitigation and visualisation of the anisotropy of diffraction limits using STARANISO. *Acta Crystallogr. D*. https://doi.org/10.1107/S090744491809640x

54. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) Phaser crystallographic software. *J. Appl. Crystallogr.* 40, 658–674. https://doi.org/10.1107/S0021889807021206

55. Vagin, A., and Lebedev, A. (2015) MoRDa, an automatic molecular replacement pipeline. *Acta Crystallogr. A.* A71, S19. https://doi.org/10.1107/S20532733150099672

56. Chojojewski, G., Choudhury, G., Haueter, P., Sobolev, E., Pereira, J., Oezugurel, U., et al. (2020) The use of local structural similarity of distant homologues for crystallographic model building from a molecular-replacement solution. *Acta Crystallogr. D* 76, 248–260. https://doi.org/10.1107/S20532733150099672

57. Murshudov, G. N., Skubak, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., et al. (2011) REFMAC5 for the refinement of macromolecular crystal structures. *Acta Crystallogr. D* 67, 355–367. https://doi.org/10.1107/S090744491001314

58. Kantardjieff, K. A., and Rupp, B. (2003) Matthews coefficient probabilities: improved estimates for unit cell contents of proteins, DNA, and protein-nucleic acid complex crystals. *Protein Sci.* 12, 1865–1871. https://doi.org/10.1101/ps.035053

59. Adams, P. D., Afonine, P. V., Bunkoczi, G., Chen, V. B., Davis, I. W., Echols, N., et al. (2010) Phenix: a comprehensive python-based system for macromolecular structure solution. *Acta Crystallogr. D* 66, 213–221. https://doi.org/10.1107/S0907444909052925

60. Krissinel, E., and Henrick, K. (2004) Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions. *Acta Crystallogr. D* 60, 2256–2268. https://doi.org/10.1107/S090744490426460

61. Emsley, P., and Cowtan, K. (2004) Coot: model-building tools for molecular graphics. *Acta Crystallogr. D* 60, 2126–2132. https://doi.org/10.1107/S0907444904019158

62. Joosten, R. P., Joosten, K., Murshudov, G. N., and Perrakis, A. (2012) PDB_REDO: constructive validation, more than just looking for errors. *Acta Crystallogr. D* 68, 484–496. https://doi.org/10.1107/S0907444911054515

63. Bricogne, G., Blanc, E., Brandl, M., Flensburg, C., Keller, P., Paciorek, W., et al. (2017) *BLUSTER Version 2.10.4*, Global Phasing Ltd, Cambridge, United Kingdom

64. Smart, O. S., Womack, T. O., Flensburg, C., Keller, P., Paciorek, W., Sharff, A., et al. (2012) Exploiting structure similarity in refinement: automated NCS and target-structure restraints in BUSTER. *Acta Crystallogr. D* 68, 368–380. https://doi.org/10.1107/S0907444911056058

65. Smart, O. S., Womack, T. O., Sharff, A., Flensburg, C., Keller, P., Paciorek, W., et al. (2021) *graece, Version 1.2.20*, Global Phasing Ltd., Cambridge, United Kingdom

66. Williams, C. J., Headd, J. J., Moriarty, N. W., Prisant, M. G., Videau, L. L., Dein, L. N., et al. (2018) MolProbity: more and better reference data for improved all-atom structure validation. *Protein Sci.* 27, 293–315. https://doi.org/10.1002/pro.3330

67. Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., et al. (2004) UCSF Chimera—a visualization system for exploratory research and analysis. *J. Comput. Chem.* 25, 1605–1612. https://doi.org/10.1002/jcc.20084

68. Krissinel, E., and Henrick, K. (2007) Inference of macromolecular assemblies from crystalline state. *J. Mol. Biol.* 372, 774–797. https://doi.org/10.1016/j.jmb.2007.05.022