Structure and activity of the DHNA Coenzyme-A Thioesterase from *Staphylococcus aureus* providing insights for innovative drug development

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Humanity is facing an increasing health threat caused by a variety of multidrug resistant bacteria. Within this scenario, *Staphylococcus aureus*, in particular methicillin resistant *S. aureus* (MRSA), is responsible for a number of hospital-acquired bacterial infections. The emergence of microbial antibiotic resistance urgently requires the identification of new and innovative strategies to treat antibiotic resistant microorganisms. In this context, structure and function analysis of potential drug targets in metabolic pathways vital for bacteria endurance, such as the vitamin K₂ synthesis pathway, becomes interesting. We have solved and refined the crystal structure of the *S. aureus* DHNA thioesterase (*Sa*DHNA), a key enzyme in the vitamin K₂ pathway. The crystallographic structure in combination with small angle X-ray solution scattering data revealed a functional tetramer of *Sa*DHNA. Complementary activity assays of *Sa*DHNA indicated a preference for hydrolysing long acyl chains. Site-directed mutagenesis of *Sa*DHNA confirmed the functional importance of Asp16 and Glu31 for thioesterase activity and substrate binding at the putative active site, respectively. Docking studies were performed and rational designed peptides were synthesized and tested for *Sa*DHNA inhibition activity. The high-resolution structure of *Sa*DHNA and complementary information about substrate binding will support future drug discovery and design investigations to inhibit the vitamin K₂ synthesis pathway.

The increase in hospital-acquired infections (HAI) is one of the major concerns for the global health system. Bacterial infections acquired during patient hospitalization contribute not only to significant mortality, but also usually require additional therapies, increasing even more the financial burden for the healthcare systems¹. Among bacterial infections involved in HAI, *Staphylococcus aureus* is a leading pathogen found in hospitals causing serious bacteraemia leading to sepsis and to infection of internal organs, such as the heart, lungs and joints, usually occurring after invasive procedures like introducing implantable medical devices².

The first successful treatment of *S. aureus* infections, in the 1940’s, involved mainly the administration of β-lactam antibiotics like penicillin G. Targeting the bacterial enzymes of the cell wall biosynthesis, called penicillin-binding proteins (PBPs), the inhibition of the PBPs induced by β-lactam antibiotics interferes with the cross-linking of peptidoglycan, making the cell wall mechanically fragile and the cell to perish³. However, since penicillin resistance was first discovered in the early 1950s due to the production of specific β-lactamases by the acquisition of the *blaZ* gene that is capable of hydrolyzing β-lactam antibiotics, the phenomenon of drug resistance has been observed for an increasing number of Gram-negative and Gram-positive pathogens⁴.

In the early 1960s, the structure of natural penicillin has been modified allowing the development of a new antibiotic named methicillin, a semi-synthetic penicillinase-resistant β-lactam antimicrobial to replace the

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conventional penicillin treatment. Methicillin was noted due to inactivation resistance by penicillinases and activity against several penicillinase-producing staphylococci. However, the widespread use of methicillin has led to the emergence of Methicillin resistant *S. aureus* (MRSA) by acquisition of a non-active gene encoding a PBP2a which has a lower affinity for β-lactam antibiotics. Nowadays, *S. aureus* exhibits resistance not only to methicillin but also to a vast number of β-lactam antimicrobial agents, including carbapenems and cephalosporins, until the most recent antibiotic such as linezolid. The increase threatening of multidrug resistant *S. aureus*, in particular MRSA, unfortunately has narrowed therapy options substantially, making the treatment of MRSA infections even more complex.

Further, *S. aureus* has a particular ability to respond quickly to new antibiotic treatments. During antimicrobial treatment, the selective pressure caused by antibiotics can induce the generation of subpopulations of *S. aureus* with slower growth compared to the wild variant, called small variant colonies (SCVs). The presence of SCVs is often associated with persistent and recurrent infections, which are difficult to diagnose and treat with antimicrobials. Studies have shown that SCVs are associated with disturbances in the electron transport chain caused by a class of antibiotics that acts on polypeptide synthesis. Deficiency in the respiratory chain can reduce the influx of antibiotics by the bacteria and, consequently their susceptibility to antimicrobial treatment. The most frequent SCV phenotypes are mutations in genes involved in the biosynthesis of components required for the electron transport chain, reducing the emergence of new therapies for MRSA.

In order to overcome bacterial resistance identification of novel drug targets as well as the development of innovative antibiotics with high specificity and effectiveness is needed. The latest drug discovery investigations are focusing on obtaining structural knowledge about enzymes involved in the bacterial cell metabolism which are critical and vital for bacteria endurance. The lipid-soluble compound vitamin K₃ (menaquinone), with about 85–90% almost entirely located in the bacterial membrane, is an important electron carrying component in the membrane-bound complexes of the electron-transport chain (ETC). Since the cellular respiration in humans does not involve the use of menaquinone and the ETC pathway in bacteria is an indispensable and essential component for ATP production, bacterial electron-transport enzymes have shown a substantial potential for novel drug development investigations. Humans entirely depend on the uptake of vitamin K which is known to improve health in cardiovascular disease, chronic kidney disease and bone metabolism.

In bacteria, several enzymes involved in menaquinone de novo biosynthesis have already been analysed, e.g. *Escherichia coli* MenD, *Mycobacterium tuberculosis* MenB and *Pseudomonas* sp. 4-Hydroxybenzoyl-CoA thioresterase. In a recent study by Smith et al. (2021) the MenI (DNHA Thioresterase) gene was deleted in *Listeria monocytogenes* and data obtained showed clearly the vital contribution of DHNA-CoA for the bacterial replication in vitro, ex vivo, and in vivo, confirming the specific role of DHNA in promoting bacterial survival in the cytosol of macrophages and demonstrating the need of the DHNA for menaquinone biosynthesis, cytosolic survival, and virulence.

Based on structural information of *E. coli* MenE, Matarlo et al. (2015) designed several o-succinylbenzoyl (OSB) secondary amine analogues (OSB-AMS) with high specificity and showing antimicrobial activity. Furthermore, the effect of these OSB-AMS on vitamin K levels of *S. aureus* can be assigned to direct interference of *S. aureus* with vitamin K which is known to improve health in cardiovascular disease, chronic kidney disease and bone metabolism.

In this context we solved and refined the tetrameric structure of 1,4-dihydroxy-2-naphthoyl coenzyme A thioresterase—SaDHNA (E.C. 3.1.2.28) from *S. aureus* to 1.3 Å resolution, which in combination with site-directed mutagenesis allowed the identification of essential residues for thioresterase activity and substrate binding.

**Results**

**Three-dimensional structure of the tetrameric SaDHNA.** Crystals of the native protein belong to the space group *P*₂₁, with unit cell dimensions of *a* = 53.61, *b* = 90.66, *c* = 75.38 Å, *α* = *γ* = 90° and *β* = 92° (°) with four molecules in the asymmetric unit. The calculated Matthews coefficient is 2.4 Å³ Da⁻¹, corresponding to an approximate solvent content of 48%. The SaDHNA monomer comprises 155 amino acids with a corresponding molecular weight of 18.1 kDa and a theoretical pl of 5.5. The structure of the SaDHNA tetramer was refined to *R*_work/*R*_free (%) values of 17.8/19.8, respectively. Data collection statistics and final refinement parameters are summarized in Table 1. Residues 156–165, corresponding to the strep-tag II at the C-terminus (SA-WSHPQFEK), as well as the residues DGGDSL at the C-terminus of chain B and D were disordered and not included in the final model. The atom coordinates of SaDHNA were deposited in the Protein Data Bank with pdb code 6FDG.

The overall structure of the SaDHNA monomer and its topology are illustrated in Fig. 1a,b, showing that SaDHNA has a high content of compact secondary structure elements (26% α-helix and 32% β-strand) with a relatively extended five-stranded antiparallel β-sheet element and one parallel β-sheet segment in the sequential order 8–1–4–5–6–3, involving the residues Val146-Ile148 (β8), Met1-Ala10 (β1), Glu72-Ser84 (β4), Arg87-Asn96 (β5), Glu100-Ile110 (β6), Pro56-Lys65 (β3) and two short antiparallel β-sheets containing the amino acid residues Gly52-Ile54 (β2) and Ile112-Glu. The β-sheets β5 and β6 are interrupted by four β-bulges at Val89, Ile94, Asn96 and Glu108. All β-sheets wrap around the four α-helices formed by residues Tyr26-Gly42 (α1), Ser44-Glu51 (α2), Arg121-Phe126 (α3) and Pro127-Glu142 (α4) and two short α-helices comprising the residues Arg11-Glu14 and (I1) and Try22-Asn25 (I2) connected by a β-turn. The turns connecting the β-strands are type I (Ser84-Arg87, 2022) 12:4313 | https://doi.org/10.1038/s41598-022-08281-2
Asn96-Gly99) and type IV (Phe69-Glu72, Lys113-Trr116). In addition, two β-hairpins between β-4/β-5 (class 2:4) and β-5/β-6 (class 3:5) and a γ-turn Ile152-Ser154 after the last β8 strand are observed within the structure.

Two monomers of SaDHNA assemble to form a homodimer by a network of nine hydrogen bonds between strand β3 of chains A or C with the adjacent β3 strand of chains B or D, respectively, producing an overall 10-stranded antiparallel β-sheet motif involving residues Thr58-Tyr64, Asp59-Asn63, Leu60-Val62, Asn61-Asn61, Tyr64-Thr58, Asn63-Asp59 and Val62-Leu60. Three further H-bonds stabilize α2, α1 and η2, involving the residue pairs Glu49-Lys17, Glu31-Tyr22 and Tyr22-Glu31 respectively, along with two ionic interactions involving Lys17-Glu49. There are two hydrogen bonds between chains A and C stabilizing the loop involving Thr15 and Lys17. Between chains A and D four hydrogen bonds involving residues Arg11-Glu14, Glu14-Arg11, Tyr12-Glu49 stabilize interactions of η1-η2 and η1-α2 and Glu31-Ala13 stabilize α1-η1 interaction. Two ionic interactions stabilize η1 between residues Arg11-Glu14. Additionally, the main α-helix (α1) is stabilized by a number of hydrophobic interactions. All SaDHNA chain interface features are summarized in Table 2. Thereby the quaternary structure of SaDHNA is formed by the assembly of four identical subunits A, B, C and D, arranged as dimer of dimers, forming a homo-tetrameric structure, as shown in Fig. 1c.

In the crystal structure four molecules of SaDHNA are present in the asymmetric unit. The oligomeric state analysis performed using PDBe PISA17 provided a value for the buried area of each monomer at the A-B (or C-D) interface of approx. 1040 Å2, corresponding to 13% of the overall surface area of each monomer. The program PBEQ solver18 was used to calculate the monomer/tetramer electrostatic surface potential to be ΔGelec of −9516.320 kcal mol−1 for the tetramer and −2676.08 kcal mol−1 for the monomer. In addition, the solvation energy was calculated to be ΔΔelec of 6.840 kcal mol−1 by utilizing the Poisson–Boltzmann Equation19, indicating a significant increase in stability upon tetramer formation.

In order to verify the oligomeric state of SaDHNA in solution, we applied dynamic light scattering (DLS) and SAXS. DLS measurements showed a monodisperse hydrodynamic radius of 4.3 ± 0.3 nm (Fig. 2a,b) and SAXS data provided, based on the p(r) function and using the Guinier approximation, a corresponding maximum diameter for SaDHNA of 10.9 nm, and the radius of gyration was calculated to be 3.27 ± 0.09 nm. Those values

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### Table 1. Data collection, processing and refinement statistics. Numbers in parenthesis refer to the outer resolution shell.

| Data collection | Native data | Deriv. data |
|-----------------|-------------|-------------|
| Beamline        | P13, PETRA III (DESY, Germany) | P14, PETRA III (DESY, Germany) |
| Wavelength (Å)  | 1.0332      | 1.072       |
| Space group     | P2_1        | P2_1        |
| Unit-cell parameters |           |             |
| a (Å)           | 53.61       | 55.20       |
| b (Å)           | 90.78       | 90.90       |
| c (Å)           | 75.38       | 74.80       |
| α = γ (°)       | 90          | 90          |
| β (°)           | 92.0        | 90.8        |
| Resolution range (Å) | 75.33–1.3 (1.33–1.3) | 55.29–2.00 (2.1–2.0) |
| Completeness (%)| 97.9 (96.1) | 98.2 (94.5) |
| Rmerge (%)      | 4.0 (123)   | 11.5 (89.8) |
|Multiplicity    | 6.9 (7.0)   | 6.9 (6.6)   |
| I(fof)/(I)     | 19.9 (2.0)  | 12.7 (2.7)  |

### Refinement

| Resolution range (Å) | 75.3–1.3 |
| No of reflections used for refinement | 164,237 |
| Rwork/Rfree (%)       | 17.8/19.8 |

### No. of atoms

- Protein: 5483
- Water: 314
- Average B value (Å²): 23.6

### R.m.s.d

- Bonds (Å): 0.03
- Angles (°): 2.86

### Ramachandran plot

- Favoured regions (%): 98.4
- Allowed regions (%): 1.6
- Disallowed region (%): 0

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Asn96-Gly99) and type IV (Phe69-Glu72, Lys113-Trp116). In addition, two β-hairpins between β-4/β-5 (class 2:4) and β-5/β-6 (class 3:5) and a γ-turn Ile152-Ser154 after the last β8 strand are observed within the structure.

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Figure 1. (a) Cartoon representation of the SaDHNA monomer. (b) The secondary structure of SaDHNA is shown schematically and annotated including the topology plot of its HotDog domain. (c) The quaternary arrangement of SaDHNA is shown for the individual monomer chains with chain A in blue, B in yellow, C in red and D in green. The figure was created applying the program PyMOL (Molecular Graphics System, Version 1.0.5.4 Schrödinger, LLC).

Table 2. Summary of SaDHNA chain interface features.

| Chains | No. of residues located in interface regions | Interface area (Å²) | No. of hydrogen bonds | No. of non-bonded contacts | No. of ionic bonds |
|--------|---------------------------------------------|---------------------|-----------------------|---------------------------|-------------------|
| A-B    | 23:20                                      | 1051:1070           | 12                    | 115                       | 2                 |
| C-D    | 22:20                                      | 1053:1058           | 12                    | 121                       | 1                 |
| A-C    | 7:8                                        | 432:432             | 2                     | 50                        | -                 |
| B-D    | 7:7                                        | 428:426             | 4                     | 52                        | -                 |
| A-D    | 9:9                                        | 611:621             | 4                     | 35                        | 2                 |
| B-C    | 10:10                                      | 614:607             | 4                     | 38                        | 2                 |
along with the calculated SAXS ab initio model (Fig. 2c) confirm a functional tetramer in solution with a slightly elongated twisted "butterfly-like" shape, as observed also for the crystal structure.

**Comparison with homologues enzymes.** A search to identify and compare homologous structures was performed applying the European Bioinformatics Institute database server [http://www.ebi.ac.uk/msd-srv/ssm](http://www.ebi.ac.uk/msd-srv/ssm). The top-scoring domain superimposition indicated homology to a hypothetical thioesterase of *Thermus thermophilus* (pdb code 1Z54) with a Cα core r.m.s.d. of 1.1 Å for 41 aligned residues and 32% amino-acid sequence identity, along with another well characterized 4-hydroxybezoyl-CoA thioesterase from *Pseudomonas sp.* strain CBS3 (pdb code 1BVQ) with a corresponding Cα r.m.s.d of 1.7 Å and 21% sequence identity. These results confirm that SaDHNA belongs to the protein family displaying a HotDog domain fold class I. The structure is most conserved in β1 and in strands β3 up to β6, in helix α1 and in the short α2 helix (Fig. 3a). The highest variability was observed in the loop region connecting β5 and β6 (Fig. 3a, light red box), as well as in the region connecting β6 and α4 (Fig. 3a, light blue box). These structural differences can be assigned to the absence of two short antiparallel β sheets in the SaDHNA structure, as well as the presence of the extra α-helix α3 and the elongated helix α4 including the residues Gln137-Lys144, the parallel strand β8 (Val146-Ile148) and a flexible loop (Met149-Leu155) at the C terminal region of SaDHNA. The protein sequence alignment of SaDHNA, shown in Fig. 3b, also revealed that Asp16 is likely to be an essential residue for a thioesterase activity (numbering according to SaDHNA). This residue is conserved among the compared proteins.
Putative active site of *Sa*DHNA thioesterase.

For future drug discovery experiments targeting *Sa*DHNA, detailed information about the location of putative active residues within the active site region and their interactions are an essential requirement. In order to obtain insights *Sa*DHNA was superimposed on the D17N mutated 4-hydroxybenzoyl-CoA thioesterase D17N mutant structure of *Pseudomonas* (PDB code 1LO9) (light grey). The substrate 4-hydroxybenzoyl-CoA (BCA) complexed with the D17N *Ps*4HBT mutant is shown in stick representation; carbon in cyan; sulfur in yellow; phosphate in orange, nitrogen in blue, oxygen in red; “N” and “C” indicate N- and C-terminus, respectively. (b) Residues at the proposed active site region with assigned thioesterase activity of *Sa*DHNA (pink), *Pseudomonas* 4HBT structure (yellow) and hypothetical thioesterase from *Thermus. thermophilus* (pdb code 1Z54) (light grey). Italic amino acid residue labels indicate corresponding residues in the *Pseudomonas* 4HBT structure (yellow).
and the benzoyl ring hydroxyl group of the amino acid residue Tyr45, as well as through the carbonyl carbon group from the amino acid Glu31 mediated by a water molecule. Also, the coenzyme A ribose of BCA molecule is positioned in a cavity located at the surface of one monomer and the remaining ligand is located in a deep cleft formed by the subunit–subunit interface. The position of the benzoyl ring hydroxyl group of the substrate may intercalate with the side chain hydroxyl group of Tyr45 from one monomer and with the side chain of His23 located at helix β2 from the corresponding subunit. Thereby the thioester carbonyl group of the substrate is located close to the N-terminal region of α1, forming a hydrogen bond with the His23 side chain imidazole ring.

**Thioesterase activity assays and peptide binding analysis by fluorescence spectroscopy.** To obtain some initial information about SaDHNA thioesterase substrate specificity, two different substrates classified according to their CoA chain length and unsaturation degree were assessed. The longest acyl-CoA chain substrate contained eighteen acyl saturated chain stearoyl-CoA (C18:0), while the shortest chain contained four acyl chain crotonyl-CoA with one unsaturation (C4:1). Thioesterase activity was observed by the increase in free thiol-CoA thioester hydrolysis formation of the 2-nitro-5-thiobenzoate anion (TNB−), resulting from the reaction of the thiolate anion (RS−) with Ellman’s reagent (DTNB−) and one mixed disulfide (R-S-TNB−). SaDHNA showed a 474-fold higher specific activity toward the extended acyl CoA chain in comparison to the short chain crotonoyl-CoA (Supplemental Table S2). Furthermore, the significance of amino acid residues Asp16 and Glu31 for thioesterase activity of SaDHNA was investigated by applying the longer acyl CoA chain as the substrate. The hydrolysis rate of stearoyl-CoA by the D16A mutant, which had the putative site carboxylate group removed, decreased 300-fold, while no activity was detected above the background level of stearoyl-CoA for the E31N mutant, indicating that both amino acid residues are essential for the activity of SaDHNA.

In parallel, in terms of structure-based computational design investigations, the atomic structure of SaDHNA was used for docking analysis applying the Bioluminate module from the Schrödinger suite, (Schrödinger, LLC, New York, 2021). Based on the structure of SaDHNA, and in particular considering the putative active site (Asp16, His23, Glu31 and Try45), we designed several peptides (ranging from 5 to 6 residues) which potentially bind to SaDHNA and inhibit its activity. Docking investigations identified two binding sites, one at the SaDHNA surface and one in the putative active site. The most effective peptide inhibitors, named Pep-1 (YGSDGR) and Pep-2 (EGYEY), showed the smallest Optimized Potentials for Liquid Simulations (OPLS) force field (potential energy OPLS2005 – 1583.93 kcal mol−1 and – 1927.27 93 kcal mol−1, respectively)33. Pep-1 (YGSDGR), with a molecular weight of 654.28 Da, was predicted to bind inside the active site region with a ΔGbind of – 81.0 kcal mol−1. According to the docking analysis, the protein-peptide complex is mediated mainly through six hydrogen bonds formed between Pep-1 and residues present in the putative active site. The benzoyl ring of the tyrosine of Pep-1 has non-covalent π-stacking interactions with the Try45 benzoyl ring of the SaDHNA structure, as well as with the side chain of Ser55 through a hydrogen bond. The residues important for the substrate binding and activity, Glu31 and Asp16, respectively, are predicted to interact with the amide of the peptide backbone and with the side chain of serine of the peptide via hydrophobic bonds as well. The second peptide (Pep-2) with a molecular weight of 623.23 Da, was predicted not to interact with the residues in the putative active site region, but with residues located on the surface of the SaDHNA structure, close to the binding site entrance, with a ΔGbind of – 41.3 kcal mol−1. Pep-2—SaDHNA interactions involve seven hydrogen bonds, as well as hydrophobic interactions between residues localized between connecting loops β2-β3, β4-β5 and β5-α3. In both cases, the predicted peptide binding free energies (∼ 81.0 kcal mol−1 and ΔGbind of – 41.3 kcal mol−1 respectively) (Fig. S2) indicate stable protein-peptide complexes28. In vitro inhibition assays were performed applying 100 μM of each peptide and 10 μM of SaDHNA and resulted in no detectable activity in comparison to control assay without peptides, confirming that the predicted peptides indeed inhibit the SaDHNA thioesterase enzyme.

Fluorescence spectroscopy was employed to analyse the inhibitory effect of peptide inhibitors on SaDHNA. The intrinsic fluorescence quenching was assessed for a gradient concentration of Pep-1 and Pep–2. The emission spectra of SaDHNA in buffer only and SaDHNA quenched with different concentrations of Pep-1 and Pep-2 are shown in Fig. 5a,b, respectively. The amino acid sequence of SaDHNA has three tryptophan residues, W29, W79 and W129 and a corresponding maximum emission near 340 nm. The gradual increase of Pep-1 to up to 1000 nM did not shift the emission spectra maximum of SaDHNA, while Pep-2 resulted in a slight blue shift of the emission, probably explained by the slight change in the polarity of the solvent surrounding the tryptophan residues25. A decrease in the fluorescence intensity was observed for both peptides. According to the quenching theory this may indicate a decrease in the lifetime of the excited state, corresponding to an additional rate process that depopulates the excited state and/or the formation of a non-radioactive ground state between the fluorophore and the quencher resulting in a non-fluorescence emission25,26.

The Stern–Volmer equation explains the relationship between fluorescence intensity and the presence of a quencher. The data obtained with different concentrations of peptides were analysed independently of the quenching process. Thereby the bimolecular quenching constant (Kq) assigned to the efficiency of quenching and the binding or affinity constant (Ka) for the associated complex were calculated27,28. SaDHNA showed a biphasic quenching behaviour, since the Stern–Volmer plot presented two linear quenching steps delimited by the red dotted line in Fig. 6a. The first one and faster quenching step was observed up to 100 nM (left side of the red dotted line), while a second and slower quenching was observed at higher concentrations of both peptides (Fig. 6a). To investigate the quenching process and to estimate Kq and Ka, the data from the second and slower step were considered (Fig. 6b,c, respectively). To estimate the bimolecular quenching constant (Kq), the Stern–Volmer quenching constant (Ksv) was determined from the slopes of Io/I versus Q (quencher concentration) (Fig. 6b) and multiplied by 10−8, which corresponds approximately to the lifetime of a biomolecule fluorophore in the absence of a quenching agent28. Therefore, Ksv [M−1 s−1] for Pep-1 and Pep-2 were 13.3 ± 1012 and 9.3 × 1012 ± 1.8 × 1012, respectively. According to the diffusion controlled quenching theory Ksv values close to 1 × 1013 were expected.
10 M$^{-1}$ s$^{-1}$ are associated with a process involving dynamic quenchers, whereas values of $K_q$ larger than the diffusive limit indicate binding interactions between the fluorophore and the quencher$^{26}$. Our results demonstrated $K_q$ values 100-fold higher than the diffusive upper limit. Therefore, it is most likely that the quenching process obeys the static mechanism through the complex association between $Sa$DHNA and the peptides.

As the estimated $K_q$ presented higher values than expected for dynamic quenching, we analysed the data applying static quenching theory to estimate the affinity constant $K_a$ for the associated complex. The intercept of log ((Io-I)/I) versus log $Q$ plot (Fig. 6c) is equal to the logarithm of $K_a$. Thereby the intercept antilog calculation determines the $K_a$ values for both peptides$^{26}$. The respective affinity constants for Pep 1 and Pep 2 were 72.7 ± 2.9 M$^{-1}$ and 3.4 ± 0.9 M$^{-1}$, indicating that Pep-1 has a 21-fold higher binding affinity to $Sa$DHNA than Pep-2. These results strongly indicate Pep-1 as a suitable candidate to be considered as a lead compound for further drug development investigations, to identify compounds that can directly interact with and inhibit $Sa$DHNA, an essential enzyme of the menaquinone pathway (Fig. S1).

Discussion

We solved and refined the crystal structure of $Sa$DHNA, and assigned $Sa$DHNA to be a member of the HotDog fold class I superfamily of proteins, where all β-strands point outwards and the long main α-helix points towards the core of the structure. $Sa$DHNA associates as a dimer of homodimers, with a face-to-face (or helix-to-helix) conformation. Leesong and co-workers firstly described the "HotDog" fold for a thiol ester dehydratase-isomerase from $E$. coli, named FabA (pdb code 1MKA)$^{29}$. Since then, a number of proteins possessing the "HotDog" fold were described for several organisms$^{30–34}$. Despite the absence of a consensus sequence and a sequence identity ranging only between 10 and 20%, the overall fold and N- or C-terminal secondary structure elements are similar and are particularly characteristic for members of the "HotDog" fold protein family. Although overall low sequence identity among all thioesterases was observed in sequence alignments, the secondary structure elements are mostly well conserved and, therefore, it is expected that the active site architecture of those enzymes remains homologous. Investigations involving protein superfamilies have shown that the mode of catalysis, the active
site location, as well as residues involved in substrate recognition and catalysis are indeed frequently conserved among these evolutionarily related proteins. This explains the relative low sequence homology between SaDHNA and other known thioesterases, even though the quaternary structures and, in particular the positions of the respective active sites and residues within the interface region forming the dimers, are homologous.

Thioesterases from *E. coli* EcYbgc as well as *Haemophilus influenzae* Hiybgc are more active for short acyl chain substrates, in contrast to *Helicobacter pylori* HpYbgc, which is more active towards long acyl chains, e.g. palmitoyl- and stearoyl-CoA. Although all of the enzymes possess the same HotDog fold class I, EcYbgc, Hiybgc, HpYbgc as well as SaDHNA have some differences in their structures, which explains a divergence in the substrate specificity. In fact, we observed the presence of a long tunnel associated with the binding site of the acyl moiety of the substrate for HpYbgc, which is absent in the Hiybgc. We detected a similar situation for SaDHNA, which is more active towards long acyl chains (stearoyl-CoA) in comparison to a short chain (crotomyl-CoA). The structure of SaDHNA reveals that activity towards long acyl chains is associated with the presence of an extended tunnel with a hydrophobic nature, involving residues Leu35, Ile38, Tyr45, Met48, Leu122, Tyr125 of the native SaDHNA. In general, stable peptide-protein interactions involve hydrogen bonds, as well as complementary interactions, such as hydrophobic van der Waals interactions, leading to a high selectivity and binding affinity. Our investigations support the general base catalysis. Required for the substrate binding and not be involved in the thioesterase activity. Initial evidence obtained from detecting activity. The orientation of the uncharged polar sidechain of an asparagine residue might interfere with the binding of substrate in the active site, in which Glu31 is more likely to act only as a supportive residue. Detectable activity. The orientation of the uncharged polar sidechain of an asparagine residue might act as a general base. In the absence of aspartic acid at the active site, nitrogen from the imidazole ring of His23 is unprotonated states at a physiological pH. This property enables histidine to participate in general acid–base catalysis and to enhance the nucleophilicity of the hydroxyl and thiol groups. Protonated nitrogen of the imidazole ring can act as a general acid while unprotonated nitrogen acts as nucleophile, and consequently, performs as a general base. In the absence of aspartic acid at the active site, nitrogen from the imidazole ring of His23 might abstract a proton of the nucleophile (a water molecule) and a general base. Indeed, by a careful inspection of the native SaDHNA structure, there is a water molecule close to BCA substrate and the sidechain of residue Asp16 located inside of the tunnel of the active site which could support the general base mechanism in SaDHNA. Interestingly, although the substitution of the aspartic acid was sufficient to completely inactivate the thioesterases from *P. profundum* and *Pseudomonas* 4HBT, for SaDHNA this mutant had substantially decreased activity but was not fully inactivated. This surprising result indicates that SaDHNA might use the aspartic acid together with another residue as an alternative during thioesterase activity. Hydrolases, including thioesterases, frequently use the Ser-His-Asp catalytic triad to perform bond cleavage. Within this triad, aspartic acid is an important activator during nucleophile attack, followed by serine and histidine residues. The imidazole ring of histidine possesses a pKₐ of approximately 6 to 7 which allows this residue to switch between protonated and unprotonated states at a physiological pH. This property enables histidine to participate in general acid–base catalysis and to enhance the nucleophilicity of the hydroxyl and thiol groups. Protonated nitrogen of the imidazole ring can act as a general acid while unprotonated nitrogen acts as nucleophile, and consequently, performs as a general base. In the absence of aspartic acid at the active site, nitrogen from the imidazole ring of His23 might abstract a proton of the nucleophile (a water molecule) and a general base. In this study we designed two peptide ligands. During docking analysis Pep-1 was predicted to bind inside the SaDHNA active site, producing a stable interaction via hydrogen bonds, as well as noncovalent interactions and via aromatic ring stacking (π stacking), which may also contribute to the peptide stability inside the binding pocket. This stable interaction might prevent the substrate from binding by blocking the active site entrance for substrates. On the other hand, Pep-2 was predicted not to bind inside the active site, but on the surface of SaDHNA. In contrast to traditional drug target sites, e.g. enzyme active sites, protein surface regions are usually more flat and mostly have less well-defined binding pockets for small molecules or peptides. Thoden and co-workers observed that the coenzyme A ribose of both 4-hydroxybenzoyl-CoA substrates and the 4-hydroxyphenacyl-CoA inhibitor were positioned in a cleft located on the solvated surface of the dimer. This important observation suggests that the interaction of Pep-2 with the SaDHNA surface might interfere with binding of the nucleotide moiety of the substrate and is reflected in the overall thioesterase catalysis...
activity. Our spectroscopy assay data provided support the inhibitory properties of the rationally designed peptides Pep-1 and Pep-2, which might be considered as potential lead compounds for further investigations to provide more insights about potential SaDHNA inhibition mechanisms.

**Conclusion**

The high-resolution structure of a 4-hydroxybenzoyl-CoA thioesterase, a key enzyme involved in the menaquinone biosynthesis pathway of *S. aureus*, resembled a Hotdog fold class I structure for the monomer. The quaternary structure of the SaDHNA homo-tetramer has four putative active sites, each located within the interface regions of two monomers, and with functionally important residues Asp16, His23 from one monomer and Glu31, Tyr45 from the adjacent monomer. Enzymatic assays and mutagenesis studies demonstrated a preference towards long chain substrates, as well as the importance of the acidic residues Asp16 and Glu31 in the active site and for substrate binding, respectively.

Targeting the menaquinone synthesis pathway of antibiotic resistant bacteria, such as *S. aureus*, can be considered as a new and innovative approach for future drug development investigations, due to the absence of the vitamin K biosynthesis in humans. Further, the peptide ligands designed for initial inhibition studies provide promising information for future inhibitor development.

**Material and methods**

**Cloning, expression and purification of SaDHNA.** The DHNA gene was amplified by PCR from *S. aureus* cDNA using the primers sequence presented in Supplemental Table S2. The PCR product was cloned via restriction enzyme *BsaI* (New England Biolabs) into *E. coli* expression vector pASK-IBA3 (IBA Lifescience) and the gene sequence was verified using automated sequencing (GATC Biotech AG, Germany). *E. coli* expression strain BL21 (DE3) was heat shock transformed with the resulting expression vector SaDHNA-IBA3 and plated on LB agar supplemented with 100 mg/mL ampicillin. For protein expression, the cells were grown in 1 L of Terrific broth media supplemented with 0.4% (v/v) glycerol (final concentration) and 100 mg/mL ampicillin at 37 °C until reaching an optical density of 0.6 applying a wavelength of 600 nm. Protein over-expression was induced with 200 ng mL⁻¹ anhydrotryptophol (IBA Lifescience) at 37 °C for 6 h. Afterwards, for protein purification, the cells were harvested for 1 h at 4000 × g, 4 °C, and then resuspended in 100 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF and sonicated twice for 8 min on ice. Soluble proteins were separated from the cell debris by centrifugation for 1 h, 18,000 × g at 4 °C and the supernatant was applied onto a gravity column containing Step-Tactin resin (IBA Lifescience) in the cold room. Unbound proteins were removed from the column by utilizing a washing buffer (WB) containing 100 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM EDTA. The protein was eluted using WB supplemented with 2.5 mM D-desthiobiotin (IBA Lifescience). Eluted protein was dialysed against 100 mM sodium phosphate buffer pH 6.0, 100 mM NaCl, applied to a pre-equilibrated HiLoad 16/600 Superdex 200 (Cytiva, former GE Healthcare) gel filtration column. After dialysis in 100 mM sodium phosphate buffer pH 6, 150 mM NaCl, SaDHNA-CoA thioesterase was concentrated for crystallization experiments until 10 mg mL⁻¹, using an extinction coefficient of 41,370 M⁻¹ cm⁻¹, provided by Protparam program (http://web.expasy.org/protparam/).

**Thioesterase activity assay.** The thioesterase activity of SaDHNA was measured according to a protocol of Rodriguez-Guilbe and co-workers. In a ELISA microplate the formation of 2-nitro-5-thiobenzoate anion provide more insights about potential SaDHNA inhibition mechanisms.

**Site-directed mutagenesis.** In order to obtain more information regarding the putative interactions identified for D16 and E31 and the substrate benzoyl-CoA obtained by superimposition using the homologous structure of 4HBT (pdb code 1LO9), as well as to obtain more insights about the structure–function relationship of SaDHNA, site-directed mutagenesis for the residues D16 and E31 was performed by plasmid PCR amplification, according to Edelheit (2009). Corresponding PCR was performed by amplification of the parental plasmid DNA in two PCR tubes and adding the forward or the reverse primer, as listed in supplemental table S2, using the Q5 High fidelity DNA polymerase (New England Biolabs). After PCR, the reaction product was combined into one single tube, denaturated by heat to separate the recently synthesized DNA strain from the template and cooled down gradually to allow the annealing of the complementary chains. The original DNA template was digested by adding the restriction enzyme Dnpl (Thermo Fisher Scientific) and as a final step, used to transform BL21 (DE3) competent cells. Sanger sequencing (GATC Biotech AG, Germany) was performed to verify the sequence of the purified DNA plasmids.
Crystallization, heavy atom derivative, data collection and structure determination. X-ray suitable crystals of SaDHNA were obtained applying the sitting drop vapor-diffusion technique mixing in a ratio of 1:1 protein solution (10 mg mL⁻¹) and reservoir solution consisting of 100 mM HEPES-Na pH 7.0, 1.0 M lithium sulphate, equilibrated against 300 mL of reservoir solution utilizing MRC Maxi 48-wells plates (Molecular dimensions, UK) at 293 K. For cryo-cooling prior to X-ray data collection crystals were transferred to a new reservoir solution containing 15% (v/v) glycerol and flash-cooled in a nitrogen stream at 100 K. Diffraction data for native SaDHNA were collected at the EMBL beamline P13 at PETRA III (DESY, Hamburg). In order to obtain phase information, heavy atom derivative soaking was performed using a final concentration of 1.25 mM potassium tetrachloroplatinate II (Hampton Research, USA), added to the crystal droplet 24 h before X-ray data collection. A platinum-SAD single-wavelength anomalous dispersion/diffraction (SAD) dataset was collected at 1.072 Å wavelength and up to 2.0 Å resolution at the EMBL beamline P14 at PETRA III (DESY, Hamburg) and integrated using the XDS software package54 and were scaled using the program AIMLESS from the CCP4i software suite55–57. In order to obtain phase information, the EMBL-HH Automated Crystal Structure Determination Platform Auto Rickshaw (EMBL, Hamburg, Germany) (www.embl-hamburg.de/Auto-Rickshaw/)58 was used. Afterwards, successive rounds of model building and refinement were performed using the program REFMAC5, version 5.8.0.1319 from CCP4 and the program Coot version 0.8.196 for model building. All structure figures were generated using the PyMOL software suite version 1.3 and the final SaDHNA structure was deposited at the Protein Data Bank (pdb code 6FDG).

Small Angle X-ray Scattering (SAXS) measurements. Small-angle X-ray scattering data of monodispers SaDHNA at concentrations from 0.8 to 2.5 mg ml⁻¹ were collected at EMBL beamline P12 at the storage ring PETRA III (DESY, Hamburg, Germany). The monodispersity of the sample solutions was verified prior to SAXS data collection applying DLS using a SpectroLight 300 instrument (Xtal Concepts, Germany). Protein was applied in 100 mM sodium phosphate buffer pH 6.0 and 150 mM NaCl with a sample volume of 25 µl at 10 °C. SAXS data were collected at a sample-detector distance of 3.1 m, a wavelength of λ=0.124 nm viand applying a 2D photon-counting Pilatus 2 M pixel detector (Dectris) with the momentum transfer ranging from 0.03 nm⁻¹ < q < 5 nm⁻¹ (s = 4π sin Λ / λ, where 2θ is the scattering angle). Data were normalized to the intensity of the transmitted beam and radially averaged. Scattering amplitudes from 20 successive X-ray exposures of 45 ms each were averaged and subtracted from the average of 40 buffer exposures. The Guinier region, radius of gyration Rg and the particle pair-distance distribution function p(r), which further provides the maximum dimension Dmax of the protein, were obtained and evaluated applying the program PRIMUSQT, part of the ATSAS software suite61. Low-resolution chain-like ab initio shapes of SaDHNA showing tetramer symmetry were subsequently generated using the composite scattering curves applying the program GASBOR62 and using a total number of 620 amino acid dummy spheres and 611 water molecules.

Docking investigations, peptide rational design and synthesis. Docking studies were carried out with SaDHNA homodimer using the BioLuminate software from the Schrödinger suite (Schrödinger, LLC, New York, 2021). The peptides were designed based on the structure of the natural substrate 1,4-dihydroxy-2-naphthoyle-CoA and used for peptide docking calculation applying the BioLuminate tool using the Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) method to calculate the free binding energy63. Afterwards, peptides, Pep-1 and Pep-2, with the lowest scores obtained from the calculated free energy binging were synthesized and used for further in vitro inhibition assays.

Investigation inhibitory activity of specific peptides Sa using fluorescence spectrophotometry. Fluorescence measurements were performed using a Cary eclipse fluorescence spectrophotometer coupled with a peltier temperature control (Agilent, USA). All assays were carried out in 1 mL cuvettes at 22 °C with excitation/emission slits at 20 nm each. The applied excitation wavelength was 290 nm and emission spectra were collected in the range of 300 up to 420 nm in increments of 1 nm. SaDHNA quenching experiments were performed with two peptides (Pep-1: YGSDGR and Pep-2: EGEYE, using a concentration range from 60 up to 1000 nM. SaDHNA at a concentration of 60 nM in 100 mM sodium phosphate buffer pH 6.0, 150 mM NaCl was applied and small volumes of the stock peptide solution were sequentially added to the cuvette for quenching analysis. Each SaDHNA-peptide assay was performed five times. As a control the fluorescence of all buffer and all peptide solutions were measured to correct the observed fluorescence accordingly. The quenching process was assessed by the Stern–Volmer theory64.

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Author contributions
A.M.M., H.B., S.F. and C.B. designed the experiment(s), A.M.M., H.B., J.L., M.P., C.M., S.F and R.S. conducted the experiment(s), A.M.M., H.B., S.F, C.W. and C.B. analysed the results. All authors reviewed the manuscript.

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Competing interests
The authors declare no competing interests.

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