Structural characterization of *Staphylococcus aureus* biotin protein ligase and interaction partners: An antibiotic target

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Received 21 January 2013; Accepted 26 March 2013

DOI: 10.1002/pro.2262

Published online 5 April 2013 proteinscience.org

Abstract: The essential metabolic enzyme biotin protein ligase (BPL) is a potential target for the development of new antibiotics required to combat drug-resistant pathogens. *Staphylococcus aureus* BPL (SaBPL) is a bifunctional protein, possessing both biotin ligase and transcription repressor activities. This positions BPL as a key regulator of several important metabolic pathways. Here, we report the structural analysis of both holo- and apo-forms of SaBPL using X-ray crystallography. We also present small-angle X-ray scattering data of SaBPL in complex with its biotin-carboxyl carrier protein substrate as well as the SaBPL:DNA complex that underlies repression. This has revealed the molecular basis of ligand (biotinyl-5'-AMP) binding and conformational changes associated with catalysis and repressor function. These data provide new information to better understand the bifunctional activities of SaBPL and to inform future strategies for antibiotic discovery.

Keywords: biotin protein ligase; biotin-carboxyl carrier protein; DNA-binding; bacterial enzyme; biotinylation; small-angle X-ray scattering

Introduction

Biotin protein ligase (BPL) is a key enzyme in biotin biology that is responsible for specifically attaching the prosthetic group biotin onto biotin-dependent

enzymes. In some organisms, it also plays a role in the regulation of biotin biosynthesis. Biotin (Vitamin H or B7) is an important micronutrient found throughout the biosphere. All living organisms use

Abbreviations: BPL, biotin protein ligase; BCCP, biotin-carboxyl carrier protein; Btyl, biotinyl-5'-AMP; SAXS, small-angle X-ray scattering; ABL, adenosine-binding loop; BBL, biotin-binding loop; *bioO*, biotin biosynthetic operon operator; *BioY*, biotin transporter; HCS, human holocarboxylase synthetase.

Additional Supporting Information may be found in the online version of this article.

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Grant sponsor: National Health and Medical Research Council (NHMRC) of Australia (application number 1011806). Grant sponsor: Adelaide Research and Innovation's Commercial Accelerator Scheme. Grant sponsor: BioInnovationSA's Commercial Development Awards.

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biotin as a prosthetic group for a family of enzymes that perform carboxylation, decarboxylation, and transcarboxylation of substrates in metabolic pathways. For example, acetyl CoA carboxylase catalyzes the first committed step in fatty acid biosynthesis, and pyruvate carboxylase is required to replenish the tricarboxylic acid cycle with oxaloacetate. Biotin is particularly important in bacterial pathogenesis. During infection, bacteria have a high demand for the micronutrient, and respond by increasing the expression of proteins required for de novo biotin synthesis and uptake.^{1–5} There is thus great interest in understanding the metabolic adaption processes that allow bacteria to survive during infection, in particular, the role of BPL.^{6–8}

The ligation reaction that BPL catalyzes is well characterized,⁹ requiring biotin and ATP and proceeding through an adenylated intermediate, biotinyl-5'-AMP, before transfer of the biotin moiety to the biotin receiving enzymes at their biotin-carboxyl carrier protein (BCCP) domain. BPL isozymes fall into three structural classes. Class I enzymes are simple modules with the sole function of biotin attachment. High-resolution crystal structures have been reported for *Pyrococcus horikoshii* OT3 (PhBPL), *Aquifex aeolicus*, and *Mycobacterium tuberculosis*.^{10–12} These proteins exist as homodimers in both solution and in their crystallized forms. Other Class I enzymes, from both *M. tuberculosis* and *A. aeolicus*, have been reported to be monomeric, even in the presence of ligands.^{12,13} The two other classes contain the conserved catalytic module of Class I but differ in their N-terminal regions. Class II enzymes have an N-terminal DNA-binding domain, enabling dual functionality. Upon ligand binding, these BPLs homodimerize, facilitating a cooperative interaction with specific DNA sequences^{14,15} as exemplified by the *Escherichia coli* BPL (EcBPL, also known as BirA) (reviewed¹⁶). The dimers of Class I and Class II BPLs are structurally distinct as they use different surfaces for homodimerization. The Class III enzymes (which include human BPL) are monomeric and contain an N-terminal domain unrelated to the DNA-binding domain of Class II BPLs—the function of which has yet to be resolved. However, it has been shown to be required for substrate recognition during catalysis using a mechanism that is distinct from Class I and II enzymes.^{17–19} These key differences point to a plausible application for bacterial BPL as a target for novel antibiotics.

Bacteraemia caused by drug-resistant strains of *Staphylococcus aureus* is a major threat to health worldwide. Common bacterial infections are becoming more difficult and expensive to treat.^{20,21} To address this, research efforts have been devoted to identifying and validating novel drug targets. For example, pathways required for virulence, such as

biotin metabolism, provide an untapped source of potential new targets.^{4,22,23} We have recently provided the first data to demonstrate it is possible to selectively inhibit SaBPL over both EcBPL and their human counterpart, holocarboxylase synthetase (HCS).²⁴ Here, structural biology played an important role in the design of small molecule inhibitors with antibacterial activity against *S. aureus*. However, a greater understanding of how these inhibitors impact on SaBPL regulated pathways at the molecular and cellular level is required.

The BPL target in *S. aureus* (SaBPL) belongs to the bifunctional Class II enzymes. Genome annotation studies have revealed that it is required for the activation of two biotin-dependent enzymes, namely acetyl CoA carboxylase and pyruvate carboxylase. Additionally, it has been proposed that it functions as a transcriptional repressor of the biotin biosynthetic operon operator (*bioO*) and the biotin transporter *bioY*.²⁵ By combining biotin ligase and transcriptional repressor activity in a single protein, BPL is uniquely placed as the key regulator of biotin metabolism in *S. aureus*. How these two activities are co-ordinated by SaBPL is still unclear. However, through extensive genetic and biochemical studies of the closely related *E. coli* BPL (EcBPL) it has been proposed that the homodimerization and protein substrate binding interfaces on BPL are mutually exclusive.²⁶ Crystal structures of the EcBPL homodimer and PhBPL in complex with the BCCP subunit of methylmalonyl-CoA decarboxylase have been reported,^{27,28} providing some molecular detail for these protein:protein interactions. However, the Class I PhBPL enzyme does not undergo the monomer–dimer transition in response to biotin binding as seen with EcBPL,²⁹ and also uses a distinctly different surface for homodimerization. Furthermore, as the Class I enzymes lack DNA-binding activity they fail to provide enough detail to elucidate the complex lifecycle of the Class II BPLs. The absence of any structural data of a Class II BPL in complex with DNA has also restricted attempts to delineate this system further.

Here, we report three X-ray crystal structures of SaBPL (1) in complex with biotin, (2) with the adenylated reaction intermediate biotinyl-5'-AMP, and (3) apo-SaBPL. The holo-structures reveal the details of the dimer formation as facilitated by the binding of the biotinyl ligand. We also present a detailed investigation of the intermolecular interactions of SaBPL with binding partners SaBCCP and the biotin biosynthetic operon operator (*bioO*) that is the DNA target of SaBPL using small-angle X-ray scattering (SAXS). This analysis illustrates the mode of DNA binding of SaBPL and confirms that the two protein:protein interactions, that is, homodimer and heterodimer, indeed compete for a common surface on SaBPL. These data provide a

Table I. Data collection and refinement statistics (molecular replacement)

	apo-SaBPL	Biotin-SaBPL	Btyl-SaBPL
Data collection ^a			
Wavelength (Å)	0.95364	0.99810	1.5418 (Cu Kα)
Space group	<i>P</i> 2 ₁	<i>P</i> 4 ₂ 2 ₁ 2	<i>P</i> 4 ₂ 2 ₁ 2
Cell dimensions			
<i>a</i> , <i>b</i> , <i>c</i> (Å)	50.1, 51.4, 67.6	94.2, 94.2, 130.9	93.6, 93.6, 130.7
α , β , γ (°)	90, 108, 90	90, 90, 90	90, 90, 90
Resolution (Å)	35.0–2.1 (2.16–2.1)	40–3.2 (3.45–3.2)	20.0–2.6 (2.67–2.6)
<i>R</i> _{sym} or <i>R</i> _{merge}	11.3 (43.3)	5.2 (44.1)	5.7 (36.2)
<i>I</i> / σ <i>I</i>	8.8 (2.8)	11.1 (2.0)	12.7 (2.1)
Completeness (%)	96.1 (96.3)	98.7 (92.6)	98.3 (94.7)
Redundancy	3.8 (3.9)	4.6 (4.3)	4.1 (3.7)
Refinement			
Resolution (Å)	35.0–2.1	30–3.2	20.0–2.6
No. reflections	17,599	9929	18,509
<i>R</i> _{work} / <i>R</i> _{free}	19.6/24.9	19.2/23.9	19.9/25.7
No. atoms			
Protein	2484	2602	2602
Ligand/ion	0	16	38
Water	212	0	305
<i>B</i> -factors			
Protein	32.1	57.6	50.8
Ligand/ion	–	58.2	55.2
Water	36.8	–	65.2
R.M.S. deviations			
Bond lengths (Å)	0.019	0.015	0.022
Bond angles (°)	1.9	1.9	1.9
% in most favored regions of a Ramachandran plot	94.0	95.0	97.1

^a Diffraction data were collected from one crystal for each structure.

molecular mechanism that underlies the dual functions of this protein, paving the way for the development of a new class of antibiotics.

Results

Structural overview of apo- and holo-forms of SaBPL

To characterize the interaction between SaBPL and its native ligand biotinyl-5'-AMP, and its impact on SaBPL dimerization, we solved its crystal structure in apo-form, in complex with biotin alone (Bt-SaBPL) and in complex with biotinyl-5'-AMP (Btyl-SaBPL) (Table I). SaBPL was observed as a crystallographic dimer when either biotin or the reaction intermediate biotinyl-5'-AMP was bound to the catalytic site. In contrast, apo-SaBPL was a monomeric molecule with an overall fold analogous to the monomeric unit of dimeric SaBPL. Similarly to *E. coli* BPL the protein can be divided into three discrete domains [Fig. 1(A)]. The N-terminal domain (residues 1–61) forms a winged helix-turn-helix DNA-binding domain. The central catalytic domain (residues 68–274) forms an α – β structure containing eight β -sheets that form a major face of the catalytic site and seven α -helices that buttress against the β -sheet scaffold. The third, C-terminal domain (residues 282–323) is characterized by a fold similar to an SH3 domain³¹ and caps the catalytic site. Two

short random coil linkers (residues 62–67 and 275–281) link these three domains. In the case of apo-SaBPL only, no electron density is visible for the loop that forms over the bound ligand, known as the biotin-binding loop ([BBL] residues 118–129 [β 5– β 6 loop]) and residue 288, due to disorder in the absence of ligand.

Structural details of biotinyl-5'-AMP and biotin interactions in holo-SaBPL

Biotinyl-5'-AMP (abbreviated “Btyl” when SaBPL complex is referred to) binds with a tight “V” shape geometry and a buried surface area of 727 Å². Figure 1(B) shows the electron density around biotinyl-5'-AMP bound in the active site of SaBPL. The key interactions between SaBPL and biotinyl-5'-AMP are summarized in Figure 1(C). These interactions are consistent with those observed in the complex of SaBPL and a chemical analogue of biotinyl-5'-AMP, biotinol-5'-AMP (PDBID 3RKW²⁴). The biotin moiety is bound in a predominantly hydrophobic pocket of SaBPL with the carbon chain of the valeric acid group and the tetrahydrothiophene component of biotin surrounded by Gly121, Trp127, Trp 136, Gly208, Gly 190, Ile209, and Gly210. Hydrogen bonds stabilize the interactions with sidechains from Ser93, Thr94, Gln116, and mainchain hydrogen bonds from Arg120 to the polar ureido head group of biotin. The oxygen atom of the tetrahydrothiophene

is solvent exposed. The BBL is positioned over the biotin-binding pocket making multiple interactions with the ligand. The basic sidechains of Arg122 and Arg125 in the BBL interact with the carboxyl group of the valeric acid tail. The adenosine-binding site is formed by residues of β 10, including Lys187, Ser128, Asn212, and the loop between α 7 and α 8, including Ile224, Arg227, and Ala228 (known as the

adenosine-binding loop [ABL]), while Trp127 positions the adenylate moiety via π - π stacking interactions.

The Bt-*Sa*BPL structure (containing biotin only) was resolved to the moderate resolution of 3.2 Å. Although not providing details of the position of some sidechains, it does confirm that there are no major differences in the positions of the domains of *Sa*BPL between the holo-forms. The R.M.S.D. of the biotin-*Sa*BPL structure compared to Btyl-*Sa*BPL is 0.4 Å for 320 C α atoms. Further, the mainchain of the ABL loop is in the same position even in the absence of the adenosyl moiety (the sidechains of this loop were not defined in the electron density). These structures, together with a recently reported structure of *Sa*BPL with biotin-based ligands bound in the active site,³² show that any biotin ligand is sufficient to stabilize the BBL and to result in the formation of an *Sa*BPL dimer.

The dimerization interface of Btyl-*Sa*BPL

The dimerization interface of Btyl-*Sa*BPL consists of α 5-helix, the β 5- β 6 BBL, the β 10- β 11 loop including regions of these β -strands, the C-terminal end of the α 6-helix, and β 15- β 16 loop from the C-terminal domain [Fig. 2(A)]. Together these form a buried surface of 1028.9 Å².³⁴ Central to the dimer interaction is the antiparallel β -strand arrangement of the β 10-strand from one monomer to its counterpart on the other monomer to create an extended, albeit distorted, β -sheet joining the two monomers. The β 10- β 11 loop is juxtaposed against the opposing monomer and all the observed hydrogen bond and electrostatic interactions emanate from residues 194-203 to their partners on the opposing monomer. The α 5-helix contributes to dimer stability via an antiparallel interaction with the α 5-helix on the opposing monomer. The DNA-binding domains make no contact with each other and are spaced at their closest point (Lys3) 63.6 Å apart.

Figure 1.

Figure 1. Crystal structure of *Sa*BPL. (A) Cartoon schematic of Btyl-*Sa*BPL monomer showing the N-terminal DNA-binding domain, residues 2-60 (blue), the central catalytic domain residues 75-268 (green), and the C-terminal cap domain 282-323 (purple). The random coil linkers residues 62-67 and 275-281 that connect the domains are in yellow. The corepressor biotinyl-5'-AMP is shown in stick representation. The BBL is highlighted in pink. (B) Cartoon schematic of the catalytic site of Btyl-*Sa*BPL and the final 2F_o-F_c map (determined to 2.6 Å resolution and contoured at the 1 σ level) showing the positioning of biotinyl-5'-AMP (stick representation). (C) A schematic representation of contacts made to biotinyl-5'-AMP within the active site of *Sa*BPL made using LIGPLOT.³⁰ Residues involved in hydrogen bonding are annotated in red and hydrophobic interactions are annotated in black with red frays. Biotinyl-5'-AMP is shown with purple bonds and hydrogen bonding distance shown in Ångstroms by green dashed lines.

Figure 2. SaBPL dimer. Cartoon schematic of the holo-SaBPL dimer. (A) The regions of secondary structure involved in forming the dimer interface are colored according to the program PROFACE³³ and shown from two angles. The central cluster including $\beta 10$ – $\beta 11$, $\beta 15$ – $\beta 16$, and $\alpha 5$ are green and elements at the rim of the interface, including Lys118, $\alpha 6$, and the BBL loop, are colored red and yellow. (B) Contacts stabilized by the biotinyl-5'-AMP ligand are shown in context of the SaBPL dimer. One monomer is colored using the same scheme as in Figure 1, while the other monomer is gray. The ligand is in red stick figure representation. Residues involved in hydrogen bonding are shown in stick figure representation. Hydrogen bonds are indicated as dotted lines.

Of particular importance for its activity as a transcriptional repressor is the manner in which the dimer is stabilized by the bound biotin or biotinyl-5'-AMP ligand. Both the C-terminal domain and the BBL, which form direct contacts with the ligand, also make contact at the dimer interface. In particular, the $\beta 15$ – $\beta 16$ loop of the C-terminal cap domain (residues 317–319) and residues Arg122 and Phe123 of the BBL reside in the dimer interface [Fig. 2(B)]. Interestingly, residues from both of these elements (Ser318 from the C-terminal domain and Arg122 from the BBL) form a hydrogen bond with Asp200 emanating from the end of the $\beta 10$ – $\beta 11$ loop from the opposing monomer. Further, upon ligand binding and dimer formation, Asp320 forms a salt bridge with Arg125 in the catalytic core. This interaction appears to stabilize the loops between $\beta 5$ – $\beta 6$ and the cap domain that forms the biotinyl-5'-AMP binding site.

Comparison between apo and ligand bound SaBPL

A comparison of the monomeric apo-SaBPL structure and dimeric holo-SaBPL reveals the structural perturbations that take place concomitant with

ligand binding and the monomer–dimer transition. Apo- and holo-SaBPL structures display highly similar folds; the R.M.S.D of apo-SaBPL superposed with holo-SaBPL is 1.2 Å (between 302 C α atoms). Apo-SaBPL, however, occurs as a monomer, with the BBL completely undefined (residues 118–129 are not defined by electron density). This is in contrast with holo-SaBPL where the BBL is positioned through contacts with biotin in the catalytic site, forming an interaction surface for dimerization. Other small differences occur in the position of loops between $\beta 7$ – $\alpha 6$, $\beta 10$ – $\beta 11$, $\alpha 7$ – $\alpha 8$, and $\beta 12$ – $\beta 13$, respectively.

Structural analysis of SaBPL in complex with its target DNA

It has previously been predicted, but not shown, that dimeric SaBPL binds to the *S. aureus bioO*.²⁵ To verify this binding interaction, we identified a 44-bp sequence containing an imperfect inverted repeat, and tested binding to holo-SaBPL using electrophoretic mobility shift assay. Binding of holo-SaBPL to this sequence (and not to a control 44-bp dsDNA of an unrelated sequence) was confirmed (Supporting Information Fig. S2 and Supporting Information Methods). The affinity of holo-SaBPL

Table II. Structural parameters calculated from SAXS data and model of *SaBPL* and complexes

Molecule	R_g (Å) ^a (Guinier)	R_g (Å) ^b ($P(r)$)	$D_{\max}$ (Å) ^c	χ^2 ^d	NSD ^e
<i>Holo-SaBPL</i>					
SAXS	30.7	30.2	97.0	0.78	0.89
Model (i)	30.7		99.8		
<i>Sa-bioO</i>					
SAXS	35.6	36.3	125.0	6.10	0.65
Model (ii)	36.7		145.0		
<i>Sa-bioO/SaBPL</i>					
SAXS	44	44.4	145	0.6	0.81
Model (iii)	40.7		153.0		
<i>Apo-SaBPL</i>					
SAXS	22.0	22.8	90	0.56	0.51
Model (iv)	22.3		86.3		
<i>SaBCCP</i>					
SAXS	16.9	16.7	57	0.77	0.60
Model (v)	16.7		54.1		
<i>SaBCCP/SaBPL</i>					
SAXS	22.5	22.8	75	0.98	0.69
Model (vi)	23.6		86.1		

^a R_g —radius of gyration given by Guinier approximation.

^b R_g —estimated from pair-wise distribution function.

^c $D_{\max}$ —maximum molecular dimension from $P(r)$ function.

^d Goodness of fit of theoretical end experimental scattering curves calculated using FOXS.³⁵

^e Normalized spatial discrepancy for ab initio SAXS models calculated from DAMAVER.³⁶

for the *Sa-bioO* DNA sequence was approximated to be nanomolar ($K_D = 100$ nM) by EMSA titration.

To obtain structural information for this interaction, we subjected a complex of holo-*SaBPL* and the 44-bp DNA (henceforth referred to as *Sa-bioO*) to SAXS. SAXS data were collected for Btyl-*SaBPL*, the *Sa-bioO*, and the Btyl-*SaBPL/Sa-bioO* complexes. The experimental and calculated scattering curves are shown in Figure 3(A), the pair-wise distance distribution function, $P(r)$, is shown in Figure 3(B) and structural parameters are reported in Table II. The radius of gyration (R_g) for Btyl-*SaBPL* was determined by Guinier analysis to be 30.7 Å and the pair-wise distribution $P(r)$ for Btyl-*SaBPL* exhibits a bimodal distribution indicative of a dimer. The R_g and maximum dimension of the particle ($D_{\max}$) derived from the function ($P(r)$) profile were 30.2 and 97.0 Å, respectively. These values are consistent with those calculated from the crystal structure of Btyl-*SaBPL*. The $D_{\max}$ derived from the $P(r)$ profile for *Sa-bioO* was 125 Å, and the $P(r)$ profile showed significant tailing towards a higher $D_{\max}$ that is consistent with an elongated molecule such as dsDNA. Interestingly, this $D_{\max}$ is shorter than the expected maximal dimension of an elongated 44-mer dsDNA (145 Å) suggesting that the dsDNA may be bent. This is possible as the *Sa-bioO* sequence contains a large number of A–T base pairs in its centre, which can increase the propensity of the dsDNA to bend. The $P(r)$ profile for the Btyl-*SaBPL/Sa-bioO* complex exhibited both a bimodal distribution as well as

tailing towards higher $D_{\max}$ values. The $D_{\max}$ of this complex was estimated to be 145 Å indicative of non-bent *Sa-bioO* upon binding of *SaBPL*.

Ab initio SAXS structures are displayed in Figure 3(C). Molecular models constructed from crystallographic structures are superimposed with the solution structure and the predicted scattering curve from this model was overlaid with the raw scattering data [Fig. 3(A)]. The ab initio SAXS structure for holo-*SaBPL* correlates remarkably well to the crystallographically determined *SaBPL* structure confirming the suitability of the system for SAXS analysis. In the case of *Sa-bioO*, the SAXS data indicate a solution structure that approximates the model based on extended B-form dsDNA [Fig. 3(C(ii))]. Third, an ab initio SAXS structure was calculated for the Btyl-*SaBPL/Sa-bioO* complex. Molecular coordinates were positioned into the SAXS structure using the program SITUS³⁷ which resulted in the recognition helices (residues 20–40) of the N-terminal domain of *SaBPL* positioned in adjacent major grooves of the DNA. This gave good consistency between the SAXS structure and the model [Fig. 3(C(iii))] with dimeric *SaBPL* straddled across the DNA at an approximately 45° angle.

Interaction of *SaBPL* with *SaBCCP*

SAXS was also used to further characterize the interaction between Btyl-*SaBPL* and *SaBCCP*. Data were collected for both apo-*SaBPL* and biotinylated-*SaBCCP* separately and together in a heterodimeric complex. The experimental and calculated scattering curves are shown in Figure 3(A), the $P(r)$ profiles shown in Figure 3(D) and structural parameters are reported in Table II. The apo-*SaBPL* SAXS data are in excellent agreement with the crystal structure. The R_g of 22.0 Å confirms the monomeric nature of this species. Likewise, the R_g and $D_{\max}$ values for the biotinylated-*SaBCCP* are consistent with the size and shape expected, including a biotinyl-lysine group that is extended away from *SaBCCP*, in a conformation ideally suited for insertion into the intact carboxylase for catalysis. This is reflected in the $P(r)$ plot as a small shoulder on the high $D_{\max}$ side. Notably, upon the addition of *SaBCCP* to BtOH-*SaBPL* (i.e., *SaBPL* in complex with biotinyl-5'-AMP, a non-hydrolysable analogue of biotinyl-5'-AMP), the SAXS data indicate the absence of dimeric *SaBPL* and instead report a shape that is slightly larger than apo-*SaBPL*. The $P(r)$ plot closely resembles that of monomeric *SaBPL*; however, the Guinier R_g of 22.5 Å is slightly larger than that determined for apo-*SaBPL*.

The ab initio SAXS structure for apo-*SaBPL* is highly comparable to the crystallographically determined *SaBPL* monomer [Fig. 3(C(iv))]. The ab initio SAXS structure for the biotinylated-*SaBCCP* also accommodates the crystallographically determined

Figure 3. SAXS analysis of apo- and holo-SaBPL, SaBPL/DNA, and SaBPL/BCCP. (A) Scattering data for the six SaBPL, SaBioO, and SaBCCP samples (black crosses) with the predicted scattering curves (gray solid line) derived from models of these molecules and complexes (a.u. corresponds to arbitrary units). (B) Pair-wise distribution ($P(r)$) profiles for holo-SaBPL, SaBioO, and SaBPL/SaBioO complexes. (C) Ab initio reconstruction of the six samples overlaid with models derived from crystallographic structural data and modeling. (D) Pair-wise distribution ($P(r)$) profiles for apo-SaBPL, SaBCCP, and the SaBCCP/SaBPL complex. Note that each of the six scattering curves, $P(r)$ profiles, and ab initio models are correlated by a Roman numeral label.

SaBCCP structure (PDB ID: 3BG5) with an extrusion from one side that we suggest represents the biotinyl-lysine group [Fig. 3(C(v))]. A molecular model of SaBPL/BCCP was built using a monomer of holo-SaBPL in complex with the SaBCCP structure by analogy to the *P. horikoshii* BPL/BCCP complex (PDB ID: 2EJG). This required the repositioning of the C-terminal cap region of SaBPL relative to the catalytic domain by 5° , to open up the binding site to accommodate the SaBCCP (Supporting Information Fig. S3). The SaBPL/BCCP model is comparable to the ab initio SAXS structure [Fig. 3(C(vi))] although the fit of the scattering data calculated from this model with the experimental data was not ideal. This could have arisen from a number of sources. The juxtaposition of SaBCCP to the SaBPL could be subtly different to that observed for the *Pyrococcus* complex, and/or, the SAXS data may reflect the presence of small amounts of dimeric

SaBPL or free SaBCCP. Importantly, it is clear that the SaBCCP disrupted the dimeric SaBPL.

Comparison with human BPL

Together the X-ray crystallographic and SAXS data verify the interactions of SaBPL with its DNA and BCCP binding partners. With this understanding of the surfaces of SaBPL important for its function, we considered the potential for SaBPL to be targeted by specific inhibitors designed to inhibit SaBPL function. In particular, we compared the SaBPL molecular surface to that predicted for human BPL (also known as human HCS). Figure 4(A) illustrates the predicted surface similarity of SaBPL and HCS, based upon a sequence alignment of catalytic and C-terminal cap domains [Fig. 4(B)]. Although the ligand-binding site itself contains conserved residues that are involved in ligand binding [identical residues are colored blue in Fig. 4(A)], a significant

Figure 4. SaBPL as a potential drug target. (A) Similarity of surface amino acid residues of human BPL mapped to the surface of SaBPL. Identical amino acids are shown in blue. Sequence similarity is shown on a ramped scale from blue through white to red scored using the BLOSUM60 matrix where white indicates sequence similarity, pink and dark pink represent lesser similarity, and red represents the highest level of dissimilarity. The N-terminal DNA-binding domain is completely unrelated to the N-terminal domain of human BPL and is shown in yellow. SaBCCP modeled in its bound position is shown in cyan ribbons. (B) SaBPL sequence alignment with human SaBPL used for the surface comparison. Indicated are SaBPL amino acid residues involved at the catalytic site or the dimer interface of SaBPL.

number of residues that immediately surround the site show considerable variation. In particular, the adjacent ABL and BBL provide distinct side-chain functionalities that could form the basis of selectivity in inhibitor molecules targeted to the ligand-binding site. Additionally, the proposed BCCP binding site identified in this study also represents a region of poor sequence similarity that could be exploited in SaBPL inhibitor development.

Furthermore, the N-terminal domains of *S. aureus* and human BPL show no sequence homology at all. The N-terminal domains of eukaryotic BPLs are thought to perform an entirely different function that does not involve DNA binding.³⁸ Truncations and mutations to the N-terminal of human BPL render an inactive enzyme, indicating an underlying reliance on this region for catalytic function that is not seen for prokaryotic BPLs.^{9,19,38,39} Together, these key differences in the isozymes from *S. aureus* and human provide a structural basis for the selective inhibition of BPL for antibiotic discovery.

Discussion

We have investigated the molecular detail of BPL from the important human pathogen *S. aureus*. The crystallographically determined structures of apo-SaBPL and SaBPL in complex with biotin and

biotinyl-5'-AMP reveal that the enzyme undergoes a monomer to dimer transition upon ligand binding. Residues 118–129, that form the BBL, become ordered, as do specific loops creating part of the surface necessary for protein:protein interaction. Upon homodimer formation, the two N-terminal DNA binding domains are optimally positioned for an interaction with the major grooves at the inverted repeat sequences of the palindromic *Sa-bioO* DNA binding site.

This study confirms experimentally that SaBPL binds to the *Sa-bioO* DNA sequence and provides the first experimentally determined structure of the SaBPL/DNA complex using SAXS. By analogy to EcBPL, this complex will repress the transcription of genes containing the recognition motif in the promoter region. *E. coli* BPL controls expression of an operon containing the genes that encode the biotin biosynthetic enzymes, whereas in *S. aureus*, it is proposed that this operon as well as the gene encoding the biotin transport protein BioY are all regulated by BPL.²⁵ Our SAXS derived structure of the holo-SaBPL/Sa-bioO complex reveals the arrangement of SaBPL straddled across the dsDNA. The relative positioning of the molecules differs from previous models proposed for EcBPL bound to dsDNA.²⁷ This is due to slight differences in the dimer

Figure 5. Schematic showing the regulation of SaBPL. The fate of SaBPL depends upon the availability of biotin for the conversion of apo-SaBPL to Btyl-SaBPL that is capable of effecting transcriptional repression and also the presence of BCCP that is able to compete for the SaBPL dimer surface becomes biotinylated resulting in the reformation of apo-SaBPL.

interface of holo-SaBPL and the positioning of the N-terminal domains revealed by X-ray crystallography compared to that of *EcBPL* (Supporting Information Fig. 4). Together these impact on their interaction at the major grooves of the DNA. Furthermore, the SAXS data reveal that the SaBPL dimer sits lower on the dsDNA than previous models have predicted, reflecting the highly complementary interface of the SaBPL dimer and target dsDNA.

This study also demonstrates that the presence of SaBCCP competes with SaBPL homodimer formation. SAXS data were consistent with the formation of a SaBPL/SaBCCP heterodimer, with no evidence of Btyl-SaBPL homodimer persisting in solution. This is consistent with a mutational study performed for *EcBPL* that tests the role of interface residues in homodimer and heterodimer formation.⁴⁰ The data also suggest that the SaBPL/SaBCCP heterodimer forms analogously to the crystal structure of the *P. horikoshii* BPL/BCCP complex⁴¹ and as modeled for the *EcBPL*/BCCP.²⁶ In our model of this complex, the reactive lysine of SaBCCP extends from the turn flanked between two β -strands. This strand-turn-strand structural element is positioned analogously to the β 10-turn- β 11 of the SaBPL partner in the dimeric form of SaBPL, albeit forming a parallel β -sheet interaction rather than antiparallel. This places the lysine towards the catalytic site where the biotin transfer can take place.

Together our data provide a molecular mechanism for biotin transfer in which the SaBCCP interacts at the dimer interface of holo-SaBPL (Fig. 5). Upon removal of the biotin from the catalytic site of SaBPL, the dimerization interface is destabilized—in particular by conformational changes to the BBL. This is predicted to free the biotinylated BCCP ready to perform its role in the biochemical pathway. In the absence of BCCP, holo-SaBPL forms a dimer

and, with two DNA-binding domains held in position, is able to form a complex with the *bioO* dsDNA resulting in transcriptional repression. This article is the first report containing structural data to support this mechanism for a complex Class II BPL. Furthermore, this study shows that, in the case of SaBPL, it is the presence of apo-BCCP that determines whether SaBPL acts in its biotin ligase role or as a transcriptional repressor. Even in the presence of a biotin ligand, BCCP outcompetes homodimer formation and thus interferes with repressor activity.

With the structural details for SaBPL, and its molecular interactions with binding partners, we have been able to assess the molecular surfaces of SaBPL and their suitability for the development of inhibitors. The biotinyl-5'-AMP binding site is clearly a critical site in SaBPL for the development of competitive inhibitors and, indeed, we have begun to exploit this site with the use of biotin analogues that bind tightly in the active site.²⁴ Here, we have shown that this site, while clearly conserved between species for binding to biotinyl-5'-AMP, possesses features that differ from human BPL that indicate the potential for the development of inhibitors specific for the bacterial BPL over the human form. Our work also confirms that the BCCP interface of SaBPL differs from that of human, suggesting it also as a site for potential inhibitor development. Third, the molecular details for the N-terminal domain of SaBPL that has no similarity at all to the N-terminal domain of human BPL may facilitate the development of yet another class of SaBPL inhibitor acting at the level of *bioO* derepression.

Because of the essential nature of BPL for survival, and *S. aureus* being one of the most clinically important pathogenic micro-organisms, our endeavor is to use the structural information reported here to target this protein for the development of new antibiotics. Targeting essential enzymes for which there are no pre-existing resistance mechanisms is a well-accepted strategy to combat the rise of drug-resistant bacteria. This data have provided a detailed understanding of the functional surfaces of SaBPL that will assist in the development of specific potent drugs against this class II BPL isoenzyme.

Materials and Methods

Preparation and crystallization of SaBPL

The cloning, purification, and crystallization of recombinant SaBPL have been previously reported.⁴² SaBPL was incubated with apo-BCCP for 1 h prior to final purification to ensure it was in the apo-form. Biotin or biotin+ATP+MgCl₂ was added to apo-SaBPL prior to sample use to form holo-SaBPL. Both the apo- and holo-SaBPL crystals were grown from similar conditions.⁴²

Preparation of SaBCCP

E. coli BL21 were transformed with a plasmid encoding GST-SaBCCP (residues 68–155 of acetyl-CoA carboxylase from *S. aureus*). The expression and purification of the biotin domain were performed as previously described.⁴³

Structure determination of SaBPL

X-ray diffraction data for crystals of biotinyl-5'-AMP/SaBPL (Bt-SaBPL) were collected in-house using a Rigaku RUH2R rotating copper anode X-ray source equipped with Osmic confocal optics, an R-Axis IV detector. Diffraction data for crystals of biotin/SaBPL (Bt-SaBPL) and apo-SaBPL were recorded at the MX1 beamline at the Australian Synchrotron using a MAR research 165 CCD detector. All data were collected with cryo-cooling using an Oxford Cryosystems 700 Series cryostream at 100 K.

The diffraction data were integrated using MOSFLM⁴⁴ and the intensities were merged and scaled using the CPP4 suite of programs.⁴⁵ Initial phases were determined for the Bt-SaBPL crystal structure by molecular replacement using PHASER⁴⁶ with the coordinates of the BPL from *P. horikoshii* OT3, PDB ID: 1WQ7, as the search model. The model was built with cycles of manual model building with COOT⁴⁵ and refinement with REFMAC.⁴⁷

The Bt-SaBPL structure, with biotinyl-5'-AMP deleted, was used as the starting model for refinement of the Bt-SaBPL. Phases for the apo-SaBPL structure determination were determined by molecular replacement PHASER⁴⁵ using holo-SaBPL as the search model. The quality of the final models was evaluated using MOLPROBITY.⁴⁸ A final cycle of refinement was performed using the program PHE-NIX.⁴⁹ Statistics for the data and refinement are reported in Table I.

Preparation of dsDNA Sa-bioO sequence

DNA of sequence: 5'-CCTTAAATGTAACTTATTAATTATAAAAGTTTACATTTGGATT-3' and its complementary strand were purchased (Geneworks) as single-stranded oligonucleotides in gel purified form. The oligonucleotides were resuspended in Tris-EDTA buffer. The annealing reaction was performed by mixing equal volumes of both strands of DNA at equimolar ratio. The mixture was heated to 95°C for 3 min, and slowly cooled down to room temperature over 1 h. The annealed Sa-bioO was then stored at 4°C. The dsDNA concentration was determined spectrophotometrically at $\lambda = 260$.

Preparation of SaBPL/SaBCCP and SaBPL/DNA samples for SAXS experiments

The SaBPL/DNA complex was formed by adding SaBPL and the Sa-bioO DNA together at 2:1 molar (dimeric SaBPL/DNA) concentrations (200 μ M) in

50 mM Tris-HCl pH 7.0, 50 mM NaCl, 2 mM ATP, 2 mM biotin (or the nonhydrolysable analogue biotinol-5'-AMP), 10 mM MgCl₂, and 5% glycerol. The SaBPL/SaBCCP complex was formed by adding the proteins at equimolar concentrations (200 μ M) in presence of biotin and ATP (or biotinol-5'-AMP) in 50 mM Tris-HCl pH 7.0, 50 mM NaCl, and 5% glycerol. Just prior to the SAXS experiments all samples were buffer exchanged and concentrated in 3 K cut-off concentrators (Centricon) with the eluant used as the buffer blank. Sample concentrations were determined prior to complex formation spectrophotometrically at $\lambda = 280$ using experimentally determined extinction coefficients.

SAXS measurements and data reduction

SAXS was conducted for SaBPL and its complex with its cognate DNA (*bioO*) or SaBCCP as well as for the individual components of these complexes. SAXS measurements were made using the SAXS-WAXS beamline at the Australian Synchrotron, Melbourne, Australia, which is equipped with a Pilatus 1M-Detector. The scattering data were collected to provide an s range of 0.015–0.3 $\text{\AA}^{-1}$. Samples flowed past the X-ray beam in a 1.5 mm quartz capillary at room temperature during data collection. Scattering was collected over a range of concentrations, five concentrations between 0.2 and 2 mg/mL for each sample. The samples and matching buffer solutions were exposed to X-rays for 10 s, subdivided into 10×1 s exposures. The scattering images were integrated, averaged, and calibrated against water using software specific to the beamline. Scattering from the buffer and empty capillaries was subtracted after scaling scattering intensities to correspond to incident beam intensities. Data analysis was performed using the ATSAS suite of software. The program AUTOPOROD⁵⁰ was used to yield the pair-wise distribution function ($P(r)$) and to determine R_g (from the second moment of the $P(r)$ function) and $D_{\max}$. The R_g values calculated from the Guinear approximation and the $P(r)$ were favorably comparable. The radius of gyration (R_g) did not vary significantly over the concentrations ranges of each species of molecule and all Guinear plots were linear for $s.R_g$ 1.3.

Modeling of SAXS data

For each molecular species, the program DAMMIF⁵¹ was used to generate 20 ab initio dummy atoms models. These models were superposed, merged, and filtered using the program DAMAVER.³⁶ Theoretical scattering curves were calculated from the model coordinates and compared to the experimental data using FOXS.³⁵

Molecular figures and sequence/structure analysis

Figures were prepared using PYMOL.⁵² LIGPLOT³⁰ was used to generate Figure 1(C). The sequence

alignment [Fig. 4(B)] was made using ESPript.⁵³ Surface areas and contents were calculated using PISA.³⁴ The surface of SaBPL shown in Figure 4(A) was colored using MULTISEQ (part of VMD⁵⁴) according to sequence similarity based upon the BLOSUM60 matrix.

ACCESSION NUMBERS

Coordinates and structure factors have been deposited in the Protein Data Bank with accession numbers: BtYL-SaBPL PDBID = 3RIR, Bt-SaBPL PDBID = 3RKY, and, apo-SaBPL PDBID = 3RKX.

Acknowledgments

MCJW is an NHMRC Senior Research Fellow and acknowledges the support of the NHMRC and the Australian Research Council. NC was a Monash Centre for Synchrotron Science Fellow. Diffraction and scattering data were collected at the Australian Synchrotron MX1 and SAXS/WAXS beamlines. We also acknowledge the computer resources of the Victorian Partnership for Advanced Computing. We wish to thank Prof John Cronan (University of Illinois) for the kind gift of biotinol-5'-AMP.

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