Structural characterization of a ribose-5-phosphate isomerase B from the pathogenic fungus Coccidioides immitis

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Abstract

Background: Ribose-5-phosphate isomerase is an enzyme that catalyzes the interconversion of ribose-5-phosphate and ribulose-5-phosphate. This family of enzymes naturally occurs in two distinct classes, RpiA and RpiB, which play an important role in the pentose phosphate pathway and nucleotide and co-factor biogenesis.

Results: Although RpiB occurs predominantly in bacteria, here we report crystal structures of a putative RpiB from the pathogenic fungus Coccidioides immitis. A 1.9 Å resolution apo structure was solved by combined molecular replacement and single wavelength anomalous dispersion (SAD) phasing using a crystal soaked briefly in a solution containing a high concentration of iodide ions. RpiB from C. immitis contains modest sequence and high structural homology to other known RpiB structures. A 1.8 Å resolution phosphate-bound structure demonstrates phosphate recognition and charge stabilization by a single positively charged residue whereas other members of this family use up to five positively charged residues to contact the phosphate of ribose-5-phosphate. A 1.7 Å resolution structure was obtained in which the catalytic base of C. immitis RpiB, Cys76, appears to form a weakly covalent bond with the central carbon of malonic acid with a bond distance of 2.2 Å. This interaction may mimic that formed by the suicide inhibitor iodoacetic acid with RpiB.

Conclusion: The C. immitis RpiB contains the same fold and similar features as other members of this class of enzymes such as a highly reactive active site cysteine residue, but utilizes a divergent phosphate recognition strategy and may recognize a different substrate altogether.

Background

Ribose-5-phosphate isomerases catalyze the interconversion of ribulose-5-phosphate and ribose-5-phosphate as an important part of the pentose phosphate pathway [1]. Ribose-5-phosphate is used in nucleotide and co-factor biosynthesis. As with other isomerases, ribose-5-phosphate catalyzes this reaction at near equilibrium. Two types of ribose-5-phosphate isomerases exist, RpiA and RpiB, which share little structural homology and have distinct active sites and mechanisms of action [2]. Most organisms such as Escherichia coli contain both RpiA and RpiB, but other organisms contain only one class of Rpi. In E. coli, a double rpiA/rpiB knockout exhibited severely impaired growth [3]. RpiB occurs almost exclusively in bacteria.

RpiB is a member of the LacAB_rpiB superfamily of proteins (PFAM PF02502). RpiB from different organisms may have different substrate specificities and several annotated RpiB enzymes act upon alloose-5-phosphate rather than or in addition to ribose-5-phosphate and are thus named AlsI. Indeed, one of the first crystal structures solved for a member of this family was the E. coli RpiB/AlsI [2]. Given the important cellular role of ribose-5-phosphate isomerases and that mammals only have RpiA, interest has been generated in RpiB enzymes as potential drug targets, especially in pathogenic organisms, many of which only have RpiB. E. coli RpiB inhibitors have been described [4] and
inhibitor-bound crystal structures have been determined for RpiB from \textit{Mycobacterium tuberculosis} \cite{5} which causes tuberculosis and \textit{Trypanosma cruzi} \cite{6}, the causative agent of trypanosomiasis.

\textit{Coccidioides immitis} is a pathogenic fungus that causes coccidioidomycosis, also known as Valley Fever \cite{7-9}. One gene (CIMG\_07932) encodes a putative uncharacterized protein that contains high sequence homology with RpiB enzymes from closely related organisms. Specifically, the CIMG\_07932 gene product contains 98\% sequence identity to an Rpi from \textit{P. posadasii}, 89\% sequence identity to an Rpi from the non-pathogenic fungus \textit{Uncinocarpus reesii}, and 78\% sequence identity to an RpiB from \textit{Paracoccidioides brasiliensis}. We have undertaken structural analysis of RpiB from \textit{C. immitis} and present here crystal structures in apo and ligand bound forms.

\textbf{Results and Discussion}

\textbf{Structure determination of \textit{C. immitis} RpiB}

Given that the \textit{C. immitis} RpiB contains modest sequence identity to other structurally characterized RpiBs, we attempted to solve the crystal structure of \textit{C. immitis} RpiB by iodide ion single wavelength anomalous dispersion (SAD) phasing \cite{10,11}, a strategy that has proven successful for structure determination of many SSGCID targets \cite{12,13}. Attempts at phasing entirely with SAD resulted in poor quality experimental phases (FOM of 0.29 prior to density modification). Attempts to solve the structure by molecular replacement with other RpiBs such as that from \textit{Clostridium thermocellum} \cite{14} yielded clear rotation and translation solutions, yet poor refinement statistics (R of 0.39 and R\text{free} 0.47). Addition of the MR solution to a SAD experiment has been shown in improve phase quality \cite{15}. Therefore the partial molecular replacement solution was combined with the SAD phases and yielded a clearly interpretable electron density map (FOM 0.53 prior to density modification) into which Buccaneer \cite{16} built both molecules in the asymmetric unit end-to-end in less than one minute (Table 1; Figure 1).

Interestingly, the active site contains electron density consistent with an oxidized cysteine residue (Cys76) in both protomers (Figure 1). Although the exact oxidation state of the cysteine residue was not determined, the electron density maps were most consistent with two conformations of the sulfenic state rather than sulfinic or sulfonic acid. The oxygen on Cys76 appears to adopt two different orientations contacting the backbone nitrogen and side chain hydroxyl of Thr78 in one orientation and the backbone nitrogens of Gly81 and Val82 in the other. The oxidized nature of Cys76 may imply that this residue, thought to be the catalytic base, is highly reactive. The high concentration solution of sodium iodide used for phasing likely contains iodine, indicated by a light yellow color. Iodine is the most probable source for the oxidation of Cys76, which was not oxidized in the other two \textit{C. immitis} RpiB structures (see below). Oxidation is unlikely to have occurred as a result of radiation damage since the data were collected in house under cryogenic temperatures. Oxidized cysteines have been observed previously in the presence of iodide ions \cite{17,18} and also for other RpiB enzymes (PDB entries 1O1X \cite{19} and 3C5Y, no primary citation) determined in the absence of iodide.

\textbf{Comparison with other ribose-5-phosphate isomerases}

The \textit{C. immitis} RpiB contains \~20-38\% sequence identity with other structurally characterized RpiB enzymes, although fewer than 10 amino acids are completely conserved across these sequences (Figure 2). The overall structure of the \textit{C. immitis} putative RpiB is quite similar to other structurally characterized RpiB enzymes from bacterial as well as eukaryotic organisms (Table 2). In addition to the conserved overall fold, certain other RpiB features are present in the \textit{C. immitis} RpiB structure such as the highly conserved cis-peptide at residue Gly43.

The \textit{C. immitis} RpiB contains the canonical open (\(\alpha,\beta\)) Rossmann fold, a common fold for proteins that bind nucleotides or nucleotide-like molecules \cite{20}. As determined by the Protein Interfaces, Surfaces and Assemblies (PISA) server \cite{21}, the quaternary structure appears to be dimeric (Figure 1) with \(7408 \text{ Å}^2\) of buried surface (\(12,690 \text{ Å}^2\) of surface area for the dimer). However, based on the structure, PISA also predicts a tetrameric structure (generated by crystal symmetry) in which \(17,610 \text{ Å}^2\) of surface area is buried (\(22,730 \text{ Å}^2\) of surface area for the tetramer). The tetrameric structure of \textit{C. immitis} RpiB is most likely a dimer of dimers with one strong dimer and the secondary weaker dimer interaction that makes the tetramer. This quaternary structure is consistent with the two ligand bound \textit{C. immitis} RpiB described below in a different crystal form, and with previous X-ray structural characterization of RpiB, such as that from \textit{E. coli} \cite{2}. Dynamic light scattering (DLS) experiments indicated a monodisperse protein with an approximate molecular weight of 80 kDa which is consistent with a tetramer in solution. DLS measurement on \textit{E. coli} RpiB also indicated a tetramer in solution \cite{2}. Thermofluor analysis indicated a stable protein with a melting temperature of 52°C. The melting temperature was unaffected by the presence of ribose-5-phosphate, ribose-5-phosphate with MnCl\(_2\), or iodoacetate.

\textbf{\textit{C. immitis} RpiB substrate recognition}

Structures of RpiB from other organisms have been obtained in the presence of ribose-5-phosphate or
inhibitors. Comparison of the active site in multiple sequence alignments showed that several residues involved in recognition of the phosphate moiety of ribose-5-phosphate or ribulose-5-phosphate are not conserved in *C. immitis* RpiB (Figure 2). While other organisms use up to five positively charged residues to recognize the phosphate moiety, only one of these residues is positively charged (Lys148) in *C. immitis*. Several other residues are small polar residues such as Ser109 and Ser145. Residue 17 is typically a histidine or asparagine, but is surprisingly a negatively charged aspartic acid residue in *C. immitis*. Because of the decreased size of several residues and the presence of Asp17, we speculated that the *C. immitis* RpiB may utilize a cation to facilitate recognition of ribose-5-phosphate. RpiB enzymes from other organisms typically recognize ribose-5-phosphate with a low affinity of *Km* ~ 1-5 mM [2,22]. Therefore, we performed co-crystallization experiments in the presence of 20 mM ribose-5-phosphate or 20 mM ribose-5-phosphate with 12 mM MnCl₂, which are in excess of the protein (~3.3 mM). We obtained a 1.8 Å resolution data set (Table 1) from *C. immitis* RpiB co-crystallized in the presence of 20 mM substrate, we only observed substantial electron density for phosphate bound in the active site, which presumably came from the 0.1 M SPG buffer (succinic acid, phosphate, glycine) buffer at pH 5.0 (Figure 3). Therefore, it appears that under the conditions of the crystallization experiment, phosphate

| Data reduction | Iodide | Phosphate | Malonic acid |
|---------------|--------|-----------|--------------|
| Space group   | C2     | F222      | F222         |
| Unit-cell parameters | *a* = 103.2 Å, *b* = 49.9 Å, *c* = 62.0 Å, *β* = 108.6° | *a* = 77.7 Å, *b* = 85.2 Å, *c* = 96.3 Å | *a* = 77.5 Å, *b* = 84.4 Å, *c* = 96.2 Å |
| Resolution range (Å) | 50-1.9 (1.95-1.90) | 50-1.8 (1.85-1.80) | 50-1.7 (1.74-1.70) |
| Unique reflections | 23,513 (1520) | 14,967 (1088) | 16,561 (1028) |
| *R*merge | 0.043 (0.286) | 0.123 (0.397) | 0.044 (0.256) |
| Mean *I*/*σ*(*I*) | 13.9 (2.9) | 17.5 (3.0) | 34.7 (6.6) |
| Completeness | 98.9% (87.5%) | 99.8% (98.7%) | 94.5% (80.2%) |
| Multiplicity | 5.8 (2.9) | 6.9 (3.9) | 9.6 (5.3) |

| Phasing | | |
|---------| | |
| Anomalous Correlation | 58% (8%) | |
| SigAno | 1.53 (0.81) | |
| Iodide Sites | 21 | |
| FOM (Phaser EP) | 0.53 | |

| Refinement | | |
|-----------| | |
| *R*_free | 0.166 (0.185) | 0.150 (0.212) | 0.144 (0.156) |
| *R*_merge | 0.205 (0.242) | 0.176 (0.270) | 0.175 (0.189) |
| RMSD bonds (Å) | 0.015 | 0.015 | 0.012 |
| RMSD angles (°) | 1.364 | 1.288 | 1.323 |
| Protein Atoms | 2415 | 1198 | 1210 |
| Waters | 213 | 173 | 169 |
| Iodide Ions | 29 | 0 | 0 |
| Mean B-factor (Å²) | 18.3 | 13.2 | 13.7 |
| Reflections | 22,275 (1434) | 14,170 (1031) | 15,677 (968) |
| *R*_free Reflections | 1205 | 752 | 832 |

| Validation | | |
|-----------| | |
| Ramachandran favored | 100% | 100% | 98.7% |
| Ramachandran outliers | 0% | 0% | 0% |
| Molprobity score [35] | 1.42 (97th percentile) | 0.98 (100th) | 1.32 (97th) |
| PDB ID | 3QD5 | 3SDW | 3SGW |

*R*_free = Σh|[*F*<sub>obs</sub> - |*F*<sub>calc</sub>|]/Σh|*F*<sub>obs</sub>|. Values in parenthesis indicate the values for the highest of twenty resolution shells.

*R*_merge was calculated using 5% of the reflections omitted from the refinement [31].
outcompeted ribose-5-phosphate for binding to the active site. Addition electron density extends from one of the oxygens of the phosphate. The crystal may contain a mixture of phosphate and ribose-5-phosphate, which could be modeled into the active site without significant steric clash. Refinement of ribose-5-phosphate alone or at 0.5 occupancy with phosphate as the other 0.5 occupancy resulted in negative density in the |Fo|-|Fc| electron density map and high crystallographic B-factors for ribose-5-phosphate. Therefore, the final model contains only phosphate. Phosphate is recognized by Ser109, Ser145 and Lys148 of the active site. Some of the negative charge may be stabilized via charge relay from Ser109 to Arg105. In other organisms, Arg105 is a leucine or methionine (Figure 2). The positioning of the phosphate moiety is slightly different than that observed in other structures, such as that of T. cruzi RpiB bound to the competitive inhibitor 4-deoxy-4-phospho-D-erythronohydroxamic acid [6] (Figure 4).

Given the substantial differences in the active site of the C. immitis RpiB in comparison with other structurally and functionally characterized RpiB enzymes, it is possible that the C. immitis enzyme utilizes a substrate different than ribose-5-phosphate/ribulose-5-phosphate. In addition to the single positively charged residue that interacts with the phosphate, the highly conserved histidine involved in ring opening in the catalytic mechanism (His102 in C. thermocellum [14]) has been replaced with Ser109 in the C. immitis structure (Figure 4). Given the position of the phosphate in the C. immitis structure, we suspect that this enzyme may isomerize shorter chain substrates, although confirmation of substrate activity will require further structural and biochemical characterization. Furthermore, C. immitis contains another gene (CIMG_09662) that encodes a putative uncharacterized protein which contains 99% sequence identity to RpiA from C. posadasii (gene CPC735_023760). Therefore, this essential function of the pentose phosphate pathway may be accomplished by RpiA, allowing RpiB to have evolved with an altered substrate specificity.

C. immitis RpiB covalent inhibition
The catalytic cysteine residue of RpiB is known to be reactive toward iodoacetate [2,23], although no crystal structure has been determined for an RpiB covalently bound to iodoacetate. Attempts at co-crystallization after incubation with 10 mM iodoacetamide did not yield diffraction quality crystals. However, a 1.7 Å resolution data set was obtained from a crystal grown from MIB buffer (malonic acid, imidazole, boric acid) at pH 5.0 which contained clear evidence for malonic acid tightly bound off Cys76 (Figure 5). The malonic acid refines with a C2-S distance of 2.2 Å, which is longer
than that expected for a covalent C-S bond (1.8 Å), but significantly shorter than that expected for van der Waals interactions (>3.3 Å); malonic acid was built into the omit |Fo|-|Fc| density and allowed to refine freely in REFMAC5 [24]. Therefore, this structure may reflect the formation of a distorted (long) covalent bond between malonic acid and Cys76. We do not know of a reasonable mechanism for the formation of such a covalent bond between malonic acid and Cys76, which rather seems counterintuitive.

The chemical reaction of Cys76 with iodoacetate is expected to form a Cys-CH₂COOH adduct. The conformation of either of the carboxylates of malonic acid may be reflective of the carboxylate of the covalent adduct formed with iodoacetate. One of the carboxylates of malonic acid forms hydrogen bonds with the backbone amides of Thr78 (conserved as a serine or threonine, 3.0 Å) and the universally conserved Gly77 (2.9 Å); these interactions are well ordered in the pH 5.0 crystal. Given an approximate pKa of ~4.8, at pH 5.0...

**Table 2** Comparison of the global structure of *C. immitis* RpiB with RpiB enzymes from other organisms

| Organism                | Reference PDB | Similar Cα atoms | R.m.s.d. (Å) | Identity (%) |
|-------------------------|---------------|------------------|-------------|-------------|
| *Clostridium thermocellum* | 3HIE [14]     | 148              | 1.17        | 34          |
| *Escherichia coli*      | 1NN4 [2]      | 143              | 1.10        | 38          |
| *Mycobacterium tuberculosis* | 2WNO [5,22]  | 141              | 1.18        | 26          |
| *Novosphingobium aromaticivorans* | 3CSY     | 149              | 1.97        | <20         |
| *Streptococcus pneumoniae* | 2PPW        | 146              | 1.91        | <20         |
| *Thermotoga maritima*   | 101X [19]     | 143              | 1.24        | 33          |
| *Vibrio parahaemolyticus* | 3ONO       | 149              | 1.86        | <20         |

| Eukaryotes              |               |                  |             |             |
|-------------------------|---------------|------------------|-------------|-------------|
| *Giardia lamblia*       | 3SSP          | 125              | 1.06        | 28          |
| *Trypanosoma cruzi*     | 2K8C [6]      | 148              | 1.40        | 29          |

Superposition calculations were done in CCP4 using the program Superposition and the secondary structure matching setting. The number of similar Cα atoms (residues) is reported. For PDB entries 3CSY (UCS6), 2PPW (Wu, R. et al.), 3ONO (Kim, Y. et al.) and 3SSP (Edwards, T.E. et al.) no primary citation has been reported.
about half of the carboxylates should be protonated. The second malonic acid carboxylate forms hydrogen bonding interactions with the backbone amide nitrogen of the universally conserved Gly81 (2.5 Å) and with the side chain of the universally conserved Asn110 of the other protomer of the obligate dimeric enzyme (3.0 Å). The interactions of this carboxylate with RpiB may reflect the interactions formed by the iodoacetate covalent adduct. There is a chloride ion in the active site 3.7 Å away from the malonic acid and near Arg105 and Arg120, although this anion is ill-suited to mimic the iodide ion leaving group of iodoacetate because it is on the opposite side of the carboxylate relative to Cys76. An iodide ion resides in the same place as this chloride ion.

Figure 3 1.8 Å resolution crystal structure of C. immitis RpiB bound to phosphate. The coloring is the same as used in Figure 1 with one protomer in gray and the other in green. The 2|Fo|-|Fc| map is shown in blue mesh contoured at 1.0 σ.

Figure 4 Comparison of active site residues of eukaryotic RpiB enzymes from C. immitis (green and gray in panel A) and T. cruzi (salmon in panel B) and overlay in panel C. The C. immitis structure contains phosphate whereas the T. cruzi structure contains the competitive inhibitor 4-deoxy-4-phospho-D-erythronohydroxamic acid.

Figure 5 1.7 Å resolution crystal structure of C. immitis RpiB bound to malonic acid. The coloring is the same as used in earlier figures with one protomer in gray and the other in green. Hydrogen bonds are shown as dashed lines. A The |Fo|-|Fc| map calculated with model phases lacking the malonic acid residue is shown in green mesh contoured at 3.0 σ. B The 2|Fo|-|Fc| map calculated with model phases containing the malonic acid residue is shown in blue mesh contoured at 1.0 σ.
in both protomers of the asymmetric unit in the iodide-phased structure.

Conclusions
Here we present crystal structures of RpiB from the pathogenic fungus *C. immitis*, which together with the structures from *T. cruzi* [6] and *G. lamblia* (Edwards, T. E. et al., unpublished) are the only eukaryotic RpiB crystal structures currently available. These structures reveal the presence of a highly reactive cysteine residue in the active site, thought to be the catalytic base in the isomerase reaction and provide insight into a possible structural mechanism for the inhibition of RpiB by iodoacetate. Furthermore, these structures reveal the basis for phosphate recognition by a single positively charged residue and may indicate that this *C. immitis* has altered substrate specificity.

Methods
Cloning, expression, and purification
The 163-residue *Coccidioides immitis* putative uncharacterized protein (C. *immitis* RpiB, UniProt ID: P0CL19, formerly Q1DP31; Gene CIMG_07932, targetDB ID: CoimA.00584.a, E. C. 5.3.1.6) was amplified from genomic DNA and cloned into an expression vector (pAYA0421) encoding an N-terminal histidine affinity tag followed by the human rhinovirus 3C protease cleavage sequence using ligation independent cloning [25]. The full length expressed protein contains the tag sequence MAHHHHHMGTLEAQTQGPGS followed by the 163-residue *C. immitis* RpiB. The plasmid is available through the BEI repository (NR-28451). The 163-residue *C. immitis* RpiB (0.4 μM for the second peak) was diluted with an equal volume of precipitant and equilibrated against 80 μL of precipitant in 96-well sitting drop vapor diffusion format using Compact Junior plates from Emerald BioSystems. Protein drops (0.4 μL at 82 mg/mL) were diluted with an equal volume of precipitant and equilibrated against 80 μL of precipitant in 96-well sitting drop vapor diffusion format using Compact Junior plates from Emerald BioSystems. A crystal grown from the JCSG+ screen condition B9 (0.1 M Na citrate pH 5.0, 20% w/v PEG 6000) was soaked into a solution containing 0.1 M Na citrate pH 5.0, 20% w/v PEG 6000, 22% v/v ethylene glycol, and 0.7 M NaCl for 1 minute, then vitrified in liquid nitrogen. A data set (Table 1) was collected at 100 K under a stream of liquid nitrogen using a Rigaku FR-E+ SuperBright Cu Ka rotating anode X-ray generator with VariMax optics and a Saturn 944+ CCD detector. Data parameters include 360 images, Δφ = 1°, 2θ = 5°, 20  s exposure times, and a detector distance of 50 mm. Data reduction with XDS [29]. The structure was solved by combined molecular replacement and iodide ion SAD phasing. First, molecular replacement was performed using the protein model from protomer A of *Clostridium thermocellum* crystal structure (PDB ID 3HEE, [14]) as a search model in Phaser [30] from the CCP4

then loaded onto a Ni-NTA His-Trap FF 5 ml column (GE Healthcare). The column was washed with 20 column volumes of wash buffer (20 mM HEPES pH 7.0, 300 mM NaCl, 5% w/v glycerol, 30 mM imidazole, and 1 mM TCEP). The bound protein was eluted with 7 column volumes of elution buffer (20 mM HEPES pH 7.0, 300 mM NaCl, 5% w/v glycerol, 1 mM TCEP, and 500 mM imidazole). The collected protein was loaded onto a Hilo load 26/60 Superdex 75 prep grade column (GE Healthcare) equilibrated in SEC buffer (20 mM HEPES pH 7.0, 300 mM NaCl, 5% w/v glycerol, and 1 mM TCEP). The protein eluted off the SEC column in a split peak, which was pooled into two samples and concentrated to 82 mg/mL for the first peak sample and 128 mg/mL for the second peak sample. Both samples were shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis to be >95% pure and contain a protein of the expected molecular weight. Therefore, the two samples may reflect different oligomeric states of the same protein in solution. The sample from the first peak resulted in the crystal structures, whereas the sample from the second peak yielded crystals which did not diffract to better than 3 Å resolution. The purified protein samples were stored at -80°C. Dynamic light scattering (DLS) was performed at 8 mg/mL in SEC buffer on a Malvern Instruments Nano series Zetasizer. Thermofluor experiments were performed in SEC buffer at 2 and 4 mg/mL as described [27].

Crystallization and structure determination
Crystallization trials were set up according to a crystallization approach [28] using the JCSG+ and PACT sparse matrix screens from Emerald BioSystems. Protein drops (0.4 μL at 82 mg/mL) were diluted with an equal volume of precipitant and equilibrated against 80 μL of precipitant in 96-well sitting drop vapor diffusion format using Compact Junior plates from Emerald BioSystems. A crystal grown from the JCSG+ screen condition B9 (0.1 M Na citrate pH 5.0, 20% w/v PEG 6000) was soaked into a solution containing 0.1 M Na citrate pH 5.0, 20% w/v PEG 6000, 22% v/v ethylene glycol, and 0.7 M NaCl for 1 minute, then vitrified in liquid nitrogen. A data set (Table 1) was collected at 100 K under a stream of liquid nitrogen using a Rigaku FR-E+ SuperBright Cu Ka rotating anode X-ray generator with VariMax optics and a Saturn 944+ CCD detector. Data parameters include 360 images, Δφ = 1°, 2θ = 5°, 20  s exposure times, and a detector distance of 50 mm. Data were reduced with XDS [29]. The structure was solved by combined molecular replacement and iodide ion SAD phasing. First, molecular replacement was performed using the protein model from protomer A of *Clostridium thermocellum* crystal structure (PDB ID 3HEE, [14]) as a search model in Phaser [30] from the CCP4

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suite [31]. Separately, twenty-one anomalous sites were identified using phenix.lysys [32]. Phases calculated from SAD were combined with the MR solution in Phaser EP [30]. After density improvement in parrot [33], the model was initially built using Buccaneer [16] using the default parameters for both programs and Hendrickson Lattman coefficients and R_free rather than Phi/FOM. Another crystal of C. immitis RpiB was grown at 66 mg/mL in the presence of 20 mM ribose-5-phosphate and 12 mM MnCl₂ in the PACT screen condition A2 (0.1 M SPG buffer pH 5.0, 25% w/v PEG 1500). The crystal was cryo-protected in a solution containing 20 mM ribose-5-phosphate, 12 mM MnCl₂, 0.1 M SPG buffer pH 4.0, 30% w/v PEG 1500 and 20% v/v ethylene glycol and vitrified. A data set was collected as described above for the iodide crystal with the exception of 2θ = 10°, 340 images and 30 s exposure times. The phosphate bound structure was solved by molecular replacement in Phaser [30] using the protein-only model of the iodide phased structure. A third crystal of C. immitis RpiB was grown at 66 mg/mL in the presence of 20 mM ribose-5-phosphate and 12 mM MnCl₂ in the PACT screen condition B2 (0.1 M MIB buffer pH 5.0, 25% w/v PEG 1500). The crystal was cryo-protected in a solution containing 20 mM ribose-5-phosphate, 12 mM MnCl₂, 0.1 M MIB buffer pH 4.0, 30% w/v PEG 1500 and 20% v/v ethylene glycol and vitrified. A data set was collected as described above for the iodide crystal with the exception of 2θ = 10°, 360 images and 6 s exposure times. This malonic acid bound structure was solved using the protein model from the phosphate-bound structure. The final models (Table 1) were obtained after numerous rounds of refinement in REFMAC5 [24] and manual re-building in COOT [34]. NCS averaging was not used due to the high resolution of each structure (sub 2 Å). Both 2|Fo|-|Fc| and |Fc|-|Fo| electron density maps were used in model building. TLS refinement was used with one group per chain. Water molecules were built that were within hydrogen bonding distance to the protein (≤3.2 Å) and showed electron density above 1.1 σ in the final 2|Fo|-|Fc| electron density map. Structures were assessed for correctness and validated using Molprobity [35]. All diffraction images are freely available (http://www.csgid.org/csgid/pages/diffraction_images).

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Authors’ contributions
TEE collected two of the data sets, solved all three structures, analyzed the data and wrote the paper. ABA purified the protein. ERS performed DLS measurements. ROB performed thermofluor analysis. JTL collected one data set. DJL performed large scale expression and KBT performed lysis. MCC verified two structures, and ASG verified the other. BLS is the SSGCID site manager. WCv is an SSGCID Co-PI. PIM is the SSGCID Co-PI. All authors read and approved the final manuscript.

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