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Structural Basis for Feedback and Pharmacological Inhibition of Saccharomyces cerevisiae Glutamate Cysteine Ligase*\textsuperscript{a}\textsuperscript{,b}

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\textsuperscript{b}The abbreviations used are: GCL, glutamate cysteine ligase; ScGCL, S. cerevisiae GCL; BSO, L-buthionine-S,R-sulfoximine; MES, 2-(N-morpholino)ethanesulfonic acid.

The central role of GCL in glutathione homeostasis makes it an attractive target for drug design. Increased glutamate cysteine ligase catalytic subunit mRNA levels and GCL activity have been frequently observed in cells derived from human tumors resistant to chemotherapeutic agents (14–16). Increased production of glutathione likely protects against reactive oxygen and nitrogen species (17, 18) and facilitates detoxification of electrophilic xenobiots by the glutathione S-transferases (19). Drug resistance in tumors can be overcome by the administration of L-buthionine-S,R-sulfoximine (BSO) (20), which inhibits GCL and subsequently depletes GSH, thus sensitizing the cancer cells to radiation treatment and chemotherapy. Administration of BSO has also been shown to prolong the survival of mice infected with the parasite Trypanosoma brucei (21), the causative agent of African sleeping sickness. Similarly, BSO-mediated depletion of glutathione inhibits the development Plasmodium falciparum in red blood cells (22). BSO presumably binds as a L-glutamate analogue with its S-butyl group extending into the L-cysteine-binding site (23). Subsequent ATP-dependent phosphorylation of the sulfoxime nitrogen by GCL leads to the formation of a tightly bound transition state analogue (20, 23).

Recently, we reported the crystal structure of Saccharomyces cerevisiae GCL (ScGCL) in complex with L-glutamate, Mg\textsuperscript{2+}, and ADP (24). As the first structure of a Group 2 glutamate cysteine ligase, examination of the model provided important molecular details of substrate recognition and led to the identification of key catalytic residues. In the current study, we have determined the crystal structures of two inhibited forms of the
enzyme. The structure of ScGCL in complex with glutathione reveals the molecular details of feedback inhibition, whereas the ScGCL-BSO complex structure details the mechanism of BSO inhibition. Examination of the available ScGCL structures provides considerable insight in the catalytic mechanism of the enzyme and suggests approaches by which GCL inhibitors with greater selectivity may be attainable.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—ScGCL was expressed in *Escherichia coli* Rosetta™ (DE3) pLysS cells (Novagen) and purified to homogeneity as described previously (24). Briefly, soluble cell lysates were cleared of debris by centrifugation and ScGCL isolated by affinity chromatography using a HisTrap Chelating HP Column (GE Healthcare). The protein was further purified by size exclusion chromatography using a Sephacryl 200 gel filtration column. Purified ScGCL was dialyzed against 20 mM Tris-HCl, pH 7.4, 2 mM dithiothreitol, concentrated (Amicon stirred cell 8050, 10-kDa cut-off), flash-frozen in liquid nitrogen, and stored at −80 °C. Point mutations were introduced at residue Cys266 (C266S and C266A) by using the QuikChange site-directed mutagenesis kit (Stratagene) following the manufacturer’s protocol. All of the constructs were verified by sequencing at the University of Nebraska Genomics Facility (Lincoln, NE).

**Kinetic Assays**—Enzymatic activity was measured using an indirect assay that couples ADP production to NADH oxidation, which was monitored at 340 nm (11). The reaction mixture contained 20 mM MgCl₂, 5 mM phosphoenolpyruvic acid, 0.2 mM NADH, and 4 units each of pyruvate kinase and lactate dehydrogenase in 1 ml of buffer (100 mM Tris, pH 8.0, 150 mM KCl). The reaction was initiated by the addition of ScGCL. To determine the apparent *Kₘ* values, two of the three substrates were added to the reaction at a saturating concentration (20 mM L-glutamate, 10 mM L-cysteine, 5 mM ATP), whereas the third was varied. At high concentrations of cysteine or ATP, substrate inhibition was observed.

To examine the mode of inhibition of glutathione, the rates for the enzyme-catalyzed reaction as a function of glutamate concentration were determined in the presence of fixed concentrations of glutathione (0, 2.5, and 5.0 mM). A general mixed model of inhibition was initially selected in Prism (Graph Pad Software) to describe the dependence of rate versus substrate concentration. This global analysis indicated that glutathione was a competitive inhibitor with respect to glutamate. Following this preliminary analysis, the data were reanalyzed designating competitive inhibition (supplemental Fig. S1). For inactivation studies, ScGCL (1.75 μM) was incubated with BSO (Sigma) in 100 mM Tris, pH 8.0, containing 150 mM KCl, 20 mM MgCl₂, and 5 mM ATP at 4 °C (20, 25). At the indicated time, an aliquot was removed, and enzymatic activity was measured at saturating substrate concentrations using the coupled assay system. Representative data from three or more determinations are plotted as a function of time with the experimental errors indicated. A single-order decay was used to describe the data using the program Prism (Graph Pad Software).

### Table 1

<table>
<thead>
<tr>
<th>Substrate</th>
<th><em>Kₘ</em> (mM)</th>
<th><em>Vₘₐₓ</em> (μmol min⁻¹ mg⁻¹)</th>
<th><em>Kᵢ</em> (mM)</th>
<th><em>Vₘₐₓ</em> (μmol min⁻¹ mg⁻¹)</th>
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<tbody>
<tr>
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<tr>
<td>l-CYS</td>
<td>0.17 ± 0.01</td>
<td>10.9 ± 0.19</td>
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<tr>
<td>ATP</td>
<td>0.08 ± 0.01</td>
<td>16.1 ± 0.56</td>
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<tr>
<td>GSH</td>
<td>12.0 ± 0.18</td>
<td>2.12 ± 0.13</td>
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</table>

Crystallization, Data Collection, Structure Determination, and Refinement—Concentrated ScGCL (7 mg/ml) was crystallized in the presence of either 5 mM reduced glutathione and 20 mM MgCl₂ or 1 mM BSO, 5 mM ATP, and 20 mM MgCl₂. Crystals were grown at 18 °C out of a solution of 12% (w/v) polyethylene glycol 400, 100 mM MES, pH 6.8, with the dimensions 0.15 × 0.15 × 0.15 mm, as described previously (24). Prior to data collection, the crystals were soaked in a stabilizing solution containing 30% polyethylene glycol 400 and the appropriate ligands and then vitrified in liquid nitrogen (26). Diffraction data for the ScGCL-glutathione complex were collected using radiation produced by a Rigaku MicroMax-007 x-ray generator fitted with confocal blue optics and an R-axis IV++ image plate system (*λ* = 1.54 Å; 100 K). For the ScGCL-BSO complex, diffraction data (*λ* = 0.9 Å; 100 K) were collected on Beamline 14-BM-C of BioCARS at Argonne National Laboratory Advanced Photon Source. All of the data were processed with the HKL2000 software package (27). The structures of the ScGCL complexes were determined by molecular replacement using the PHENIX software suite (28) with the previously determined ScGCL structure (Protein Data Bank code 3IG5) as the search model. Iterative rounds of model building and refinement were carried out using Coot (29) and Refmac5 (30), respectively. As the protein models neared completion, water molecules obeying proper hydrogen-bonding constraints with electron density greater than 1.0 σ on a 2Fo − Fc map and 4.0 σ on an Fo − Fc map were also included in the final structure. Model geometry was monitored using MOLPROBITY (31), and the figures were produced using Chimera (32).

**RESULTS AND DISCUSSION**

**Kinetic Characterization of ScGCL**—Previously reported structural and biochemical data indicate that ScGCL likely functions as a monomer both *in vitro* and *in vivo* (24). To investigate its kinetic parameters, ScGCL was purified to homogeneity, and enzymatic activity was assessed using a coupled enzyme system that monitors the production of ADP (11). Apparent kinetic constants for the enzyme-catalyzed formation of γ-glutamylcysteine were determined (Table 1) and are comparable with those reported for other eukaryotic GCL (1, 11–13, 33). Inhibition by glutathione, a feedback inhibitor of ScGCL, was also examined. Glutathione is a competitive inhibitor with respect to the glutamate substrate (supplemental Fig. S1), with an apparent *Kᵢ* (GSH) of 2.12 ± 0.13 mM, similar to other Group 2 GCL holoenzymes (11–13, 33).

BSO is one of the most commonly used pharmacological inhibitors of glutathione biosynthesis, and its efficacy with respect to inhibition of ScGCL was examined (Fig. 1). A time-dependent loss of enzymatic activity was observed in the presence of Mg²⁺ and ATP at each of the BSO concentrations.
S. cerevisiae Glutamate Cysteine Ligase Inhibition

FIGURE 1. Time-dependent inactivation of ScGCL by the inhibitor BSO. ScGCL was incubated with a given concentration of BSO in the presence of Mg\textsuperscript{2+} and ATP at pH 8.0 and 4°C. Relative enzymatic activity was monitored as a function of time. The activity measurements were made in triplicate, and the data for a given BSO concentration fit to a single exponential decay. The curves are shown for the control (filled circles) and six experimental BSO concentrations (5 \( \mu \)M, filled squares; 7.5 \( \mu \)M, filled triangle; 10 \( \mu \)M, filled inverted triangle; 15 \( \mu \)M, filled diamonds; 20 \( \mu \)M, open circles; 50 \( \mu \)M, open squares).

FIGURE 2. Ribbon representations of the crystal structures of ScGCL in complex with inhibitors. An ScGCL monomer is contained in the asymmetric unit, and the N- and C-terminal residues are indicated. \( \beta \)-Strands are colored in yellow, and \( \alpha \)-helices are depicted in green. A, bound GSH is shown in space filling representation with carbon atoms colored in gray, oxygen atoms are in red, sulfur atoms are in yellow, and nitrogen atoms are in blue. The glutathione-binding site overlaps the glutamate binding site within the active site funnel. B, ADP and the transition state analogue, phosphorylated BSO (BSO-P), are shown in space filling representation. Phosphorus and magnesium atoms are colored in orange and purple, respectively, with the remaining atoms colored as in A.

TABLE 2

<table>
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<tr>
<th>Data collection and refinement statistics</th>
<th>ScGCL-GSH</th>
<th>ScGCL-BSO</th>
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tested (5 \( \mu \)M to 50 \( \mu \)M). A near linear dependence on the inactivation rate as a function of BSO concentration was observed (data not shown). At 50 \( \mu \)M BSO, ScGCL activity was reduced nearly 10-fold in \( \sim 5 \) min. Unfortunately, reliable rate measurements above this concentration of BSO could not be made because of the limitations of the assay. Nonetheless, BSO is clearly a potent inhibitor of ScGCL. Previous studies of related GCL indicated that \( \alpha \)-l-buthionine-\( S \)-sulfoximine is the relevant stereoisomer and that its enzymatic phosphorylation generates a high affinity transition state analogue (23, 34). As discussed below, the ScGCL-BSO structure supports these findings.

**Overall Structures of ScGCL-Glutathione and ScGCL-BSO Complexes**—The structures of ScGCL in complex with either glutathione or BSO were determined by molecular replacement using the apo form of ScGCL as a probe (24). In the ScGCL-glutathione complex, reduced glutathione was readily modeled into the strong positive density observed within the enzyme active site (supplemental Fig. S2). The glutamate portion of glutathione is located at the base of the active site funnel (Fig. 2A). The cysteine moiety occupies a relatively hydrophobic binding pocket, whereas the terminal glycine is near the outer edge of the active site and is solvent-exposed. In the ScGCL-BSO complex, the electron density supports the modeling of phosphorylated BSO, ADP, and three Mg\textsuperscript{2+} ions (supplemental Fig. S3). The adenine ring of ADP is located at the lower lip of the active site cavity and is solvent-exposed (Fig. 2B). The phosphorylated BSO occupies a site comparable but distinct from the glutathione-binding site, as discussed below. The overall ScGCL-glutathione and ScGCL-BSO structures are very similar to that of ScGCL in complex with glutamate and Mg\textsuperscript{2+} with an root mean square deviation for \( C\text{a} \sim 0.2 \) Å (24). Refinement statistics for the final ScGCL-glutathione and ScGCL-BSO models are provided in Table 2. The refined ScGCL-gluta-
the bound glutamate substrate. In the ScGCL/Glu/Mg\(^{2+}\) structure, the \(\gamma\)-carboxylate of the glutamate substrate occupies one of the coordination sites of the bound M1 Mg\(^{2+}\) (24). However, in the ScGCL-glutathione structure, the \(\gamma\)-carboxylate has been assimilated into the \(\gamma\)-glutamyl peptide bond and can no longer promote Mg\(^{2+}\) binding. Glu\(^{52}\) and Glu\(^{96}\), which also coordinate the Mg\(^{2+}\), maintain comparable positions in both structures, but Glu\(^{103}\) has shifted away from the M1 binding site (not shown). Loss of the M1 binding site causes the \(\gamma\)-glutamyl portion of glutathione to be shifted 0.3 Å out from the base of the active site, limiting interactions between its \(\alpha\)-carboxylate and the side chain of Arg\(^{413}\). In addition, the M2 and M3 binding sites are not significantly occupied in the absence of ATP or ADP.

An intriguing feature of the \(\gamma\)-glutamyl-binding pocket is the conserved cysteine residue, Cys\(^{266}\), which is in close proximity to the \(\alpha\)-carboxylate of glutamate. Previously, mutation of the equivalent cysteine residue in \(T.\ breuc GCL\) to an alanine had little effect on the specific activity or the substrate binding affinity of the enzyme (35). In ScGCL, substitution of this residue with either a serine (C266S) or an alanine residue (C266A) had a modest but reproducible effect on glutamate and glutathione binding (Table 3). For both mutants, the apparent \(K_m\) (Glu) and the apparent \(K\) (GSH) increased ~2-fold relative to the wild-type enzyme. Studies to examine the impact of these mutations on overall glutathione production in \(S.\ cerevisiae\) are ongoing.

**Molecular Details of the \(\beta\) Thionine-\(S\)-sulfoximine-binding Site**—In addition to glutathione, all three families of GCL can be inhibited by \(S\)-alkyl-\(\beta\)-homocysteine sulfoximines (36). As discussed above, BSO is a potent mechanism-based inhibitor of ScGCL. The enzyme catalyzes the ATP-dependent phosphorylation of BSO to form BSO phosphate and ADP, which mimic the transition state. Phosphorylated BSO binds tightly and dissociates very slowly (20, 37), making this compound pharmacologically important for development of treatments against cancer and certain parasites (21, 22, 38).

Phosphorylated BSO occupies the \(\gamma\)-glutamate and the presumed \(\beta\)-cysteine-binding sites of ScGCL (Fig. 4A). The \(\alpha\)-carboxylate and \(\alpha\)-amino groups of BSO are virtually superimposable on the comparable functional groups of the glutamate substrate (not shown). BSO is phosphorylated on the sulfoximine nitrogen, and the S-butyl group of BSO mimics \(\alpha\)-cysteine, occupying a relatively hydrophobic pocket within the enzyme active site. Arg\(^{472}\) is within hydrogen bond distance of the sulfoximine oxygen and an oxygen of the newly added phosphate group and likely stabilizes the transition state. In support of a direct role in catalysis, mutation of the equivalent arginine, Arg\(^{491}\) in \(T.\ breuc GCL\), decreased enzymatic
Phosphorylation by ATP and subsequent tight inhibitor-enzyme interaction is dependent on the metal ion binding (1). The precise locations of the three metal-binding sites are discussed below.

The crystal structures of *E. coli* (40) and *Brassica juncea* (41) GCL in complex with alkyl sulfoximine inhibitors have also been reported. Comparison with the ScGCL-BSO complex reveals a dramatic conservation of active site functionality across bacteria, plants, and nonplant eukaryotes. In these three structures, the γ-glu-tamyl-binding sites are superimposable, with the α-carboxylate adjacent to a conserved arginine residue (Arg313 in ScGCL) and the α-amino group within hydrogen bond distance of a bound water, the backbone carbonyl of residue 264, and the carboxylate of Glu52 (Fig. 4A). The proposed catalytic arginine residue, Arg472 in ScGCL, is also conserved and suggests that all three enzymes function using a similar mechanism.

**Description of the ADP-binding Site of ScGCL**—Previously, we described the structure of ScGCL in complex with glutamate, ADP, and Mg^{2+} to 2.7 Å resolution (24). The current ScGCL-BSO complex structure has been refined to significantly higher resolution (2.2 Å) and provides additional details regarding ADP binding (Fig. 4B). As described previously, the 2′ and 3′ hydroxyls of the ribose are involved in an extended hydrogen bond network. The oxygen of the furanose ring forms a hydrogen bond with an ordered water molecule that is positioned by the side chain of Arg468. Substitution of the equivalent residue in *T. brucei* GCL, Arg487, with an alanine increases the *K_m*(ATP) 15-fold (39). The C6 amino group and N7 nitrogen of the adenine ring are within hydrogen bond distance of the side chain of Arg472 in ScGCL, and the proposed catalytic arginine residue, Arg317, and phosphorylated BSO represented as solid black lines. Three Mg^{2+} ions, designated as M1, M2, and M3, are shown as purple spheres, and dashed black lines illustrate their likely coordination (interatomic distances of <2.2 Å).
importance of binding determinants. In *T. brucei* GCL, mutation of Thr\(^{323}\) (Thr\(^{370}\) in ScGCL) to an alanine dramatically increased the apparent $K_m$ for ATP (39). Interestingly, the imidazole ring of His\(^{94}\) moves ~1.4 Å toward the ADP molecule and forms hydrogen bonds with an $\alpha$-phosphate oxygen and the $\gamma$-carboxylate of Glu\(^{103}\) (not shown). Three bound Mg\(^{2+}\) ions provide additional stabilizing interactions as described below.

Three Bound Mg\(^{2+}\) Ions Contribute to the Formation and Binding of the Transition State Analogue.—In the ScGCL-BSO structure, three octahedrally coordinated Mg\(^{2+}\) ions are observed (Fig. 4C). The first metal-binding site, M1, is formed by the side chain carboxylates of Glu\(^{52}\), Glu\(^{96}\), and Glu\(^{103}\), the sulfoximine nitrogen; an oxygen of the covalently attached phosphate group; and an ordered water molecule. The M2 site is fashioned from the side chains of Gln\(^{268}\), Glu\(^{50}\), and Glu\(^{470}\), as well as from oxygen atoms from the $\beta$ phosphate of ADP and the phosphoryl group of the transition state analogue. The M3 site is in contact with oxygen atoms from each of the three phosphate groups, the carboxylates of Glu\(^{50}\) and Glu\(^{103}\), and a bound water molecule. This constellation of Mg\(^{2+}\)-binding sites facilitates the binding of ATP and positions the $\gamma$-phosphate of ATP for in-line nucleophilic attack by the $\gamma$-carboxylate of the glutamate substrate. As mentioned above, Arg\(^{472}\) is likely a key residue in this initial step of catalysis.

The coordination of these critical Mg\(^{2+}\) ions appears to be highly conserved. A similar arrangement of active site Mg\(^{2+}\) ions is observed in the equivalent *E. coli* GCL structure (40), despite less than 10% sequence identity between the Group 1 and 2 enzymes. Mutation of glutamate residues 55 and 100 in *T. brucei* GCL (equivalent to Glu\(^{52}\) and Glu\(^{103}\) in ScGCL) to alanine led to a striking loss of enzyme activity, suggesting that Glu\(^{52}\) and Glu\(^{103}\) are indispensable for catalysis (42). Substitutions at either residue likely result in the loss of Mg\(^{2+}\) binding at the M1 site. Interestingly, mutation of Glu\(^{96}\) in *T. brucei* GCL to alanine (equivalent to Glu\(^{96}\) in ScGCL) resulted in an enzyme capable of ATP hydrolysis. However, the E93A mutant could not catalyze the peptide bond formation between $\alpha$-glutamate and $\alpha$-aminobutyrate (a surrogate for $\gamma$-cysteine), suggesting that this glutamate residue may instead facilitate the nucleophilic attack of $\gamma$-cysteine on the $\gamma$-glutamylphosphate intermediate (42).

**Identification of the Cysteine-binding Site of ScGCL.—**Attempts to crystallize a pseudo-Michaelis complex have been unsuccessful. In each case, the electron density for the cysteine substrate has been quite poor, precluding the direct identification of the cysteine-binding pocket. To overcome this limitation, the ScGCL-glutathione and ScGCL-BSO structures were superimposed, and the environment surrounding the cysteine or cysteine mimic was examined (Fig. 5). The thiol group of glutathione and the $S$-butyl group of BSO overlap reasonably well and are located in a hydrophilic pocket in the ScGCL active site.
Perhaps additional conformational changes would occur if this moiety were present.

Implications for Catalysis and Inhibitor Design—The available biochemical and structural data provide many of the details of the catalytic mechanism of the enzyme (Fig. 6). Glutamate binds at the base of the enzyme active site with its side chain carboxylate occupying one of the coordination sites of the M1 Mg$^{2+}$. The nucleophilicity of the γ-carboxylate is likely increased by the adjacent Mg$^{2+}$ as well as the side chain of Arg472. The addition of Mg$^{2+}$/ATP leads to the formation of two additional magnesium-binding sites, M2 and M3, which orient the phosphate groups of ATP, placing the γ-phosphate in position for in-line attack by the activated glutamate substrate. This leads to the formation of a γ-glutamyl phosphate intermediate, which is tightly anchored in the enzyme active site, and the eventual displacement of ADP. The incoming cysteine nucleophile is potentially activated by the side chain carboxylate of Glu96, and the developing negative charge on the γ-carboxylate oxygen of the glutamate substrate is stabilized by the side chain of Arg472. Collapse of the tetrahedral intermediate leads to the expulsion of the phosphate group and the formation of the γ-glutamyl peptide bond. Additional biochemical and mutational studies to examine this proposed mechanism are ongoing. However, the essential features of catalysis appear to be conserved in related enzymes such as glutamine synthetase (43, 44), glutathione synthetase (45–47), and homoglutathione synthetase (48).

Elucidation of the detailed catalytic mechanism of GCL in conjunction with the structural studies of the inhibited ScGCL may lead to improved glutathione biosynthesis inhibitors. The
alkyl sulfoximine-based inhibitors are excellent transition state mimics that dramatically reduce enzymatic activity. Examination of the ScGCL-BSO complex suggests that additional functionalities may be engineered to increase selectivity. ScGCL and human GCL share >40% sequence identity, with nearly complete conservation of active site architecture (24), suggesting that the insights garnered from the study of ScGCL will facilitate the development of improved therapeutics that modulate glutathione production in mammalian systems.

Acknowledgments—We thank the BioCARS staff for assistance in x-ray data collection, Dr. Mark Wilson (University of Nebraska) for helpful discussions, and Dr. Melanie Simpson (University of Nebraska) for thoughtful insights and review of the manuscript.

REFERENCES

Supplemental data.

**Supplemental Figure 1.** To examine the mode of inhibition of glutathione, rates for the enzyme-catalyzed reaction as a function of glutamate concentration were determined in the presence of fixed concentrations of glutathione (0, 2.5, and 5.0 mM). Global analysis of the dependence of rate versus substrate concentration indicated that glutathione was a competitive inhibitor with respect to glutamate, with an apparent $K_i$ (GSH) of $2.12 \pm 0.13$ mM.
Supplemental Figure 2. The structure of ScGCL in complex with glutathione was determined by molecular replacement using the apo form of ScGCL as a probe. Shown in the stereodiagram is the calculated difference map prior to the inclusion of ligands and solvent in the model. Positive and negative peaks are contoured at 3.0 σ and shown in blue and red respectively. The final ScGCL-GSH model corresponding to this region of the map is shown in stick representation with carbon atoms colored in green, oxygen in red, nitrogen in blue, and sulfur in yellow. Glutathione is shown in ball and stick representation with carbon atoms colored grey.
Supplemental Figure 3. The structure of ScGCL in complex with phosphorylated BSO and ADP was determined by molecular replacement using the apo form of ScGCL as a probe. Shown in the stereodiagram is the calculated difference map prior to the inclusion of ligands and solvent in the model. Positive and negative peaks are contoured at 3.0 \( \sigma \) and shown in blue and red respectively. The final ScGCL-BSO model corresponding to this region of the map is shown in stick representation with carbon atoms colored in green, oxygen in red, nitrogen in blue, sulfur in yellow, magnesium in magenta, and phosphorus in orange. BSO and ADP are shown in ball and stick representation with carbon atoms colored grey.