RhoGC is a fusion protein from the aquatic fungus Blastocladiella emersonii, combining a type I rhodopsin domain with a guanylyl cyclase domain. It has generated excitement as an optogenetic tool for the manipulation of cyclic nucleotide signaling pathways. To investigate the regulation of the cyclase activity, we isolated the guanylyl cyclase domain from Escherichia coli with (GCwCC\textsubscript{Rho}) and without (GC\textsubscript{Rho}) the coiled-coil linker. Both constructs were constitutively active but were monomeric as determined by size-exclusion chromatography and analytical ultracentrifugation, whereas other class III nucleotidyl cyclases are functional dimers. We also observed that crystals of GC\textsubscript{Rho} have only a monomer in an asymmetric unit. Dimers formed when crystals were grown in the presence of the non-cyclizable substrate analog \(2',3',5\)''-dideoxyguanosine-5'\'''-triphosphate, MnCl\textsubscript{2}, and tartrate, but their quaternary structure did not conform to the canonical pairing expected for class III enzymes. Moreover, the structure contained a disulfide bond formed with an active-site Cys residue required for activity. We consider it unlikely that the disulfide would form under intracellular reducing conditions, raising the possibility that this unusual dimer might have a biologically relevant role in the regulation of full-length RhoGC. Although we did not observe it with direct methods, a functional dimer was identified as the active state by following the dependence of activity on total enzyme concentration. The low affinity observed for GC\textsubscript{Rho} monomers is unusual for this enzyme class and suggests that dimer formation may contribute to light activation of the full-length protein.

The rhodopsin-guanylyl cyclase fusion protein RhoGC\textsuperscript{3} was first identified in the phototactic fungus Blastocladiella emersonii by Avelar et al. (1). It is a unique protein composed of a type I (microbial) rhodopsin (2, 3) domain fused to a guanylyl cyclase catalytic domain showing homology to the rod outer segment guanylyl cyclase from vertebrate retina. It was the first retinylidene protein described that is directly coupled to an enzyme. The protein is localized to the \(B.\ emersonii\) eyespot and is essential for zoospore phototaxis, a pathway known to signal through the second messenger cGMP. RhoGC contains four domains (Fig. 1): an intracellular N-terminal domain of unknown function (N-term); a type I rhodopsin domain (Rho); a CC domain; and a guanylyl cyclase domain (GC).

RhoGC is of considerable interest as an optogenetic tool for control of cyclic nucleotide-signaling pathways (4, 5), and it has now been joined by another type I rhodopsin fusion protein, RhoPDE (6, 7), with potential as an optogenetic tool. The gene for RhoPDE was found in the genome of the choanoflagellate Salpingoeca rosetta and encodes a fusion of the rhodopsin domain with a cGMP-specific phosphodiesterase catalytic domain.

We have developed an expression/purification system and performed preliminary characterization for RhoGC in preparation of studies to elucidate the mechanism by which light controls guanylyl cyclase activity in the enzyme (8). We now turn our attention to the individual domains to more fully identify their functional contribution to the full-length RhoGC. We begin here with the guanylyl cyclase domain, GC\textsubscript{Rho}.

Crystal structures of GC catalytic domains have been determined for bacterial (9), algal (10), and human (11) proteins (see Fig. S1 for sequence alignment). GC domains from the cyanobacterium Synechocystis PCC6803 (GC\textsubscript{Cya2}) (9) and the unicellular algae Chlamydomonas reinhardtii (GC\textsubscript{Cyg12}) (10) were crystallized as homodimers, whereas the GC domain (GC\textsubscript{Hum}) from the human soluble guanylyl cyclase, sGC (11), was crystallized as both homodimer and heterodimer, although the enzyme is active only in the heterodimeric form. The active sites form at the dimer interface where catalytic residues are contributed by both monomers. Hence, dimerization is necessary for catalysis in this class of enzymes (12, 13). All three GC
Transmembrane helices were predicted and drawn by Protter (proter/start) and without (GCRho, residues 443–627) the connecting linker (CC, residues 397–442), from full-length RhoGC (Fig. 1).

Expression and purification of GCRho and GCwCCRho

We have expressed the catalytic domain of RhoGC (GCRho) in Escherichia coli, purified the protein by nickel-affinity chromatography, and show here that the isolated protein is constitutively active in catalyzing the formation of cGMP. It is a monomer in solution and crystallizes in the asymmetric unit, but it has a non-linear dependence of activity on enzyme concentration that is consistent with transient formation of an active-state dimer. Crystals of a dimeric form of GCwCCRho, were obtained from crystallization trials in the presence of the non-cyclizable substrate analog 2′,3′-dideoxyguanosine-5′-triphosphate (ddGTP), Mn$^{2+}$, and added potassium sodium tartrate, but the two protomers are arranged in a head-to-head conformation distinct from the canonical head-to-tail wreath-like structures first described for adenyl cyclases (13, 15) and then later for the guanylyl cyclases (9–11). We anticipate that both monomer and dimer structures reported here for GCwCCRho will help guide mutagenesis experiments focused on the mechanism by which light controls activity of the cyclase domain in full-length RhoGC.

Results

Expression and purification of GCRho and GCwCCRho

Conventional PCR was used to construct genes for the isolated guanylyl cyclase domain, with (GCwCC$\text{Rho}^\circ$, residues 397–627) and without (GC$\text{Rho}^\circ$, residues 443–627) the connecting linker (CC, residues 397–442), from full-length RhoGC (Fig. 1). Both constructs contained a His$_6$ tag on the C terminus for purification of the protein by nickel-affinity chromatography. Initial tests in E. coli showed that the proteins were expressed to a high level in T7-express cells under standard induction conditions (1 mM IPTG at $A_{600}$ 0.4–0.7 followed by incubation for 3 h at 37 °C), but neither protein was found in the soluble fraction after cell lysis and centrifugation. Subsequent attempts varying induction conditions, growth and expression temperature, and a change of cell lines met with limited success. In the end, coexpression with the molecular chaperones GroEL and GroES (16) dramatically increased the amount of both GCwCC$\text{Rho}^\circ$ and GC$\text{Rho}^\circ$ in the soluble fraction such that we could purify (Fig. 2A) on average about 50 mg of protein (determined spectrophotometrically using an extinction coefficient $e_{280}$ nm $= 31,065 \text{ M}^{-1} \text{ cm}^{-1}$) per liter of cell culture, a quantity sufficient to undertake biochemical and structural characterization of the proteins.

GCwCC$\text{Rho}^\circ$ and GC$\text{Rho}^\circ$, both displayed constitutive guanylyl cyclase activity that varied depending on whether Mn$^{2+}$ or Mg$^{2+}$ was used for the metal ion. As is shown in Fig. 2B, GC$\text{Rho}^\circ$ displayed much higher activity in the presence of Mn$^{2+}$ than in Mg$^{2+}$, consistent with observations from other isolated guanylyl (9–11, 16) and adenyl cyclase (12) domains. However, this is not the case when the CC linker was included. GCwCC$\text{Rho}^\circ$ displays about the same activity in Mn$^{2+}$ as in Mg$^{2+}$, and both are significantly less active than the GC domain alone in Mn$^{2+}$. These data seem to suggest that the role of CC might be to attenuate the constitutive activity of the GC$\text{Rho}^\circ$ domain, as has been suggested for other systems, but this is clearly not the case if GCwCC$\text{Rho}^\circ$ is compared with GC in the presence of Mg$^{2+}$, where GCwCC$\text{Rho}^\circ$ displays the higher activity. At 10 μM enzyme concentration, the activities were as follows: GC$\text{Rho}^\circ$ with Mn$^{2+}$ = 21 ± 1 μM/s; GCwCC$\text{Rho}^\circ$ with Mn$^{2+}$ = 1.0 ± 0.1 μM/s; GCwCC$\text{Rho}^\circ$ with Mg$^{2+}$ = 1.0 ± 0.1 μM/s, and GC$\text{Rho}^\circ$ with Mg$^{2+}$ = 0.064 ± 0.002 μM/s.

The dependence of activity on GTP concentration for GC$\text{Rho}^\circ$ in the presence of MnCl$_2$ is presented in the plot of Fig. S2, where the data are fit with a Hill-type equation using the parameters $S_{0.5} = 0.9 ± 0.1$ mm, $V_{max} = 16 ± 1$ μM/s, and $n = 1.2 ± 0.3$.

All known class III cyclases function as dimers (12, 17, 18). They can be either hetero- or homodimers, with active sites formed at the dimeric interface. Given the robust constitutive activity of both GCwCC$\text{Rho}^\circ$ and GC$\text{Rho}^\circ$, we anticipated that the proteins were assembled as homodimers in solution. Contrary to expectations, both proteins ran as monomers by size-exclusion chromatography (SEC) on a Superdex-200 column (Fig. 2C), with molecular mass estimates for the peak elution fractions from standards within 5% error of the calculated molecular mass of monomeric GC$\text{Rho}^\circ$ (21.5 kDa) and GCwCC$\text{Rho}^\circ$ (26.5 kDa).

Structure of GC

Initial crystallization trials were conducted with GCwCC$\text{Rho}^\circ$. After 3 months, a small rectangular-shaped crystal appeared in a PEG3350 solution. The structure of the enzyme was determined to 1.6 Å resolution by molecular replacement using human soluble guanylyl cyclase (PDB entry 2WZI) as a search model. The protein had crystallized in space group P2$_1$2$_1$2$_1$, with one molecule in the asymmetric unit. Although the full-length GCwCC$\text{Rho}^\circ$ construct was purified and used in the crystalliza-
Structure and monomer/dimer equilibrium of guanylyl cyclase

**Figure 2. Purification and initial characterization of the isolated GCwCCRho and GCRho domains.** A, purification of GCwCCRho and GCRho. Left panel, Coomassie-stained SDS-polyacrylamide gel showing fractions for purification of the isolated GCwCCRho domain from transformed E. coli BL21(DE3)-pGro7 cells (co-expressing GroEL/ES) using nickel-affinity chromatography. Right panel, Coomassie-stained SDS-polyacrylamide gel showing fractions from purification of GCRho. The last lane contains purified GCwCCRho for size comparison. B, initial rate data for guanylyl cyclase activity from GCRho and GCwCCRho in the presence of MnCl₂ (left panel) or MgCl₂ (right panel). Reactions were performed as described under “Experimental procedures” and contained 10 μM enzyme, 5 mM GTP, and 10 mM divalent cation. C, Äktá FPLC profiles for size-exclusion chromatography of GCRho (solid line) and GCwCCRho (dashed line) on a Superdex-200 10/300 GL column in 25 mM HEPES buffer, pH 7.0, containing 100 mM NaCl. Both proteins were loaded onto the column at a concentration of 200 μM. Molecular mass standards: 1, blue dextran, 2000 kDa, 8.5 ml (void volume); 2, aldolase, 158 kDa, 13.15 ml; 3, albumin, 67 kDa, 14.57 ml; 4, ovalbumin, 43 kDa, 15.41 ml; 5, chymotrypsinogen-A, 25 kDa, 16.8 ml; and 6, ribonuclease-A, 14 kDa, 18 ml. Both GCRho and GCwCCRho elute as monomers under these conditions.

...tion trials, electron density was not observed for the CC linker, likely as a result of proteolysis or disorder in the crystal. Repeated trials in the presence of sodium azide to prevent bacterial growth did not yield crystals, and further attempts to optimize crystallization conditions were performed with GCRho alone. Crystals of GCRho formed in the same crystallization conditions after about 3 weeks, still in space group P2₁2₁2₁ with one molecule in the asymmetric unit, and the structure was determined to 1.13 Å resolution and refined to an R_work and R_free of 0.188 and 0.195, respectively. Importantly, crystal packing did not reveal dimer formation with symmetry-related molecules.

The overall structure of the GCRho catalytic domain has five α-helices and eight β-strands (Fig. 3), as has been reported previously for other GC (9–11) and AC structures (13, 15). Clear electron density was observed for residues Thr-443 through Lys-626, with the exception of two missing loop regions not modeled in the final structure: Gly-559–Asn-562 in the β₄–β₅ loop, which has a major role in dimer formation, and Val-608–Lys-616 in the β₇–β₈ loop, which carries one of the eight conserved catalytic residues, Lys-612.

**Comparison with other guanylyl cyclases**

*Synechocystis* guanylyl cyclase Cya2. The catalytic domain (GRCya2) of the Cya2 guanylyl cyclase from the cyanobacterium *Synechocystis PCC6803* crystallized as a homodimer, and its structure was determined at 2.3 Å resolution (9).
the GC\textsubscript{Rho} monomer and molecule A of GC\textsubscript{Cy2} shows that the two proteins are very similar in overall fold with r.m.s.d. for C\textsubscript{a} atoms of 0.9 Å. The α3–β4 loop in GC\textsubscript{Rho} is four residues shorter than the corresponding loop in GC\textsubscript{Cy2}, and the α3 helix is two turns longer in GC\textsubscript{Cy2}. This structural feature appears to be unique to GC\textsubscript{Cy2} because the respective regions in Chlamydomonas (10) and human sGCs (11) were similar to that of the GC\textsubscript{Rho} monomer. The GC\textsubscript{Rho} β4–β5 and β7–β8 loops are each one residue shorter than in GC\textsubscript{Cy2}, whereas GC\textsubscript{Cy2} has an extended C terminus.

C. reinhardtii guanylyl cyclase CYG12—The catalytic domain (GC\textsubscript{CYG12}) of the soluble guanylate cyclase from the green algae C. reinhardtii crystallized as a homodimer, and its structure was solved at 2.6 Å resolution (10). A superpose of the GC\textsubscript{Rho} monomer and molecule A of GC\textsubscript{CYG12} shows that the two proteins are very similar in overall fold with r.m.s.d. for C\textsubscript{a} atoms of 1.2 Å. The α2–β2 and α5–β7 loops in GC\textsubscript{Rho} are one and two residues shorter, respectively, than the corresponding regions in GC\textsubscript{CYG12}. The C. reinhardtii protein also has an additional short α6 helix, which is not observed in other GC structures.

Human soluble guanylyl cyclase—The catalytic domain (GC\textsubscript{Hum}) of the human soluble guanylate cyclase crystallized as a heterodimer, and its structure was solved at 1.6 Å resolution (11). A superpose of the GC\textsubscript{Rho} monomer with the α-subunit of the human enzyme shows that the two proteins are very similar in overall fold with r.m.s.d. for C\textsubscript{a} atoms of 1.2 Å but that they differ in conformation of the α3–β4 loop. In addition, the α1–α2, α2–β2, and α6–β7 loops in GC\textsubscript{Rho} are five, three, and eight residues shorter than respective regions in GC\textsubscript{Hum}.

**Dimeric GC\textsubscript{Rho}**

Given that the active state of GC\textsubscript{Rho} was expected to be a dimeric enzyme, we explored other crystallization conditions that might trap a dimer in the asymmetric unit. Because the enzyme displayed significant guanylyl cyclase activity when assayed in solution (Fig. 2B), we reasoned that substrate and metal ion might facilitate formation of the dimer. We used the substrate analog 2′,3′-dideoxyguanosine 5’-triphosphate (ddGTP), which lacks the ability to be cyclized due to the absence of a hydroxyl group at the 3′-position of the sugar ring. GC\textsubscript{Rho} was mixed with ddGTP and Mn\textsuperscript{2+} and then monitored for formation of dimer by analytical ultracentrifugation (AUC). Sedimentation velocity profiles showed the existence of two separate sedimenting species consistent with the molecular mass expected for monomeric and dimeric GC\textsubscript{Rho} (Fig. 4A). Notably, GC\textsubscript{Rho} sediments exclusively as monomer in the absence of ddGTP and Mn\textsuperscript{2+}. When a similar sample was applied to the Superdex-200 column, three major species were observed, corresponding to dimer, monomer, and free nucleotide (Fig. 4B). The percentage of dimer in the SEC experiment was less than that observed in AUC likely due to slight differences in sample preparation.

**Crystal structure of the dimer**

GC\textsubscript{Rho} crystallized in space group C222\textsubscript{1}, from PEG3350 in the presence of 1 mM ddGTP, 10 mM MnCl\textsubscript{2}, and 200 mM potassium sodium tartrate additive. The structure was solved to 1.7 Å resolution by molecular replacement using the GC\textsubscript{Rho} monomer (Fig. 3) as search model and yielded two molecules in the asymmetric unit (Fig. 5A). The final model was refined to R\textsubscript{work} and R\textsubscript{free} of 0.164 and 0.196, respectively. Clear electron density was observed for residues Met-442 to Met-627 in molecule A and Ala-445 to Lys-626 in molecule B. The β7–β8 loop region residues Glu-610–Gly-615 in molecule A and Lys-612–Lys-614 in molecule B were highly disordered and not modeled in the final structure.

Superposition of the GC\textsubscript{Rho} monomer with both monomers of the dimer showed that there are no significant structural differences in sample preparation.
Figure 5. Structure of the GCRho homodimer. A, overall structure of the GCRho homodimer. Schematic representations of molecule A and B are colored green and gold, respectively. Selected secondary structure elements are labeled according to convention. Tartrate is modeled as sticks and shown in yellow. Manganese ions are shown as green spheres. Cysteine residues are shown as sticks and colored according to the respective main chain coloring. B, structural superposition of the GC homodimer with the “active conformation” of the human AC heterodimer (PDB entry 1CJU). Schematic representations are color-coded as follows: GCRho molecule A, green; GCRho molecule B, gold; and human AC molecules A and B, blue. N- and C-terminal amino acids are marked and color-coded according to the coloring of the respective molecule. C, disulfide cross-link at the dimer interface. F₀ − Fᵢ omit map is contoured at 3σ cutoff. Parts of the protein backbone have been omitted for clarity. D, tartrate-binding site at the dimer interface. F₀ − Fᵢ omit map is contoured at 4σ cutoff. Metal ion coordination and hydrogen bonding are indicated with dotted lines. Parts of the protein backbone and additional solvent molecules have been omitted for clarity.
Structure and monomer/dimer equilibrium of guanylyl cyclase

Figure 6. Analysis of disulfide cross-linking by SDS-PAGE. Protein samples (GC\textsubscript{Rho} or GC\textsubscript{wCCRho}) were incubated for 20 h under the conditions indicated in the figure before being loaded onto 10% polyacrylamide gels so as to simulate the AUC conditions in Fig. 4A. All samples were prepared in 25 mM HEPES, pH 7.0, 100 mM NaCl using either 1 mM GC\textsubscript{Hum}, or GC\textsubscript{wCCRho}. Top panels; GC\textsubscript{Rho}, bottom panels; GC\textsubscript{wCCRho}, left panels, without DTT; and right panels, with 5 mM DTT. Other conditions are as indicated in the figure: Mn, 2 mM MnCl\textsubscript{2}; Hi Mn, 20 mM MnCl\textsubscript{2}; Mg, 10 mM MgCl\textsubscript{2}; cGMP, 1 mM cGMP; GTP, 1 mM GTP; and PPI, 1 mM pyrophosphate.

| Protein Alone | GC\textsubscript{Rho} - DTT | GC\textsubscript{Rho} + DTT |
|---------------|------------------------------|---------------------------|
| 42 kDa        | 42 kDa                       | 42 kDa                     |
| 21 kDa        | 21 kDa                       | 21 kDa                     |
| 52 kDa        | 52 kDa                       | 52 kDa                     |
| 26 kDa        | 26 kDa                       | 26 kDa                     |

| Protein Alone | GC\textsubscript{wCCRho} - DTT | GC\textsubscript{wCCRho} + DTT |
|---------------|-------------------------------|--------------------------------|
| 42 kDa        | 42 kDa                        | 42 kDa                        |
| 21 kDa        | 21 kDa                        | 21 kDa                        |
| 52 kDa        | 52 kDa                        | 52 kDa                        |
| 26 kDa        | 26 kDa                        | 26 kDa                        |

*Figure 6. Analysis of disulfide cross-linking by SDS-PAGE. Protein samples (GC\textsubscript{Rho} or GC\textsubscript{wCCRho}) were incubated for 20 h under the conditions indicated in the figure before being loaded onto 10% polyacrylamide gels so as to simulate the AUC conditions in Fig. 4A. All samples were prepared in 25 mM HEPES, pH 7.0, 100 mM NaCl using either 1 mM GC\textsubscript{Hum}, or GC\textsubscript{wCCRho}. Top panels; GC\textsubscript{Rho}, bottom panels; GC\textsubscript{wCCRho}, left panels, without DTT; and right panels, with 5 mM DTT. Other conditions are as indicated in the figure: Mn, 2 mM MnCl\textsubscript{2}; Hi Mn, 20 mM MnCl\textsubscript{2}; Mg, 10 mM MgCl\textsubscript{2}; cGMP, 1 mM cGMP; GTP, 1 mM GTP; and PPI, 1 mM pyrophosphate.*

changes in the monomer units, with r.m.s.d. for C\textalpha{} atoms of 0.4 and 0.3 Å for monomer A and monomer B, respectively, although minor changes were observed in part of the dimer interface around the α1 helix (Asn-460–Ser-469) and β4–β5 loop (Leu-558–His-564). Nonetheless, the monomer units do not form the signature head-to-tail wreath-like conformation observed in other GC (9–11) and AC (13–15) dimeric structures. Instead, the two monomers are in a head-to-head arrangement. Superposition of the GC\textsubscript{Rho} dimer with the active arrangement. Superposition of the GCRho dimer with the active conformation of the mammalian AC heterodimer (PDB entry 1CJU (14)), using monomer A as a reference, shows that monomer B of GC\textsubscript{Rho} is rotated by 101° and displaced by 6.3 Å with respect to AC monomer B (Fig. 5B). The large rotation of monomer B compared with the AC monomer suggests that the GC\textsubscript{Rho} dimer is non-functional.

The GC\textsubscript{Rho} dimer has a buried surface area of 1273 Å\textsuperscript{2} and geometric surface complementarity (S\textsubscript{g}) (19) of 0.63. The buried surface area is comparable in magnitude to the buried surface area for the C. reinhardtii cyclase GC\textsubscript{CYG12} (1370 Å\textsuperscript{2}; 3ET6), for the cyclase GC\textsubscript{CYa2} from Synechocystis (1548.9 Å\textsuperscript{2}; 2W01), and for the human sGC heterodimer GC\textsubscript{Hum} (1230 Å\textsuperscript{2}; 3UVJ). The shape complementarity statistic, S\textsubscript{sh}, is also similar in magnitude to those of other adenylyl and guanylyl cyclases (AC, 0.59; GC\textsubscript{CYG12}, 0.64; GC\textsubscript{CYa2}, 0.67; GC\textsubscript{Hum}, and 0.74).

Part of the dimer interface is formed by an unusual interaction of β-strands β4 and β5 of monomer A with those of monomer B. In other GC dimeric structures (9–11), this β-sheet interacts with strands β2 and β3 to form the dimer interface. Significantly, monomers A and B of GC\textsubscript{Rho} are connected by a disulfide bridge involving the catalytically important residue Cys-566 (8, 20, 21) in β5 from both monomers (Fig. 5C), reinforcing the conclusion that this is an inactive form of the protein.

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After initial refinement, difference Fourier density appeared at the cavity formed by the catalytically important residues Asp-457, Asp-501, and Arg-545 (12–14) from both monomers A and B (Fig. 5D). The density was further resolved into two 22 σF\textsubscript{o} − F\textsubscript{c} peaks and an overlapping, elongated ~8 σF\textsubscript{o} − F\textsubscript{c} peak. Both 22 σ peaks were modeled with a Mn\textsuperscript{2+} ion. The 8 σ peak electron density was not fit well by ddGTP, cGMP, or PP\textsubscript{i}, but was modeled well by the tartrate additive. Mn\textsuperscript{2+} is also hexa-coordinate with Asp-457 O\textsubscript{62} of molecule B, Asp-501 O\textsubscript{61} of molecule A, O2 and O6 atoms of the tartrate molecule, and two water molecules as ligands. Mn\textsuperscript{2+} is also hexa-coordinate with Asp-457 O\textsubscript{62} and Asp-501 O\textsubscript{61} and O\textsubscript{62} of molecule B, O1 and O3 atom of the tartrate molecule, and a single water molecule as ligands. Tartrate is perfectly sandwiched between the side chains of the catalytically important residue R545 of both monomer A and monomer B. Apart from metal ion coordination, tartrate also forms hydrogen bonds with amino acids D457, T462, and D501 of molecule A, D457, I458, T462, and D501 of molecule B, and with several water molecules.

dependence on Mn\textsuperscript{2+}

Discovery of the disulfide cross-link in the GC\textsubscript{Rho} dimer caused us to revisit the AUC experiments where we could show that no dimer species was formed in the presence of a reducing agent (5 mM DTT). Likewise, subsequent crystal trials with ddGTP and Mn\textsuperscript{2+} in the presence of DTT produced only crystals with monomer in the asymmetric unit and cell dimensions and space group identical to those observed in the absence of nucleotide. Therefore, we conducted a series of experiments to determine what factors were important for formation of the disulfide cross-linked GC\textsubscript{Rho} dimer as assayed by SDS-PAGE. As is shown in Fig. 6, the single factor most important was the...
Structure and monomer/dimer equilibrium of guanylyl cyclase

Figure 7. GCAC activity data and omit map. A, initial rate data for catalytic activity of GCAC when provided ATP (black circles) or GTP (red circles) as a substrate. The reactions contained 5 mM substrate (GTP or ATP) and 10 mM MnCl2. B, omit map contoured at 3σ cutoff shows electron density for the two mutated active site residues Lys-497 and Asp-566 in GCAC. Secondary structure elements are colored green and labeled according to convention. Amino acid side chains are shown as sticks. Hydrogen bonding is indicated by the dotted line. Part of the protein backbone has been removed for clarity.

Indirect evidence for functional dimerization

Indirect evidence for formation of an active dimer was obtained by following the dependence of enzyme activity on the concentration of protein in the assays. As shown in Fig. 8, guanylyl cyclase activity showed a distinctly nonlinear dependence on total enzyme concentration that was well fit by Equation 1 under “Experimental procedures” for a monomer/dimer equilibrium in which only the dimer is active. The parameters used to fit Equation 1 to the data are presented in Table 1. The inclusion of DTT in the reaction had no effect on activity demonstrating that the disulfide cross-linked dimer observed in the crystal structure does not form during the short incubation times used here in the activity assays. It is evident from the data in Fig. 8 and Table 1 that the effect of the CC domain on quaternary structure of the protein is a rather modest 3–4-fold decrease in $K_D$ for formation of the dimer.

To eliminate concern that the C-terminal His6 tag might interfere with GC-Rho dimerization, a new construct was generated with an N-terminal His6 tag and TEV-proteolysis site inserted between the His6 tag and the remainder of the GC-Rho catalytic domain, and the dependence of activity on enzyme concentration repeated with the TEV-proteolysed enzyme. The data were again well fit by Equation 1 with an obviously non-linear dependence of activity on enzyme concentration giving a $K_D$ value of $64 \pm 9 \mu M$, well within experimental error of the $K_D$ values for GC-Rho reported in Table 1.

Active-state dimer

Because we could not crystallize an active-state dimer of GC-Rho, we modeled what the dimer might look like based upon superposition with the published structure of the mammalian adenyl cyclase VC1IC2 catalytic domain in complex with Mn$^{2+}$ and ddATP (Fig. S4) (14). The active-site residues at the dimer interface align nicely between the two structures, although side chain conformations show some differences, as is expected given that GC-Rho crystallized as a monomer.
domains suppress constitutive activity of the catalytic domains (9–11, 16).

Although there were clear similarities of GC_Rho and GCwCC_Rho in comparison with other guanylyl cyclases, a striking difference was encountered in crystal structures of the GC_Rho domain where the protein crystallized under standard conditions with only a single subunit in the asymmetric unit. This was unexpected because both GCwCC_Rho and GC_Rho display significant guanylyl cyclase activity. All other guanylyl cyclases crystallize as dimers with two monomers in the asymmetric unit (9–11), and the active sites of adenylyl and guanylyl cyclases are known to be formed at the interface of the two protomers in a dimeric complex (17, 18). The unusual crystallization results for GC_Rho were supported by SEC and AUC experiments showing that the protein was 100% monomer in solution. To be sure, monomers of other guanylyl cyclases have been observed in solution studies (9, 16), but the monomer/dimer equilibria are more heavily shifted toward the dimer, and, as noted above, the other guanylyl cyclases all crystallized with a dimer in the asymmetric unit. There are two reports in which adenylyl cyclases crystallized as a monomer: one for a Phe to Arg mutant involving a residue in the dimer interface of the Mycobacterium tuberculosis guanylyl cyclase Rv1625c catalytic domain (22), and the other for the catalytic domains of the adenylyl cyclases, GRESAG4.1 and GRESAG4.3, from Trypanosoma brucei (23). Rv1625c forms an inactive dimer in solution (22) whereas the T. brucei enzymes display weak subunit affinity and transient formation of catalytically active dimers in solution (23).

We were able to crystallize a GC_Rho dimer in the presence of dGTP, Mn^{2+}, and added potassium sodium tartrate. The two subunits of the dimer are nearly identical in overall fold to the isolated GC_Rho monomer and to the individual subunits found in dimer structures of other guanylyl cyclases, but the arrangement of the two subunits is very different from the canonical wreathlike structure first described for adenylyl cyclase (13–15) and later found in the guanylyl cyclases (9–11). The subunits of

Table 1

| Construct and condition |  \( K_D \) \( \mu M \) |  \( k_{cat} \) \( s^{-1} \) |
|-------------------------|-----------------|-----------------|
| GC_Rho–MnCl\(_2\)      | 78 ± 12         | 10 ± 1          |
| GC_Rho–MnCl\(_2\) and DTT | 83 ± 30     | 9.6 ± 1.6       |
| GC_Rho–MgCl\(_2\)      | 76 ± 25         | 0.16 ± 0.02     |
| GCwCC_Rho–MnCl\(_2\)  | 23 ± 15         | 2.5 ± 0.5       |

Figure 8. Guanylyl cyclase activity as a function of enzyme concentration. A, rate of cGMP formation as determined by the HPLC assay is plotted as a function of total enzyme concentration showing a non-linear dependence that is well fit by Equation 1 under “Experimental procedures” relating initial rate to a monomer/dimer equilibrium model in which only the dimer is active. The construct and condition for each set of data are as indicated in the figure. B, data for GC_Rho with MnCl\(_2\) are re-plotted from A on a more sensitive scale to show clearly the non-linear dependence on enzyme concentration. All reactions contained 10 mM GTP and 20 mM metal ions (Mn\(^{2+}\) or Mg\(^{2+}\)). The dotted curves are fits of Equation 1 to the data using the parameters of \( K_D \) and \( k_{cat} \) listed in Table 1. The enzyme concentration is total enzyme concentration showing a non-linear dependence that is well fit by Equation 1 under “Experimental procedures” relating initial rate to a monomer/dimer equilibrium model in which only the dimer is active. The construct and condition for each set of data are as indicated in the figure.
GC\textsubscript{Rho} dimer are displaced by 6 Å and rotated by 101° with respect to the arrangement of subunits in the adenylyl cyclase dimer (14) such that the conserved active-site residues are dispersed in the protein and are unlikely to support binding of nucleotide. In addition, the GC\textsubscript{Rho} dimer contains a disulfide bond involving Cys-566, an active-site residue known to be critical for activity of the protein (8). Thus, it is clear that this dimer cannot be part of the catalytic cycle of the enzyme.

Unusual dimer structures have been described for both the Rv1264 (24) and Rv1625 (22) adenylyl cyclases from \textit{M. tuberculosis}, and in this context the domain-swapped head-to-head dimer observed for WT Rv1625 (22) shares some similarity with the head-to-head dimer we see here for GC\textsubscript{Rho}. It is unlikely that the disulfide in GC\textsubscript{Rho} would form under intracellular reducing conditions, and it seems most probable that the disulfide forms subsequent to dimerization as a consequence of a high local concentration of the two Cys residues in an oxidizing environment. Thus, the disulfide could function as a kinetic trap for the dimer. The dimer structure does not appear to result from two monomers that were brought together in a random and haphazard association as would be expected if initial formation of a disulfide cross-link were followed by formation of the dimer. To begin, the GC\textsubscript{Rho} dimer has a buried surface area and geometric surface complementarity (\(S_g\)) comparable in magnitude to those of other guanylyl and adenylyl cyclases. The C2 axis of the GC\textsubscript{Rho} dimer is rotated by 90° with respect to the canonical AC dimer such that six of the conserved active-site residues, three identical residues from each subunit (Asp-457, Asp-501, and Arg-545), come together to form a ligand-binding pocket for tartrate and two Mn\textsuperscript{2+} ions. The Asp residues bind the Mn\textsuperscript{2+} ions much as do the corresponding residues in adenylyl cyclase, and the conserved Arg residues bind to the tartrate carboxylates much as the corresponding residue in adenylyl cyclase binds the terminal phosphate in dATP (14). Interestingly, the disulfide bond and Mn\textsuperscript{2+}/tartrate ligand-binding site are located on the C2 axis, at opposite ends of the GC\textsubscript{Rho} dimer interface. Nonetheless, the disulfide-linked dimer is likely a crystallization artifact, and it remains to be determined experimentally whether this particular oligomeric state has any relevance for regulation of guanylyl cyclase activity.

Irrespective of whether or not the observed dimer is involved in GC\textsubscript{Rho} function, it seems clear that the catalytically active state must be a dimer, similar in arrangement of subunits to that of adenylyl cyclase (13, 14). We have not been able to provide evidence for such a dimer by direct methods such as SEC, AUC, and crystallography, but we could provide evidence through the non-linear dependence of guanylyl cyclase activity on enzyme concentration. It is likely that Mn\textsuperscript{2+} and substrate assist in formation of the dimer under these conditions, but even so, the \(K_D\) value for formation (20–80 \(\mu\text{M}\)) is much higher than for other guanylyl cyclases (e.g. the \(K_D\) value for the catalytic domain of the mammalian soluble guanylyl cyclase is 0.45 \(\mu\text{M}\)) (16). The extremely high \(K_D\) value suggests that formation of the active-state dimer might be part of the light-activation mechanism. Future work in the laboratory will explore the role of domain dimerization in the light-dependent activation of full-length RhoGC.

**Experimental procedures**

**Materials and methods**

Unless specified otherwise, all materials were as described previously (8).

**Protein expression and purification**

Two constructs containing the GC domain of RhoGC were used in these studies: one composed of the GC domain alone (“GC\textsubscript{Rho}”; amino acids 443–627), and the other that included the CC domain as well (“GCwCC\textsubscript{Rho}”; amino acids 397–627). Both were cloned into a pET15b vector for expression in \textit{E. coli} BL21(DE3) cells and included a C-terminal His\textsubscript{6} tag for purification on a nickel-affinity column. Both genes also included a Val codon after the first methionine to improve bacterial expression. The BL21(DE3) cell line harbored an additional pGro7 plasmid (Takara Bio Inc.) containing the genes for the GroEL and GroES molecular chaperone proteins under the control of the AraB promoter. Competent cells were transformed with either pET15b-GC\textsubscript{Rho} or pET15b-GCwCC\textsubscript{Rho} according to standard procedures and incubated overnight at 37 °C on LB-agar plates containing 100 \(\mu\text{g}/\text{ml}\) ampicillin and 20 \(\mu\text{g}/\text{ml}\) chloramphenicol. Single colonies were used to inoculate small growth cultures (10 ml of LB containing 100 \(\mu\text{g}/\text{ml}\) ampicillin and 20 \(\mu\text{g}/\text{ml}\) chloramphenicol), which were grown for 14–18 h with shaking at 220 rpm and 37 °C. One ml of the overnight culture was used to inoculate a liter of LB containing both antibiotics. 500 mg of 3-(−)-arabinose (Acros Organics) was added upon inoculation to begin induction of GroEL/ES. Cells were grown at 37 °C until an \(A\text{\textsubscript{600}}\) 0.4–0.7 was reached, at which point expression of the protein was induced by addition of IPTG to a final concentration of 250 \(\mu\text{M}\). Cells were then incubated with shaking at 20 °C for 20 h and harvested by centrifugation at 4000 rpm in a Beckman Coulter JLA-8.10000 rotor for 15 min at 4 °C. Cell pellets were stored at −80 °C until needed.

The frozen cell pellets were thawed on ice and resuspended to a final volume of 50 ml with lysis buffer (25 mM HEPES, pH 7.0, 100 mM NaCl, and 20 mM imidazole) containing 1 mM PMSF. The cell suspension was sonicated on ice with a Misonix Sonicator 3000 at 70-watt power with a cycle of 20 s on and 20 s off for a total of 4 min. Sonicated cells were centrifuged in a Beckman Coulter JA-20 rotor at 16,000 rpm for 30 min at 4 °C to pellet cell debris, and the supernatant fraction was passed through a 0.22-\(\mu\text{m}\) filter before loading onto a pre-equilibrated 5-ml prepacked HiTrapFF nickel-Sepharose column (GE Healthcare) at 1 ml/min. The column was washed with 10 column volumes of lysis buffer followed by 20 volumes of lysis buffer containing 40 mM imidazole, and the protein was eluted with an 80-ml linear gradient of 40–500 mM imidazole in lysis buffer. Protein was monitored by absorbance at 280 nm, and fractions containing GC\textsubscript{Rho} (or GCwCC\textsubscript{Rho}) were identified by SDS-PAGE and pooled before concentration with an exchange of buffer to remove imidazole (10-kDa molecular mass cutoff Amicon Ultra Centrifuge Filter from EMD Millipore). Purified GC\textsubscript{Rho} and GCwCC\textsubscript{Rho} were frozen in small concentrated aliquots at −80 °C until needed.

Size-exclusion chromatography (SEC) on an Äkta FPLC system (Amersham Biosciences, Uppsala, Sweden) equipped with
Structure and monomer/dimer equilibrium of guanylyl cyclase

A Superdex-200 10/300 GL gel filtration column (GE Healthcare) was used to determine the oligomeric state of the proteins. 300 μL of 200 μM purified GC_Rho (or GCwCC_Rho) was loaded onto the Superdex column that had been pre-equilibrated with 25 mM HEPES, pH 7.0, and 100 mM NaCl at 4 °C, and the column was run at a constant rate of 0.5 mL/min. Protein was monitored by absorbance at 280 nm, and elution volume was compared with a standard curve using soluble protein standards to estimate the molecular mass of the peak fraction.

A separate GC_Rho construct, GC_NH4TEV, was designed with a TEV-protease site (ENLYFQG) inserted immediately after the N-terminal His6 tag and followed by a Met and the remainder of GC_Rho sequence to determine whether the affinity tag interfered with dimerization of the protein in solution. The protein was expressed and purified as presented above. The purified protein was proteolyzed with a 30:1 ratio of GC_NH4TEV to TEV-protease by nickel-affinity chromatography, as confirmed by SDS-PAGE and anti-His6 Western blot analysis. Both TEV-cleaved and -uncleaved GC_NH4TEV standards were used to estimate the molecular mass of the peak fraction.

Analytical ultracentrifugation

Sedimentation velocity experiments were designed to identify factors (e.g. salt, enzyme concentration, divalent cation, etc.) that could impact the oligomeric state of isolated GC_Rho protein (25). All centrifugation steps were carried out using a ProteomeLab Optima XL-A ultracentrifuge (Beckman Coulter). In all cases, 395 μL of protein was loaded into double sector centerpieces balanced by 400 μL of reference solution containing all components of the sample solution except the protein. All cell housings were loaded into a four-hole An-60 Ti rotor and spun at 35,000 rpm at 22 °C for 15 h. Absorbance at 280 nm was measured to track protein concentration during sedimentation. Data were processed and analyzed using SEDFIT software (National Institutes of Health, Version 14.4b). Data were fit using a continuous c(s) distribution model based on theory for a soluble and globular (spherical) protein with self-association. Parameters used to fit the raw absorbance data to a confidence level of 95% included assumptions for average protein partial specific volume (0.73) as well as for buffer density (1.00 g/mL) and buffer viscosity (0.01002 units) being identical to pure water as no viscous agents such as glycerol were used to stabilize the protein in solution.

Enzymatic activity assays

Guanylyl cyclase activity was measured using reversed-phase (RP)-HPLC to follow formation of cGMP and disappearance of GTP. 100-μL reactions were prepared in 50 mM Tris buffer, pH 7.6, containing 50 mM NaCl and 0.5 mM EDTA. Reactions contained GTP and metal ion (MgCl2 or MnCl2) at the concentrations indicated in the figures and were initiated by addition of enzyme. 20-μL aliquots were quenched at specified time points (30 s to 5 h) by combining with an equal volume of 1 n HCl. Precipitated protein was removed with 0.22-μm Spin-X centrifugal filters (Corning Costar) by centrifugation at 5000 rpm for 5 min in a tabletop centrifuge. The samples were then neutralized with 20 μL of 1 M potassium phosphate, pH 8.5, and applied to a 250 × 2.1-mm ACE 5 C18-AR reversed-phase 5 μm column connected to an Agilent 1260 Infinity HPLC system with a G136D 1260 multiwavelength detector. Nucleotides were separated by isocratic elution with a 100 mM potassium phosphate buffer, pH 6.2, at a flow rate of 0.4 mL/min and monitored by absorbance at 254 nm. Peaks were integrated with OpenLab CDS ChemStation software and compared with peaks from standards for GTP and cGMP of known concentration.

Assays for adenylyl cyclase activity were performed identically except that the reaction contained ATP instead of GTP, and the HPLC column running buffer was 100 mM potassium phosphate, pH 6.2, containing 10% (v/v) methanol.

The concentration of stock solutions was determined spectrophotometrically using extinction coefficients of 13,700 M−1 cm−1 at 252 nm for ATP, 12,320 M−1 cm−1 at 260 nm for cGMP, 15,400 M−1 cm−1 at 259 nm for ATP, and 15,000 M−1 cm−1 at 260 nm for cAMP.

The dependence of activity on enzyme concentration was evaluated through a non-linear least-squares fit (MATLAB) of the rate data to Equation 1 for a reaction in which the only active species is a dimer of the enzyme in a monomer/dimer equilibrium,

\[

\nu = \frac{k_{cat} \times (4E + K_D) - \sqrt{K_D^2 + (8E \times K_D)}}{8}

\]

where \( \nu \) = reaction rate (μM/s); \( k_{cat} \) = turnover number (s−1); \( E \) = total enzyme concentration (μM); and \( K_D \) = the equilibrium dissociation constant for dimerization of the enzyme, defined as \( K_D = [\text{monomer}]^2 / [\text{dimer}] \) with units of μM. \( K_D \) and \( k_{cat} \) are parameters determined through the fitting process. These reactions contained 10 mM GTP and 20 mM metal ion (Mn2⁺ or Mg2⁺), which was saturating for all enzyme concentrations tested.

SDS-PAGE analysis of disulfide cross-links

Samples of GC_Rho and GCwCC_Rho were incubated for 20 h under various conditions, as indicated in Fig. 6, to test for the formation of intermolecular disulfide bonds. Each sample contained 1 μM GC_Rho or GCwCC_Rho in 25 mM HEPES, pH 7.0, and 100 mM NaCl. Metal ion (Mn2⁺ or Mg2⁺ ), GTP substrate, and cGMP and inorganic pyrophosphate products were included as indicated in the figure. Each sample was diluted 2:1 with gel load buffer, with or without 5 mM DTT, and then loaded onto a 10% Mini-PROTEAN TGX polyacrylamide gel (Bio-Rad) for analysis. Protein bands were visualized with Coomassie Brilliant Blue stain.

Crystallization

Crystallization trials were performed by sitting drop vapor diffusion at room temperature using Hampton (Hampton Research, CA) and Jena Bioscience (Jena Bioscience, Jena, Germany) sparse matrix crystallization screens. Drops were set with a Phoenix robot (Art Robbins Instruments, CA) by mixing...
protein (GCRho, GCwCCRho, or GCAC; 20 mg/ml) in 25 mM HEPES, pH 7.0, containing 100 mM NaCl with crystallization mother liquor in a 1:1 ratio. Crystals of GCRho appeared after about 3 weeks in 25% w/v PEG3350, 100 mM BisTris, pH 5.5, and 200 mM NaCl, whereas GCAC crystals formed in 15% w/v PEG20000 and 10 mM potassium hydrogen tartrate. These crystals contained a single subunit in the asymmetric unit. In some trials, the protein solution also contained 10 mM MgCl2 (or MnCl2) and 1 mM ddGTP. In this case (MnCl2), crystals appeared in 20% (w/v) PEG3350 and 200 mM potassium sodium tartrate and contained a disulfide cross-linked dimer in the asymmetric unit.

**Data collection, processing, and refinement**

Crystals were soaked in reservoir solution containing 15% glycerol as cryoprotectant before flash-freezing in liquid nitrogen. Diffraction data were collected at 100 K with beamline 8.2.1 at the Advanced Light Source (Lawrence Berkeley National Laboratory, Berkeley, CA) using an ADSC Q315R CCD detector (Area Detector Systems Corp.). The best crystals diffracted up to 1.1 and 1.4 Å resolution for the GCRho CCD detector (Area Detector Systems Corp.). The Berkeley National Laboratory, Berkeley, CA) using an ADSC nitrogen. Diffraction data were collected at 100 K with glycerol as cryoprotectant before flash-freezing in liquid.

**Table 2**

| Crystallographic data collection and refinement statistics |
|----------------------------------------------------------|
| **GC**<sub>Rho</sub>-monomer | **GC**<sub>Rho</sub>-dimer | **GC**<sub>AC</sub>-monomer |
| PDB ID | 6AO9 | 6AOB | 6AOA |
|---------|------|------|------|
| **Data collection statistics** | | | |
| Space group | P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> | C2<sub>2</sub>2<sub>1</sub> | P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> |
| Resolution range (Å) | 20 – 1.13 | 20 – 1.70 | 20 – 1.40 |
| Highest resolution shell (Å) | 1.19 – 1.13 | 1.79 – 1.70 | 1.48 – 1.40 |
| Unit cell parameters (Å) | a = 34.5, b = 65.6, c = 90.6 | a = 91.8, b = 95.9, c = 100.1 | a = 34.4, b = 65.4, c = 88.4 |
| Total reflections | 980,795 | 617,510 | 280,921 |
| Unique reflections | 77,710 | 48,964 | 40,049 |
| Completeness %<sup>a</sup> | 99.9 (99.7) | 99.3 (99.7) | 99.8 (100) |
| R<sub>merge</sub> (%) | 7.7 (100) | 10.6 (130) | 11.1 (42.3) |
| R<sub>merge</sub> (%)<sup>b</sup> | 15.2 (2.2) | 16.1 (2.0) | 8.7 (2.9) |
| Redundancy | 12.6 (10.2) | 12.8 (12.3) | 7.0 (7.0) |
| **Refinement statistics** | | | |
| Resolution range (Å) | 20 – 1.13 | 20 – 1.70 | 18 – 1.40 |
| No. of reflections used | 77,532 | 48,333 | 39,937 |
| R<sub>work</sub> % | 18.8 | 16.4 | 19.0 |
| R<sub>free</sub> % | 19.5 | 19.6 | 20.8 |
| Protein atoms | 1384 | 2842 | 1388 |
| Ligand atoms | 0 | 10 | 0 |
| Metal atoms | 0 | 2 | 0 |
| Water molecules | 338 | 507 | 320 |
| r.m.s.d. in bond lengths (Å) | 0.005 | 0.006 | 0.005 |
| r.m.s.d. in bond angles (°) | 0.8 | 0.8 | 0.8 |

<sup>a</sup> Highest-resolution shell values are given in parentheses.

Rigid body refinement followed by positional and B-factor refinement was carried out using phenix.refine (31) from the PHENIX software suite version 1.11 (32). Simulated annealing was included in earlier refinements to minimize the initial model bias. Manual model building was done using COOT version 0.8 (33). Water molecules were included in the final refinement after satisfying the criteria of 3<sub>Fo</sub> – 2<sub>Fc</sub> and 1<sub>σ</sub> 2<sub>Fo</sub> – 2<sub>Fc</sub>. Several iterative cycles of refinement were carried out before final submission of data. Data collection and final refinement statistics are given in Table 2. Data sets for the GCRho monomer (PDB entry 6AO9), GCAC (PDB entry 6AOA), and GCRho dimer (PDB entry 6AOB) have been submitted to the Protein Data Bank. All crystal structure figures in this paper were prepared using PyMOL version 1.8 (Schrödinger LLC, Portland, OR).

**Author contributions**—D. D. O. conceived and coordinated the study. R. P. K. and B. R. M. were responsible for all aspects of the experimental program. J. F. was involved in the initial development of the expression and purification system for the guanylyl cyclase domain. M. M. T. was involved in the development of the HPLC method for enzymatic assays and in the construction of the GCAC mutant. D. H. Z. performed assays for dependence of activity on enzyme concentration (Fig. 8). M. O. L. developed and performed disulfide cross-linking assays (Fig. 6). D. D. O., R. P. K., and B. R. M. wrote the paper. All authors discussed and commented on the manuscript.

**Acknowledgments**—This research used resources of the Advanced Light Source, which is a Department of Energy Office of Science User Facility under Contract No. DE-AC02-05CH11231. We are grateful to the staff at the Advanced Light Source–Berkeley Center for Structural Biology for their assistance in X-ray data collection. We thank the Adar family and friends for continued support throughout this work. We also thank Prof. Timothy Street and Jackson Halpin for their HPLC and providing technical advice. We thank Prof. Bruce Foxman for helpful discussions on crystallography.
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