Cobalamin-dependent methionine synthase (MetH) is a modular protein that catalyzes the transfer of a methyl group from methyltetrahydrofolate (CH$_3$-H$_4$folate) to homocysteine (Hcy), forming tetrahydrofolate (H$_2$folate) and methionine. The enzyme from *Escherichia coli* is one of the best-studied members of a large class of cobalamin- and corrinoid-dependent methyltransferases (2). In this class of enzymes, the cobalamin cofactor plays a crucial role in catalysis, acting both as a methyl donor and acceptor. During the MetH reaction cycle the methylcobalamin form of the cofactor [MeCo(III)Cbl] is demethylated by Hcy. The electrons of the carbon–cobalt bond remain on the cofactor during catalysis, oxidizing the cobalt to cob(II)alamin (Co(I)Cbl). Co(I)Cbl is subsequently remethylated by CH$_3$-H$_4$folate regenerating the MeCo(III)Cbl cofactor (Fig. 1A). However under aerobic conditions, the cobalamin cofactor is oxidized to an inactive cob(II)alamin [Co(II)Cbl] form about once in every 2,000 turnovers (3). To avoid accumulation of this inactive species, the enzyme undergoes a reductive reaction, in which Co(II)Cbl is reduced to Co(I)Cbl with an electron supplied by flavodoxin (Fld), and Co(I)Cbl is then remethylated using S-adenosylmethionine (AdoMet) (4, 5). Reductive reactivation is a challenging reaction because the reduction potential of Co(II)Cbl in solution is out of the range of physiological reducing agents (6–8).

MetH has 4 functional units that bind Hcy, CH$_3$-H$_4$folate, Cbl, and AdoMet, respectively (1). During catalysis and reactivation, the appropriate substrate-binding module must be positioned close to the cobalamin to allow methyl transfer. A major challenge is to understand what guides the enzyme through its complex conformational changes without permitting futile cycling. Although the structure of the full-length enzyme has not yet been determined, there are structures of the individual cobalamin-binding (9) and AdoMet-binding modules (10) from *E. coli*. In addition, 2 multimodular fragments have been characterized: an N-terminal polypeptide (Hcy and CH$_3$-H$_4$folate modules) from *Thermotoga maritima* (11, 12) and a C-terminal polypeptide (cobalamin and AdoMet modules) from *E. coli* (12, 13). During catalysis the enzyme cycles between conformations in which the cobalamin-binding module is juxtaposed alternatively with the Hcy-binding module and the CH$_3$-H$_4$folate-binding module, whereas for the reductive reactivation the enzyme assumes a conformation in which the cobalamin-binding module is juxtaposed with the AdoMet-binding module. Access to this reactivation conformation is rigorously controlled to

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**Fig. 1.** (A) Reaction cycle of MetH. The primary catalytic cycle is shown in red whereas the secondary reactivation cycle is shown in blue. (B) MeCo(ll)Cbl in the free state with a N atom of the dimethylbenzimidazole group coordinat- ing Co in the lower axial position. A methyl ligand occupies the upper axial position. (C) MetH-bound MeCo(ll)Cbl with H759 as the lower axial ligand. (D) Schematic drawings of the preferred coordination numbers and geometries for the Cbl oxidation states that are relevant to MetH function. The superscripts a, b, and c in part A indicate the locations of these forms of the cofactor in the catalytic and reactivation cycles.

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**Author contributions:** M.K., S.D., and R.G.M. designed research; M.K., S.D., and K.A.P. performed research; M.K., S.D., K.A.P., J.L.S., and R.G.M. analyzed data; and M.K., S.D., and R.G.M. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: The structure coordinates reported in this study have been deposited in the Protein Data Bank, www.rcsb.org [PDB ID codes 3IVA for the MetH Co(II)Cbl I690C/G743C MetH(649–1227) and 3IV9 for the AquoCo[l]Cbl I690C/G743C MetH(649–1227)].

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**Insights into the reactivation of cobalamin-dependent methionine synthase**

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avoid futile cycling (14). A major factor in controlling access to the catalytic and reactivation cycles of the enzyme is the coordination of the Cbl, which in turn is responsive to the positive charge and the oxidation state of the Co.

The free Co(II)Cbl cofactor in solution is 5-coordinate, with a nitrogen ligand in the lower axial coordination site. In this state, a N atom of the dimethylbenzimidazole nucleotide substituent of the corrin macrocycle is directly coordinated to the Co atom resulting in an intramolecular linkage (Fig. 1B). At pH values <2.9, where the dimethylbenzimidazole is protonated and dissociates, it is replaced by a water ligand with the resulting shift of the reduction potential from −610 mV to −500 mV (6).

When MeCo(III)Cbl is bound to MetH, the dimethylbenzimidazole ligand that is coordinated to the cobalt in the free cofactor is displaced by a histidine from the corrin-binding domain (H759 in E. coli MetH) (9) (Fig. 1C). As shown in Fig. 1D, MeCo(III)Cbl, Co(II)Cbl, and Co(I)Cbl differ in their preferred coordination states. In particular, the affinity of the cobalt for a nitrogen ligand in the lower axial position is directly proportional to the net positive charge on the cobalt (15–17). Entrance into the reactivation conformation is associated with dissociation of the histidine ligand from the Co(II)Cbl (18, 19).

We previously described the generation of a C-terminal fragment of MetH (MetHCT) that contains the Cbl- and AdoMet-binding modules (18). In the MeCo(III)Cbl form this fragment undergoes temperature-dependent spectral changes, indicating that it interconverts between forms in which H759 is coordinated to the cobalt (His-on) or dissociated (His-off). To reduce conformational flexibility a disulfide cross-link was introduced by mutating Ile-690 and Gly-743 to cysteines (s,S-MetHCT). The X-ray structure of the MeCo(III)Cbl form of s,S-MetHCT revealed that the MeCo(III)Cbl cofactor had been photolyzed by the X-ray beam during data collection, and that the cobalt appeared 4-coordinate with no electron density evident for the methyl group (12). Four-coordinate Co(II)Cbl has never been observed in aqueous solution. Follow-up studies by Brunold and coworkers (20) of the Co(II)Cbl form of s,S-MetHCT using a variety of spectroscopic approaches revealed that the Co(II)Cbl form of the isolated s,S-MetHCT is 5-coordinate, with an axially coordinated water molecule. Following addition of AdoMet to s,S-MetHCT, the Co–OH2 bond is dramatically lengthened such that the Co(II)Cbl is effectively 4-coordinate.

The work presented here explores the mechanisms by which MetHCT stabilizes the 4-coordinate form of Co(II)Cbl. The structure of a binary complex of s,S-MetHCT in the Co(II)Cbl form with adenosylhomocysteine (AdoHcy) has been determined, revealing that Y1139 facilitates the lengthening of the Co–OH2 distance such that the corrin is effectively 4-coordinate. This is corroborated by the mutation of Y1139 to phenylalanine (Y1139F), which lowers the reduction potential from −490 mV to −540 mV. We have also examined the surprising finding that s,S-MetHCT remains capable of converting between His-on and His-off states, even though its conformational flexibility is greatly reduced by the disulfide cross-link. We present an X-ray structure of the aquocobalamin [AquoCo(III)Cbl] form of s,S-MetHCT, which crystallizes in the same conformation as the His-off MeCo(III)Cbl s,S-MetHCT and which spectral studies show to be His-on. This His-on form may resemble a transient intermediate in the reductive reactivation of the enzyme.

Results

Activation Complex with Co(II)Cbl and AdoHcy Bound. Statistics for the X-ray structure of s,S-MetHCT in the Co(II)Cbl form with adenosylhomocysteine (AdoHcy) bound are shown in Supporting Information (SI) Table S1. This is the first structure to reveal the positioning of an AdoMet analogue with respect to the corrin cofactor in the reactivation complex (Fig. 2). AdoHcy was built into clear electron density at 2.7 Å resolution following molecular replacement and initial refinement without a bound ligand (Fig. 2). The orientation of the AdoHcy and its interactions with the activation domain are essentially the same as those of the AdoMet in the structure of its excised binding domain (10).

The environment of the Co(II)Cbl cofactor represents the state of the enzyme as it is ready to accept an electron from Fld (Fig. 3), and in this structure the cobalt appears to be 4-coordinate. H759, the lower axial ligand in other forms of MetH, is dissociated and interacts with the AdoMet domain, with no evidence for a water or any other ligand in the upper or lower axial position. However, density near the upper axial position was assigned to a water molecule stabilized by a hydrogen bond with the side chain of E1097. Compared to the previously determined structure of the MeCo(III)Cbl form (12), the side chain of E1097 is repositioned by AdoHcy binding, allowing formation of a hydrogen bond to the water molecule. Moreover,
the distance between the cobalt and the Y1139 OH moiety has decreased from 4.5 Å to 4.0 Å. A model methyl group was added to the AdoHcy (Fig. S1), but the resulting Co–Me distance of 5.3 Å and the position of Y1139 between Cbl and AdoHcy suggest that further conformational changes would be necessary to accomplish methyl transfer.

**Effect of a Y1139F Mutation on Cbl Reduction Potential.** To determine the role of Y1139 in the generation of the Co(I)Cbl intermediate, a Y1139F/I690C/G743C triple mutant was constructed (s,S-MetHCT/Y1139F). The Y1139F mutation was incorporated into the double mutant background (I690C/G743C). We measured the reduction midpoint potentials of the Co(II) in both the double (s,S-MetHCT) and triple mutant (s,S-MetHCT/Y1139F) constructs (Fig. 4A and B). The Co(II)/Co(I) Cbl midpoint potential was −490 mV for s,S-MetHCT, a value identical to that reported for the full-length wild-type enzyme. In contrast, the potential was lowered to −540 mV for the s,S-MetHCT/Y1139F mutant (Fig. 4C and D). This result can be rationalized in the context of the s,S-MetHCT structure with AdoHcy bound (Fig. 3). The hydrogen bond between the Y1139 OH and the axial water observed in the structure cannot form in s,S-MetHCT/Y1139F, which could thus impede the formation of 4-coordinate Co(II)Cbl and subsequent reduction of the Co(II) to Co(I)Cbl. The 50-mV decrease in reduction potential of s,S-MetHCT/Y1139F is consistent with the theoretical prediction that a hydrogen bond between water and the Y1139 OH should increase the reduction potential (20).

**Activation Complex with Aquocobalamin [AquoCo(III)Cbl] Bound.** Previous attempts to crystallize the MetHCT–AquoCo(III)Cbl fragment failed, but the disulfide-stabilized form of the crystallographically determined s,S-MetHCT/Cbl crystallographic statistics (Table S1). These crystals do not contain the substrate/product in the X-ray beam, as seen in other Co(III)Cbl structures (12, 21). Density for the Y1139 side chain indicates partial disorder, but nonetheless clear evidence of movement away from the cobalamin toward the empty AdoHcy/AdoMet binding pocket. The AquoCo(III)Cbl axial water ligand was omitted from the original model, but following refinement, prominent density above the Co atom was assigned to a water ligand. After further refinement, the 2.1-Å distance is consistent with water coordination to the Co atom.

Comparison of the AquoCo(III)Cbl s,S-MetHCT structure with the MeCo(III)Cbl form of s,S-MetHCT (PDB ID 3BUL) (12) reveals a small but significant shift of the cobalamin domain relative to the AdoMet domain (Fig. 5B, Fig. S2). When the AdoMet domains of the 2 structures are aligned, the average motion of the cobalamin domain is ∼1 Å, with a maximum displacement of 2.1 Å at the outer edge. Surprisingly, the corrin ring system does not move with its binding domain but maintains its position relative to the AdoMet domain.

**Discussion**

**Activation for Electron Transfer.** In the absence of the protein scaffold, the reduction potential for a 5-coordinate Co(II)Cbl cofactor is thermodynamically unfavorable relative to those of physiological reductants. Thus, the occasional oxidation to the Co(II) state should result in very low concentrations of Co(I)Cbl at equilibrium, and extremely slow rates of reduction and subsequent methylation by AdoMet. In conjunction with recently reported spectroscopic data (20), the structure of the Co(II)Cbl s,S-MetHCT provides a clear picture of how MetH is able to move the potential of the Co(II)Cbl/Co(I)Cbl couple into the physiological range. The enzyme elongates the Co–H2O...
bond to a formally 4-coordinate species, thereby raising the reduction potential of Co(II)Cbl (20, 22), and facilitating reduction by the physiological partner Fld.

Fig. 3 presents the crystal structure of \textit{S.s}MetH\textsuperscript{C}\textsubscript{T} in the conformation required for Co–H\textsubscript{2}O bond elongation. This structure reveals that elongation of the Co–H\textsubscript{2}O bond is enabled by the contributions of 2 invariant residues, Y1139 and E1097 (sequence alignment shown in Fig. S3). Following AdoMet/AdoHcy binding, these amino acids are repositioned to interact with the water molecule that was coordinated to Co in the 5-coordinate Co(II)Cbl species as seen in Fig. 3. Comparison of this structure to previously reported structures of MetH\textsuperscript{C} (12, 13) in absence of the AdoMet cofactor indicates that the Y1139/E1097 water-binding pocket is formed only when the AdoMet/AdoHcy cofactor is present. In the previous structures, E1097 was oriented differently, and only in the present structure it is poised to interact with the water molecule (Fig. 5). Further evidence for the repositioning of E1097 following AdoMet binding is found in the structure of the isolated MetH reactivation domain with AdoMet bound, in which the E1097 is pointed toward the water-binding pocket (10). The reduction potential of Y1139F \textit{S.s}MetH\textsuperscript{C}\textsubscript{T} is lowered and reduction of Co(I-)Cbl by Fld becomes less favorable. The effects of the Y1139F mutation can be rationalized in the context of the structure and the proposed reactivation cycle (Fig. 6). The structure reveals that the

AdoMet ribose moiety is perpendicular to the Y1139 aromatic group with the ribose ring oxygen pointing directly toward the center of the \sigma-aromatic cloud. This interaction allows Y1139 to move closer to the Co atom (Co/Y-OH distance 4.0 Å), and positions it for a hydrogen bonding interaction to the water formerly bound to Co. The importance of Y1139 in lowering the reduction potential via this mechanism is corroborated by spectroscopic data (20).

Structural superpositions of the available C-terminal \textit{S.s}MetH fragments demonstrate that the position of Y1139 varies significantly between structures (Fig. S4). In the absence of the AdoMet/AdoHcy ligand, the distance between the hydroxyl of Y1139 and the cobalt increases and the tyrosyl ring approaches the binding site for the adenosine of AdoHcy (Fig. 5B). The binding of AdoMet allows Y1139 to move closer to the water ligand and the Co atom, thus enabling the elongation of the H\textsubscript{2}O–Co bond. This strained state would not be possible in the absence of AdoMet or its analogue AdoHcy. This elegant safety mechanism discourages futile electron transfer from Fld, in the absence of AdoMet.

**Rearrangement Following Methyl Transfer.** When MetH enters the reactivation conformation, H759 dissociates from the cobalamine and forms a hydrogen bond with D1093 in the AdoMet domain that stabilizes this conformation (11, 12). Following reduction and methyl transfer, a 5-coordinate MeCo(III)Cbl species is formed (Fig. 6). We propose that this species is in a conformation similar to the Co(II)Cbl structure presented here [Intermediate conformation (2 or 6) in Fig. 6]. After methyl transfer,
the affinity of H759 for the cobalt of MeCo(III)Cbl increases (17) and allows H759 to break its interaction with the AdoMet domain and bind to Co. However, a domain motion is required to position H759 to bind the cobalt. A comparison of the Co(II)Cbl and AquoCo(III)Cbl structures provides insight into this domain motion. The AquoCo(III)Cbl structure represents an open conformation (Figs. 5, 6, Fig. S2) in which the cobalamin domain has moved away from the AdoMet domain to allow for H759 coordination. The formation of AquoCo(III)Cbl s,s-MetH^CT permits us to trap the enzyme in this open conformation that mimics the last step of the reactivation cycle (Fig. 6).

Conclusions

Our findings emphasize the importance of the coordination state of the cobalamin cofactor in governing entrance into and exit from the reactivation conformation. We propose a tug of war between coordination of H759 by the cobalt of Cbl and its participation in intramodular hydrogen bonding interactions. We have previously proposed that when cob(I)alamin is formed during the catalytic cycle, such intramodular interactions are participation in intramodular hydrogen bonding interactions. We propose a tug of war between coordination of H759 by the cobalt of Cbl and its participation in intramodular hydrogen bonding interactions. Our findings emphasize the importance of the coordination state of the cobalamin cofactor (3). We have previously proposed that when cob(I)alamin is formed during the catalytic cycle, such intramodular interactions are participation in intramodular hydrogen bonding interactions. We propose a tug of war between coordination of H759 by the cobalt of Cbl and its participation in intramodular hydrogen bonding interactions. Our findings emphasize the importance of the coordination state of the cobalamin cofactor (3).

Methods

Chemicals/Cofactors. AdoMet, glycerol, hydrochloric acid (HCl), 2,2,6,6-tetramethylpiperidine-1-oxyl, MeCbl, and potassium ferricyanide were purchased from Sigma-Aldrich and used without further purification. The 5-deazafavin-3-sulfonate was a gift from the late Vincent Massey (University of Michigan). E. coli Fid was purified according to published procedures (23, 24).

Construction of an Expression Plasmid for s,s-MetH^CT and Y1139F s,s-MetH^CT. The I680C/G743C double mutation was introduced into the pVB8 vector containing an E. coli wild-type catalytic domain and the QuikChange site-directed mutagenesis kit (Stratagene). The primers were obtained from Invitrogen. The sequence was confirmed by complete sequencing at the Biomedical Research Core Facility of the University of Michigan. The resulting plasmid was named pSD-3.

The Y1139F mutation was introduced into pSD-3 by ligation of a fragment of DNA from the pCS-15 vector containing the mutation. Briefly, pCS-15 was digested with HindIII and BbvCI at 37 °C. The 330-bp fragment was purified from an agarose gel and ligated into the similarly digested pSD-3 vector, yielding pSD-4. The presence of this mutation was verified by sequencing at the Biomedical Research Core Facility of the University of Michigan.

Expression and Purification of s,s-MetH^CT and Y1139F s,s-MetH^CT. The enzymes were expressed in cells of E. coli Hms174(DE3) (Novagen) and purified by nickel affinity chromatography using a 5-mL Hi-trap and a MonoQ 16/10 column (Amersham Biosciences).

Reduction and Photolysis of Cbl Bound to s,s-MetH^CT, s,s-MetH^CT was converted to the MeCo(III)Cbl form through reductive methylation by AdoMet in an electrochemical cell. The Co(II)Cbl-bound MetH^CT forms were generated via photolysis of the corresponding MeCo(III)Cbl-bound species, as described previously (26). Protein concentrations were determined spectrophotometrically by using the extinction coefficients of the enzyme-bound MeCo(II)Cbl cofactor (ε420 [s,s-MetH^CT] = 10.2 mM⁻¹ cm⁻¹) (26). The AquoCo(III)Cbl forms of the mutants were prepared by treating the enzyme with 50 μM of potassium ferricyanide in 10 mM phosphate buffer, pH 7.2 in a cuvette immersed in ice-water and exposing to a tungsten-halogen lamp (650W) at a distance of 5 cm. The formation of the aquo species was monitored in a spectrophotometer (352 nm) for completion of the reaction. The excess reagents were removed by the use of a gel filtration column. Protein concentrations were determined spectrophotometrically by using the extinction coefficients of the enzyme-bound AquoCo(II)Cbl cofactor (ε320 [s,s-MetH^CT] = 21.2 mM⁻¹ cm⁻¹ and ε320 [Y1139F s,s-MetH^CT] = 20 mM⁻¹ cm⁻¹).

Spectrophotometric Determination of the Co(II)/Co(III)Cbl Midpoint Potential. The midpoint potential was measured as described previously (3). A 1-mL solution of 30 μM enzyme, 100 μM methyl viologen, and 5 μM 5-deazafavin-3-sulfonate in buffer (100 mM potassium phosphate, 100 mM KCI, and 25 mM EDTA, pH 7.2) was equilibrated with Ar(g) in an anaerobic glass cuvette. The enzyme was reduced by irradiation with a 600 W tungsten/halogen lamp, and the slow oxidation at 37 °C was monitored by absorption spectroscopy. The concentration of reduced methyl viologen was calculated on the basis of the absorbance at 600 nm (ε400 = 13,600 M⁻¹ cm⁻¹) (27), taking into account that the enzyme also weakly absorbs at this wavelength. These values were used in the Nernst equation, along with the midpoint potential for methyl viologen (~446 mV vs. SHE), to calculate the system potential at each time point. Then, the absorbance at 468 nm was corrected for contributions from methyl viologen and used to calculate the concentrations of Co(II)Cbl and Co(III)Cbl:AdoMet. The increased positive charge on MeCo(III)Cbl then allowed H759 to break its interaction with the AdoMet module (12), allowing H759 to position itself to bind the cobalt. A comparison of the Co(II)Cbl and AquoCo(III)Cbl structures provides insight into this domain motion. The AquoCo(III)Cbl structure represents an open conformation (Figs. 5, 6, Fig. S2) in which the cobalamin domain has moved away from the AdoMet domain to allow for H759 coordination. The formation of AquoCo(III)Cbl s,s-MetH^CT permits us to trap the enzyme in this open conformation that mimics the last step of the reactivation cycle (Fig. 6).


Supporting Information
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Fig. S1. AdoMet was modeled in the AdoMet/AdoHcy pocket on the basis of the position of the AdoHcy in the Co(II)Cbl$_{5,8}$MeH$^+$ structure.
Fig. S2.  Superposition of the Co(II)Cbl$_{5}$MeH$^{CT}$ shown in green with (A) MeCo(III)Cbl$_{5}$MeH$^{CT}$ (PDB ID 3BUL) [Datta S, Koutmos M, Pattridge KA, Ludwig ML, Matthews RG (2008) A disulfide-stabilized conformer of methionine synthase reveals an unexpected role for the histidine ligand of the cobalamin cofactor. Proc Natl Acad Sci USA 105:4115–4120] colored as in Fig. 2, and with (B) AquoCo(III)Cbl$_{5}$MeH$^{CT}$, colored as in Fig. 2. The superpositions are based on selected residues from the β-strands of the AdoMet domain.
Fig. S3. Sequence alignment of MetH enzymes from various sources. Invariant and conserved residues are highlighted according to the clustalx coloring scheme (1). Invariant residues D1093, E1097, and Y1139 in E. coli are indicated in the figure. These alignments are selected from a multiple alignment performed in CLUSTALW (2) using MetH sequences obtained from the NCBI database.

Fig. S4. Overlay of the Co(II)Cbl $S_S$MeH<sup>CT</sup> structure, in green, with the AquoCo(III)Cbl $S_S$MeH<sup>CT</sup> structure, in light gray, and with the MeCo(III)Cbl $S_S$MeH<sup>CT</sup> structure (PDB ID 3BUL) [Datta S, Koutmos M, Pattridge KA, Ludwig ML, Matthews RG (2008) A disulfide-stabilized conformer of methionine synthase reveals an unexpected role for the histidine ligand of the cobalamin cofactor. Proc Natl Acad Sci USA 105:4115–4120], in blue. Residues from the β-strands of the AdoMet domain were used for the superposition. Of note are (i) the 2 different positions of H759, a “His-on” position in $S_S$MeH<sup>CT</sup>/AquoCo(III)Cbl and a “His-off” position in the other 2 structures in which H759 hydrogen bonds to D1093; (ii) the relative movement and different positions of Y1139 in all 3 structures; and (iii) the movement of the cobalamin domain but not the cobalamin cofactor.
**Fig. S5.** Electron density and ball and stick model of the AquoCo(III)Cbl cofactor. The blue (at 1σ) and red (at 3σ) contours represent electron density from a weighted $2F_{\text{obs}}-F_{\text{calc}}$ and $F_{\text{obs}}-F_{\text{calc}}$ omit map, respectively. His-759 was omitted from the model before the calculation of the composite omit map.
Table S1. Data collection and refinement statistics

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aStatistics for the highest resolution shell are enclosed in parentheses.
bR_{sym} = Σ|I − <I> |/ΣI, where I = observed intensity, and <I> = average intensity obtained from multiple measurements.
R_{cryst} = Σ|F_{obs} − F_{calc}|/ΣF_{obs}, where F_{calc} and F_{obs} are the calculated and observed structure factor amplitudes, respectively.
R_{free}, R-factor based on 5% of the data excluded from refinement.
RMSD, root mean square deviation.