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FOR THE RECORD

Expression, crystallization, and preliminary X-ray analysis of a sialic acid-binding fragment of sialoadhesin in the presence and absence of ligand

A.P. MAY,[1,4] R.C. ROBINSON,[1,4,5] R.T. APLIN,[3,4] P. BRADFIELD,[2]
P.R. CROCKER,[2] AND E.Y. JONES[1,4]

[1]Laboratory of Molecular Biophysics, University of Oxford, Oxford, United Kingdom
[2]I.C.R.F. Laboratories, University of Oxford, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, United Kingdom
[3]Dyson Perrins Laboratory, University of Oxford, Oxford, United Kingdom
[4]Oxford Centre for Molecular Sciences, University of Oxford, Oxford, United Kingdom

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Abstract: Sialoadhesin is a macrophage-restricted cell surface receptor, consisting of 17 immunoglobulin domains, which mediates cell adhesion via the recognition of specific sialylated glycoconjugates. A functional fragment of sialoadhesin, comprising the N-terminal immunoglobulin domain, has been expressed in Chinese hamster ovary cells as both native (SnD1) and selenomethionyl (Se-SnD1) stop protein. The successful production of 86% selenomethionine-incorporated protein represents a rare example of production of selenium-labeled protein in mammalian cells. SnD1 and Se-SnD1 have been crystallized in the absence of ligand, and SnD1 has also been crystallized in the presence of its ligand 2,3 sialyllactose. The ligand-free crystals of SnD1 and Se-SnD1 were isomorphous, of space group $P3_121$ or $P3_221$, with unit cell dimensions $a = b = 38.9$ Å, $c = 152.6$ Å, $\alpha = \beta = 90°$, $\gamma = 120°$, and diffracted to a maximum resolution of 2.6 Å. Cocrystals containing 2,3 sialyllactose diffracted to 1.85 Å at a synchrotron source and belong to space group $P2_12_12_1$, with unit cell dimensions $a = 40.9$ Å, $b = 97.6$ Å, $c = 101.6$ Å, $\alpha = \beta = \gamma = 90°$.

Keywords: cell adhesion; complex; crystallization; lectin; selenomethionine; sialic acid

Sialoadhesin is a cell surface receptor that mediates cellular interactions by recognizing specific sialylated glycans in cell surface glycoconjugates with terminally exposed Neu5Acα2,3Gal, the minimal oligosaccharide required for recognition (Crocker et al., 1991; Kelm et al., 1994b). It is expressed only on resident macrophage subpopulations (Crocker et al., 1994), and cell binding studies have shown that, among hemopoietic cells, it binds preferentially to granulocytes (Crocker et al., 1995). In the bone marrow, Sn is found selectively at contact sites with developing myeloid cells (Crocker et al., 1990, 1991), and it has been suggested that, in lymphoid tissues, it acts as a lymphocyte adhesion molecule (Van den Berg et al., 1992). On the basis of sequence analysis, Sn has been shown to be a member of the immunoglobulin superfamily (IgSF) (Williams & Barclay, 1988; Williams et al., 1989) consisting of 17 immunoglobulin-like domains, a membrane-spanning helix, and a short cytoplasmic domain (Crocker et al., 1994). The immunoglobulin domains are arranged in a linear array made up of 16 C2-set domains and one N-terminal, membrane distal, V-set domain. Sn is most closely related in sequence in its N-terminal two domains to the myelin-associated glycoprotein (MAG), CD22, and CD33, and, with these, it forms the sialoadhesin family, a subgroup of the IgSF involved in the recognition of specific sialylated glycans (Kelm et al., 1994a; Freeman et al., 1995). It has been shown by domain deletion experiments that the membrane distal N-terminal domain is both necessary and sufficient to mediate sialic acid-dependent adhesion (Nath et al., 1995). A V-set immunoglobulin domain consists of nine β strands arranged into two antiparallel β sheets, termed the ABED and GFCC′C″ sheets (Williams & Barclay, 1988). Site-directed mutagenesis studies on the N-terminal V-set domain located a number of residues, predicted to lie in a discrete region on the surface of the GFCC′C″ β sheet, that were thought to form the sialic acid-binding site (Vinson et al., 1996). It has been suggested that the large number of subsequent domains may extend the sialic acid-binding site away from the plasma membrane to minimize *cis*-interactions with oligosaccharides in the macrophage glycocalyx (Crocker et al., 1994).

The N-terminal, sialic acid-binding domain of sialoadhesin, consisting of the first 119 amino acids, has been expressed in soluble

Reprint requests to: E.Y. Jones, Laboratory of Molecular Biophysics, Rex Richards Building, University of Oxford, Oxford. OX1 3QU. United Kingdom; e-mail: yvon@biop.ox.ac.uk.
[5]Present address: Structural Biology Laboratory, The Salk Institute, La Jolla, California 92037.

Abbreviations: MAG, myelin-associated glycoprotein; IgSF, immunoglobulin superfamily; VCAM-1, vascular cell adhesion molecule 1; CHO, Chinese hamster ovary; DTT, dithiothreitol; PEG, polyethylene glycol; MPD, 2-methyl-2,4-pentanediol; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; PBS, phosphate buffered saline; OPD, *o*-phenylenediamine dihydrochloride; Sn, sialoadhesin.

form as both native (SnD1) and selenomethionyl (Se-SnD1) stop proteins in CHO cells. Selenomethionine incorporation into protein expressed in mammalian cells for successful use in structural studies has been reported previously only once (Lustbader et al., 1995). The results presented here demonstrate the successful use of alternative cell culture protocols and confirm the general efficacy of CHO cells for production of selenomethionine-labeled protein. SnD1 is functional (Nath et al., 1995; P.R. Crocker, unpubl. results), and contains no glycosylation sites. Here we report on crystals and preliminary X-ray diffraction data for both SnD1 and Se-SnD1 in the absence of ligand, and SnD1 in the presence of its ligand, 2,3 sialyllactose.

A number of solution and crystal structures exist for soluble fragments of the extracellular portions of cell-surface IgSF molecules. Structural studies on both rat and human CD2 (Jones et al., 1992; Bodian et al., 1994) have provided information on the adhesive interactions between IgSF domains; studies on VCAM-1 (Jones et al., 1995) gave insight into IgSF/integrin adhesion. It is hoped that the structural analysis of this fragment of Sn will lead to information on the details of IgSF/carbohydrate interactions and their role in intercellular adhesion, and provide a template for studying other members of the recently characterized sialoadhesin family (Kelm et al., 1994a; Freeman et al., 1995). The structure of the N-terminal domain of sialoadhesin will be the first of a new class of carbohydrate-binding proteins. With 2,3 sialyllactose, it will also be the first structure of a cell-surface IgSF molecule in complex with a heterotypic, functional ligand.

Results and discussion: CHO-expressed SnD1 and Se-SnD1 could be purified in milligram quantities using a two-step procedure: 3D6 mAb affinity chromatography followed by FPLC gel filtration with Superdex 75. The gel filtration step showed that SnD1 existed as both a monomer and a dimer (data not shown), the ratios varying from one preparation to another, but, in most cases, being at least 10:1 monomer:dimer. Analysis of dimeric fractions by SDS-PAGE under reducing and nonreducing conditions showed that the dimers were noncovalently bound (data not shown). SnD1 was folded correctly, as determined by binding of the anti-sialoadhesin mAb 3D6, and was able to bind human erythrocytes in a sialic acid-dependent manner (P.R. Crocker, unpubl. data).

Analysis of SnD1 by mass spectrometry confirmed that it consisted of a highly homogeneous polypeptide chain of the expected 119 amino acid sequence (calculated average mass 13,303.16 Da; observed mass 13,301.88 ± 1.04 Da) (Fig. 1A). SnD1 contains two methionine residues, which, when replaced by selenomethionine are predicted to produce a mass change of 46.89 Da per selenium substitution. The mass spectrum of Se-SnD1 (Fig. 1B) showed that Se-SnD1 used for crystallization consisted of 9% unsubstituted SnD1, 11% monosubstituted Se-SnD1, and 80% disubstituted Se-SnD1, indicating that there had been an 86% overall incorporation of selenium in place of sulfur.

Isomorphous SnD1 and Se-SnD1 crystals grew reproducibly with typical dimensions of 1.0 × 0.1 × 0.7 mm^3 (Fig. 2B). The crystals belonged to either space group $P3_121$ or its enantiomorph $P3_221$, with room temperature unit cell dimensions of $a = b = 38.9$ Å, $c = 152.6$ Å, $\alpha = \beta = 90°$, $\gamma = 120°$. Diffraction data to 2.6 Å resolution, collected on three Se-SnD1 crystals at the Photon Factory, were 94% complete, with an $R_{merge(F)}$[6] on structure factors of 5.9%. SnD1 crystals did not diffract as well as Se-SnD1 crystals, a typical SnD1 data set having an $R_{merge(F)}$ on structure factors of 25.4% in the resolution range 2.67–2.58 Å, compared with 18.5% for the best Se-SnD1 data set. If it is assumed that the crystals contained one molecule in the crystallographic asymmetric unit, this gives a Matthews coefficient (V_m) of 2.9 $Å^3$/Da,

[6] $R_{merge(F)} = \Sigma|F - \langle F\rangle|/\Sigma\langle F\rangle$.

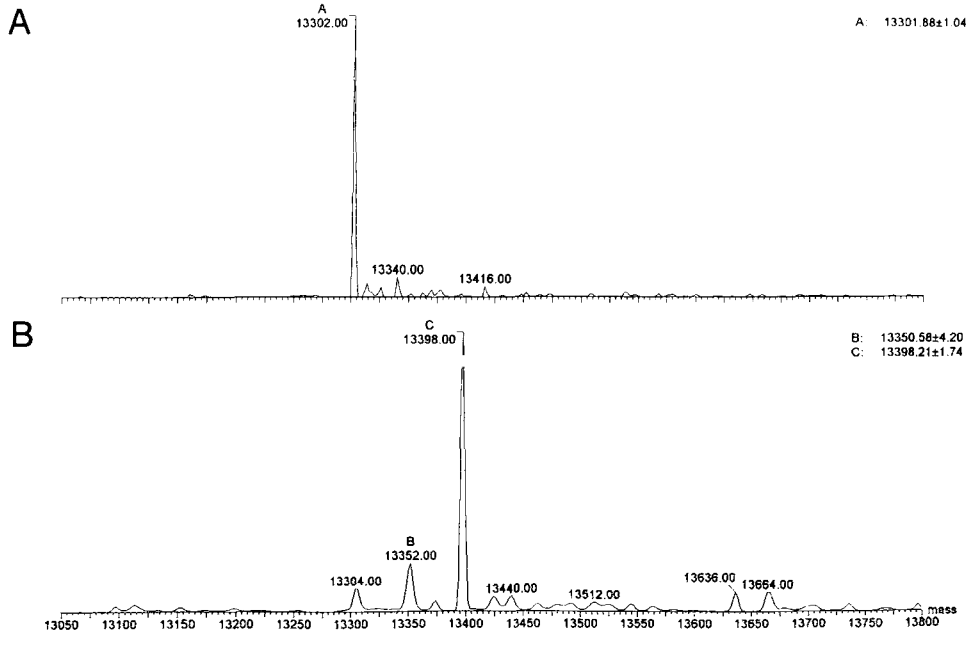

Fig. 1. Results of electrospray ionization mass spectrometry. **A:** SnD1; the native protein consists of a single homogeneous species of observed mass 13,301.88 ± 1.04 Da. **B:** Se-SnD1; peaks A, B, and C in the spectrum correspond to unsubstituted SnD1, monosubstituted Se-SnD1 (13,350.58 ± 4.20 Da), and disubstituted Se-SnD1 (13,398.21 ± 1.74 Da), respectively.

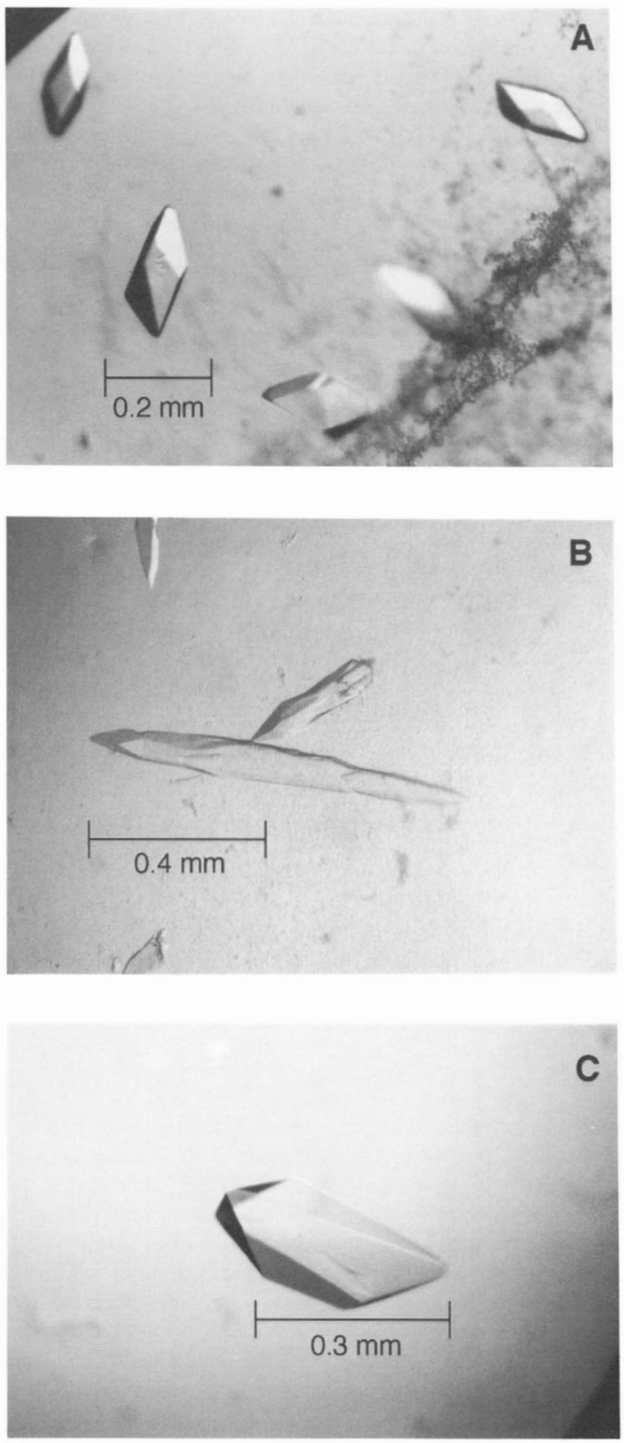

Fig. 2. Crystals of sialoadhesin. **A:** Initial crystals of SnD1 grown at 17 °C. **B:** SnD1 crystals used for synchrotron data collection grown at 4 °C. **C:** Cocrystal of SnD1 complexed with 2,3 sialyllactose grown at 17 °C.

which corresponds to a solvent content of approximately 58% (Matthews, 1968). The crystals were unstable over several months, with a loss in the maximum resolution of observed diffraction to around 4.0 Å. Occasional variation in room temperature unit cell dimensions was observed, typically in the order of 1 Å in each dimension.

Initial efforts to obtain a SnD1/ligand complex centered on soak experiments using the native crystals. Native crystals were soaked with reservoir solutions containing 1.25, 2.5, 5, 10, and 25 mM 2,3 sialyllactose for 6 h. After this time, crystals in the 25 mM solution had completely dissolved and those at 10 mM were cracked extensively. Those in the other solutions remained intact. Data sets collected from these crystals revealed small isomorphous differences (commonly <10%) that were accounted for by unit cell differences on freezing. Because of the dramatic effect of the 25 mM soak on the native crystals, it was decided to proceed with cocrystallization at this concentration. This choice of concentration was also supported by the known dissociation constant of 2,3 sialyllactose from influenza hemagglutinin (K_d = 3.2 mM; Sauter et al., 1992), which has similar affinity and binding specificity to sialoadhesin (Crocker et al., 1991). Cocrystals grew typically in 48 h and reached their maximal dimensions of 0.3 × 0.2 × 0.4 mm^3 in four days (Fig. 2C). The crystals are of space group $P2_12_12_1$, with room temperature unit cell dimensions of a = 40.9 Å, b = 97.6 Å, c = 101.6 Å, $\alpha = \beta = \gamma = 90°$, which gives a V_m of 2.54 $Å^3$/Da (solvent content approximately 52%) for three copies in the asymmetric unit (Matthews, 1968). Calculation of a native Patterson map and self rotation function did not provide any further information on the arrangement of the molecules in the asymmetric unit. Data collected at the Photon Factory were 94.6% complete to 1.85 Å and had an $R_{merge(I)}$[7] on intensities of 7.2%.

This study, along with that of Lustbader et al. (1995), shows that selenomethionine may be incorporated successfully into recombinant proteins in mammalian expression systems as well as the more commonly used bacterial systems. Growth of CHO cells in L-selenomethionine-containing media led to premature cell death in five days, thus reducing yield relative to the native protein. By this time, however, sufficient quantities of labeled protein had been produced for use in crystallographic studies. Selenomethionine may be used as a method of incorporating heavy atoms into a protein in order to provide a rapidly available, highly isomorphous heavy-atom derivative, and substituted proteins may be used for structure determination by multiwavelength anomalous diffraction (Hendrickson et al., 1990). We have exploited this strategy using Se-SnD1 and, as a result, the structure determination and analysis of ligand-free and complexed SnD1 are underway and will be reported elsewhere.

Materials and methods: Unless stated otherwise, all reagents and chemicals were purchased from Sigma (Poole, UK or St. Louis, Missouri). 2,3 Sialyllactose was purchased from Oxford Glycosystems (Oxford, UK). Crystalscreen sparse matrix sampling crystallization kit and microbridges for sitting drop vapor diffusion were purchased from Hampton Research (Laguna Hills, California).

Expression and purification of native and selenomethionyl protein: Subcloning of SnD1 stop protein in pEFBOS into pEE14: SnD1 was cloned initially in pEFBOS and expressed transiently in monkey COS cells (Nath et al., 1995). In order to produce sufficient quantities for crystallographic studies, SnD1 was subcloned from pEFBOS into pEE14 for stable expression in CHO cells. The *Eco*R I fragment containing the entire open reading frame and some 3′ untranslated sequence was excised from pEFBOS and

[7] $R_{merge(I)} = \Sigma|I - \langle I\rangle|/\Sigma\langle I\rangle$.

inserted in the correct orientation into the pEE14 glutamine synthase expression system (Bebbington, 1991). CHO cells were transfected by electroporation and colonies secreting SnD1 were selected in methionine sulfoxamine and screened by ELISA as follows. Ninety-six well microtiter plates (Immulon 3; Dynatech, Chantilly, Virginia) were coated sequentially with 3D6 mAb, supernatant from CHO cells diluted 1:1, a rabbit polyclonal anti-sialoadhesin antiserum (described in Crocker et al., 1991) and a goat anti-rabbit peroxidase conjugate, followed by OPD as the peroxidase substrate and optical density determination at 450 nm. Positives were then amplified by selection in 400 μM methionine sulfoxamine. The clone that was finally selected was designated 6G7 and was found to secrete SnD1 at levels up to 10 mg/L as determined by ELISA, using purified SnD1 as a standard.

Purification from CHO cell supernatants: CHO cells were cultured in 15-cm diameter gridlock petri dishes (Falcon). Each dish contained 40 mL supplemented GMEM (Bebbington, 1991) containing 5% FCS and 400 μM methionine sulfoxamine. At ~75% cell confluence, sodium butyrate was added to 2 mM and the plates left for up to 10 days before harvesting the supernatant. Cell debris was removed by centrifugation at 5,000 g and the supernatants were filtered through 0.2-μm bottle-top filter units (Falcon). Supernatant containing up to 5-mg SnD1 stop, as determined by ELISA, was passed through a 10-mL 3D6 mAb Sepharose 4B affinity column (5 mg 3D6 IgG/mL wet resin). After washing with 500 mL PBS, the bound protein was eluted with 0.1 M glycine, pH 2.5, and fractions of 3 mL were neutralized immediately with 0.3 mL 1.0 M Tris, pH 8.0. Fractions containing more than 0.1 mg/mL protein were pooled and dialyzed against 20 mM Tris, pH 8.0. The protein was then concentrated to 5 mg/mL using Amicon centricon miniconcentrators (MW cutoff, 10-kDa) and 2 mL passed down a Hi-Load 16/60 Superdex 75 Prep-grade FPLC column (Pharmacia) equilibrated in 20 mM Tris + 150 mM NaCl, pH 8.0. Fractions containing the monomeric form of SnD1 were pooled and prepared for crystallization as described below.

Production of L-selenomethionine-labeled SnD1 protein (Se-SnD1): CHO cells expressing SnD1 were grown on 60 plates as described above. One day after adding the sodium butyrate, the plates were rinsed once with PBS and the medium replaced with methionine-free Dulbeco's modification of Eagle's medium containing 30 mg/L L-selenomethionine (Sigma) and 5% FCS (Gibco BRL) dialyzed against PBS. After five days, most of the cells had died and, at this point, the supernatant was harvested and the labeled Se-SnD1 protein was purified as described above.

Molecular characterization of SnD1 and Se-SnD1: Mass determinations by electrospray ionization were performed on a Micromass BioQ II triple, quadruple, atmospheric pressure mass spectrometer equipped with an electrospray interface operating in the positive ion mode. The instrument was calibrated using horse heart myoglobin (12 pmol/mL, average molecular mass 16,951.5 Da) and scanned over the mass range 650–1,550 Da.

Crystallization of SnD1 and Se-SnD1: Both SnD1 and Se-SnD1 showed a propensity to dimerize both covalently and noncovalently over time. In order to isolate the monomeric species, the material was treated by gel filtration (Hi-Load 16/60 Superdex 75 Prep-grade FPLC column [Pharmacia], 150 mM NaCl, 20 mM Tris, pH 8.0) immediately prior to crystallization. For crystallization, the protein was concentrated and buffer exchanged using Amicon centricon tubes (MW cut off, 10 kDa) to a concentration of 10 mg/mL in 10 mM Hepes buffer, pH 7.5, containing 10 mM DTT. Crystallization trials were performed using a sparse matrix screen (Jancarik & Kim, 1991; Hampton Research CrystalScreen) to identify suitable conditions. Crystallizations were all set up by the sitting-drop vapor diffusion method using micro-bridges (Harlos, 1992).

Ligand-free crystals: Crystals were obtained within 24 h under initial trial conditions at 17 °C (Fig. 2A) using Crystalscreen solution number 41. Subsequent optimization of crystal growth led to final conditions based on sitting drops set up at 4 °C as 2 μL of (10 mg/mL) protein solution plus 2 μL of reservoir solution containing 16% (w/v) PEG 4000, 8% (v/v) propan-2-ol, 10 mM DTT, 80 mM Hepes, pH 7.5 (Fig. 2B). Se-SnD1 produced similar quality crystals under these same conditions.

Cocrystals: Initial crystals were grown in three days in the presence of 25 mM 2,3 sialyllactose using Crystalscreen solution number 9 under initial trial conditions at 17 °C. The final conditions used were sitting drops at 17 °C consisting of 2 μL (10 mg/mL) protein solution plus 2 μL reservoir solution containing 30% (w/v) PEG 4000, 0.1 M sodium citrate, pH 5.6, 0.2 M ammonium acetate (Fig. 2C).

X-ray diffraction and analysis: Ligand-free crystals: Initial characterization and data collection were performed in-house using a 30-cm MAR-research imaging plate system on a Rigaku rotating anode (CuKα X-rays, graphite monochromator). Medium resolution data to 2.6 Å were collected using 0.97 Å wavelength synchrotron radiation (0.1 mm collimation) at 14 °C on beam line BL6A2 (Sakabe, 1991) of the Photon Factory, Tskuba, Japan. Apart from oscillations taken in order to facilitate auto-indexing, data collection employed the Weissenberg method, typically 4° oscillations with a coupling constant of 0.8°/mm or 2.0°/mm. X-ray diffraction data were recorded on BASIII imaging plates and scanned off line with Fuji BA100 IP scanners.

Cocrystals: Initial data were collected from a single cocrystal flash frozen using an Oxford Cryosystems Cryostream on beam line BL19 (Thompson et al., 1996) at the ESRF, Grenoble. Crystals were soaked in a cryoprotectant solution containing 15% MPD (v/v) 30% PEG4000 (w/v), 0.1 M sodium citrate, pH 5.6, 0.2 M ammonium acetate, 25 mM 2,3 sialyllactose prior to freezing in the dry air stream at 104 K; 0.979-Å wavelength synchrotron radiation was used and data recorded using a CCD-based detector (Moy, 1994). X-ray data were collected by the oscillation method using 1° oscillations, calibrated, and corrected for spatial distortion (Hammersley et al., 1995; Moy et al., 1996). High-resolution data to a maximum resolution of 1.85 Å were collected using 1.0-Å wavelength synchrotron radiation (0.1-mm collimation) at 14 °C on beam line BL6A2 (Sakabe, 1991) of the Photon Factory, Tskuba, Japan. Data collection employed similar methods to the medium resolution data collected for the native crystals, but used 3.5° oscillations and a coupling constant of 1.5°/mm.

All data were auto-indexed, integrated, and corrected for Lorentz and polarization effects with the program DENZO and scaled and merged using the program SCALEPACK (Otwinowski, 1993). Separate data sets collected from different ligand-free crystals were scaled and merged together using the program 3D-SCALE (Stuart et al., 1979).

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