Mannose 6-phosphate receptors (MPRs) participate in the biogenesis of lysosomes in higher eukaryotes by transporting soluble acid hydrolases from the trans-Golgi network to late endosomal compartments. The receptors release their ligands into the acidic environment of the late endosome and then return to the trans-Golgi network to repeat the process. However, the mechanism that facilitates ligand binding and dissociation upon changes in pH is not known. We report the crystal structure of the extracytoplasmic domain of the homodimeric cation-dependent MPR in a ligand-free form at pH 6.5. A comparison of the ligand-bound and ligand-free structures reveals a significant change in quaternary structure as well as a reorganization of the binding pocket, with the most prominent change being the relocation of a loop (residues Glu134–Cys141). The movements involved in the bound-to-free transition of the cation-dependent MPR are reminiscent of those of the oxy-to-deoxy hemoglobin transition. These results allow us to propose a mechanism by which the receptor regulates its ligand binding upon changes in pH; the pKₐ of Glu₁³² appears to be responsible for ligand release in the acidic environment of the late endosomal compartment, and the pKₐ values of the sugar phosphate and His₁⁰⁵ are accountable for its inability to bind ligand at the cell surface where the pH is about 7.4.

P-type lectins function as an essential part of the degradative pathway of higher eukaryotic organisms. A well-characterized function of this receptor family is its ability to target newly synthesized acid hydrolases to the lysosome. During their transport through the Golgi complex, acid hydrolases acquire a mannose 6-phosphate (Man-6-P) recognition marker on their N-linked oligosaccharides that serves as a high affinity ligand for the receptors. The resulting receptor-acid hydrolase complex is transported to late endosomal compartments. Upon encountering the lower pH of this prelysosomal compartment, the complex dissociates. The hydrolytic enzymes are then packaged into lysosomes, and the uncomplexed receptors move back to the Golgi where the process is repeated numerous times for a single receptor (1–3).

The 46-kDa cation-dependent mannose 6-phosphate receptor (CD-MPR) and the 300-kDa insulin-like growth factor II/cation-independent mannose 6-phosphate receptor (IGFII/CI-MPR) are the two known members of the P-type lectin family, both of which are type I membrane glycoproteins. Carbohydrate-binding proteins have historically been divided into two subgroups. The group I carbohydrate-binding proteins, which include periplasmic transport proteins and some enzymes, typically envelop their ligands in deep pockets. In contrast, group II carbohydrate-binding proteins, which include lectins, typically bind their ligands in shallow clefts on the protein surface (4). Our recent crystallographic studies of the CD-MPR in complex with ligands containing phosphomannosyl residues reveal that this P-type lectin is unusual with respect to the architecture of its carbohydrate-binding pocket when compared with the majority of animal and plant lectins; the CD-MPR has a relatively deep binding pocket, which essentially buries the terminal sugar ring and phosphate group (5, 6). Thus, the CD-MPR displays similarities with the group I, but not the group II, carbohydrate-binding proteins. Another feature commonly observed among both animal and plant lectins is the lack of any conformational change evoked upon binding ligand. To determine whether the conformation of the CD-MPR is dependent upon the presence of bound carbohydrate, we have solved the structure of the extracytoplasmic domain of the CD-MPR in a ligand-free state at pH 6.5. Our results demonstrate that, unlike other lectins, the CD-MPR undergoes a dramatic change both in its quaternary structure and in the positioning of a loop (residues Glu₁³⁴–Cys₁⁴¹) upon carbohydrate binding.

**EXPERIMENTAL PROCEDURES**

**Protein Expression, Purification, and Crystallization**—The glycosylation-deficient extracytoplasmic domain (Asn₁⁵⁰Thr¹⁵⁰) of the bovine CD-MPR was generated as described previously (7). Recombinant protein was grown in *Trichoplusia ni* SB1–4 (High Five) insect cells and purified to near homogeneity by pentamannosyl-phosphate-agarose affinity chromatography as described previously (8). Protein was dialyzed extensively against buffer containing 150 mM NaCl, 10 mM MnCl₂, 5 mM β-glycerophosphate, and 50 mM imidazole, pH 6.5, to remove the Man-6-P present from the purification procedure. Crystallization was carried out at 19 °C by vapor diffusion using the hanging drop method (9) by mixing equal volumes of the purified protein solution (10 mg/ml) with the precipitating solution (25% (w/v) polyethylene glycol 5000 monomethyl ether, 0.2 M ammonium acetate in 0.1 M cacodylate buffer, pH 6.5).

The crystal was mounted in a thin-walled glass capillary tube, and
diffraction data were collected at 4°C on an R-AXIS IIC image plate detector system with a Rigaku RU200 rotating anode generator operating at 50 kV and 100 mA with a graphite monochromator. The crystal belongs to the I4 space group with unit cell parameters a = 108.7 Å and c = 72.6 Å. Assuming two monomers/asymmetric unit, the calculated Matthews’s coefficient is 3.16 Å³/da (10). One data set was collected, the crystal was then translated, and another data set was collected from the same crystal. The two data sets were then merged to yield a final set at 2.2 Å resolution. The diffraction data were processed and scaled using the programs DENZO (11) and SCALEPACK (11); the statistics are given in Table I.

Structure Determination and Refinement—The structure of the CD-MPR in the absence of ligand was solved using the molecular replacement protocol in the CCP4 (12) version of AmoRe (13) using one monomer of the dimeric structure of the ligand-bound form of Asn⁸¹/STOP¹⁵⁵ (Protein Data Bank code 1M6P) as the search model. Two solutions were obtained with correlation factor values of 27.4 and 21.1 and R-factor values of 52.5 and 53.3%, respectively. The resulting model was refined using CNS (14) in conjunction with manual model adjustments using Turbo-Frodo (15). The statistics for the final model (Protein Data Bank code 1KEO) are given in Table I.

RESULTS AND DISCUSSION

Overall Structure—We have previously reported the structures of the extracytoplasmic domain of the bovine CD-MPR bound to either Man-6-P (5) or pentamannosyl phosphate (6) at pH 6.5 (Fig. 1a). This 154-residue soluble, glycosylation-deficient form of the receptor (Asn⁸¹/Stop¹⁵⁵), which lacks both the 25-residue transmembrane and 67-residue carboxyl-terminal domains and contains substitutions (Asn replaced with Gln) at four (residues 31, 57, 68, and 87) of the five potential N-glycosylation sites (Fig. 1b), has previously been shown to bind the lysosomal enzyme β-glucuronidase with an affinity identical to that of the full-length receptor (8). We now report the structure of Asn⁸¹/STOP¹⁵⁵ in the absence of ligand at pH 6.5, which has been refined to 2.2 Å resolution with good geometry (Fig. 2a). Electron densities of all but the first two amino acid residues are clearly defined. Only one N-acetylglucosamine residue of the oligosaccharide chain is visible on Asn⁸¹, suggesting that the remainder of the oligosaccharide is flexible. The overall topology of the monomer polypeptide fold (Fig. 2e) is the same as that of the ligand-bound form (Fig. 1a); it consists of a flattened β-barrel made of nine strands and a short α-helix located at the amino terminus. As in the structure of the ligand-bound forms of the CD-MPR, crystals of the ligand-free receptor also contain one dimer per asymmetric unit. A comparison of the two-monomer peptide chains reveals them to be essentially identical with a root-mean-square (r.m.s.) deviation of 0.15 Å for 151 Ca atoms. However, in the absence of ligand the individual monomer peptide chain shows a significant difference from that of the pentamannosyl phosphate-bound structure (Protein Data Bank code 1C39), having an r.m.s. deviation of 2.6 Å for 151 Ca atoms, with the main differences occurring in the positioning of loops A, C, and D (Fig. 2b). In the absence of ligand, loop A and loop C move away from the dimer interface. Ser⁴¹ on loop A is displaced by 4.1 Å from its position in the pentamannosyl phosphate-bound structure whereas His¹⁰⁵ on loop C is relocated by 2.9 Å. However, by far the most dramatic change is observed in the position of loop D. In the absence of ligand, loop A and loop C move away from the dimer interface. Ser⁴¹ on loop A is displaced by 4.1 Å from its position in the pentamannosyl phosphate-bound structure whereas His¹⁰⁵ on loop C is relocated by 2.9 Å. However, by far the most dramatic change is observed in the position of loop D. In the absence of ligand, residues Glu¹³⁴–Cys¹⁴¹ of loop D along with Glu¹³⁴ of β-strand 8 and Phe¹⁴² of β-strand 9 comprise a flap that flips toward the binding pocket in the absence of ligand (Fig. 2, b and c), with Val¹³⁶ displaying the largest displacement (Co–Co distance of 16.4 Å).

This transition of loop D from the “open” conformation in the bound state to the “closed” conformation in the ligand-free state has intriguing consequences for the binding site. Previous mutagenesis studies have shown that residues Glu⁶⁶, Arg¹¹¹, Glu¹³³ and Tyr¹⁴⁵ are essential for binding to pentamannosyl phosphate, whereas mutation of residues Tyr⁴⁶, His¹⁰⁵, or Arg¹³⁵...
results in only partial inhibition of carbohydrate binding (16). In the bound state, these seven residues are all within hydrogen-bonding distance of the terminal sugar moiety (Fig. 3a). If the CD-MPR followed the precedent set by the other lectins, one would expect these essential residues to be within hydrogen-bonding distance to water molecules, which occupy the binding pocket in the ligand-free structure (17). However, this is not the case. There are four detectable water molecules in the binding pocket of the ligand-free form of the receptor (Fig. 3c). Of the four seen, one (W1) is also present in the pentamannosyl phosphate-bound CD-MPR (Fig. 3a). Of the remaining three water molecules, W2 contacts Asn104, W3 bridges Glu134 to Gly136 (main chain carbonyl oxygen), and W4 is within hydrogen-bonding distance of Tyr45. However, none of these three water molecules (W2, W3, W4) are in the same position otherwise held by a hydroxyl group of the sugar in the ligand-bound structure. This is due to relocation of residues in loop A (containing Tyr45), loop C (containing Asn104), and loop D (containing Glu134 and Gly136) when the receptor releases its ligand.

The CD-MPR appears to use a series of hydrogen bonds among residues in and near the binding pocket to maintain the integrity of the site in the absence of ligand. Residues of loop D are in position to form essential hydrogen bonds in this network because of the reorientation of this loop (Fig. 3, a–c). In the ligand-free conformation (Fig. 3c), the carboxyl group of Glu134 bridges between the guanidinium group of Arg135 and the hydroxyl of Tyr45 (β-strand 2). The carboxyl group of Glu134 of loop D is able to hydrogen bond to both the backbone nitrogen of Cys141 as well as the δ nitrogen of the imidazole ring of His105. Meanwhile the backbone carboxyl of Glu134 is in position to form hydrogen bonds to both the hydroxyl of Tyr143 and the γ-amide group of Gln66. In both the bound and unbound forms the hydroxyl of Tyr143 is within hydrogen-bonding distance to the ε nitrogen of Arg111. These results demonstrate that the side chains of three (Gln66, Arg111, and Tyr143) of the four residues that are critical for carbohydrate recognition are held in nearly identical positions in both the absence and presence of the ligand. In the ligand-free state, this balance is accomplished by an intricate network of hydrogen bonds, with important roles being played by Glu134 (the fourth critical residue for carbohydrate recognition), Glu114, and Arg135.

Influence of Ligand Binding on the Dimer Interface—Biochemical studies on the full-length CD-MPR (18, 19) along with our biochemical (8) and crystallographic (5, 6) studies on a soluble form of the receptor, Asn81/STOP155, demonstrate that the CD-MPR is a homodimer composed of identically folded
resulting from this scissoring and twisting motion preserves the exact molecular 2-fold symmetry of the dimeric molecule. This is very reminiscent of the quaternary structural change observed in the deoxy-to-oxy transition of hemoglobin (20, 21). The more closed structure of the ligand-free CD-MPR is analogous to the T-state of deoxy hemoglobin, and the open structure of the ligand-bound receptor compares to the R-state of oxyhemoglobin. However, it is not known at the present time whether there is any allosteric effect in the ligand binding mechanism of CD-MPR. The open/close effect of the dimer interface is also illustrated by the change in the distance between the individual ligand binding sites; the Cα atoms of His105 located at the tip of loop C are 26.6 and 34.3 Å apart, respectively, in the absence and presence of ligand (Fig. 4, a and b).

The scissoring and twisting motion the monomers undergo relative to each other also causes a change in the size of the interface area (Fig. 5, a and b). In the presence of ligand, either Man-6-P or pentamannosyl phosphate, the CD-MPR has a buried surface at the interface of ~2000 Å²/dimer and corresponds to a total of 30 residues being solvent-inaccessible (Fig. 1b). In the absence of ligand, the dimer interface decreases by ~500 to 1536 Å² and corresponds to a total of 24 residues being solvent-inaccessible (Fig. 1b). The changes in the interface area are also reflected in changes to the overall shape of the interface. In the presence of ligand, the inaccessible molecular surface may be described as “Y”-shaped with the two “arms” positioned ~45° apart on a central circular region (Fig. 5a). In the absence of ligand, the interface loses both arms and condenses down to an oval-shaped region (Fig. 5b). Two regions are responsible for the major changes observed in the solvent accessibility status of the ligand-bound versus ligand-free forms of the receptor; residues Val19, Gly10, Ser16, and Lys18 located within/near the amino-terminal loop and residues Glu133, Glu134, Gly136, Lys137, and Val138 located within/near loop D become solvent-accessible in the absence of ligand (Figs. 1b and 5, a and b).

In addition to the changes observed in the solvent accessibility of a number of residues, the nature of the amino acids comprising the interface of this receptor differ depending on whether the protein is in a ligand-bound or ligand-free state. The CD-MPR as a whole is composed of 43% nonpolar residues (Gly, Ala, Val, Leu, Ile, Phe, Met, Tyr, Trp, and Pro), 28% polar residues (Ser, Cys, Thr, Asn, and Gln), and the remaining 29% charged residues (His, Lys, Arg, Asp, and Glu). The buried interface in the presence of ligand is very similar in composition to that of the whole protein: 43% nonpolar, 27% polar, and 30% charged. In the absence of ligand, the interface becomes less nonpolar (38%) and contains more polar (29%) and charged (33%) residues, reflecting the change in solvent accessibility of the residues of the amino-terminal loop and loop D. Therefore, the release of ligand increases the hydrophilicity of the buried interface, which may increase the propensity of the two polypeptide chains in the dimer to move relative to each other depending on the pH and ionic strength of the environment.

Currently, there are more than 100 lectin structures available in the Protein Data Bank representing a wide range of lectin family members. Of the structures we have examined in which there are both a ligand-bound and ligand-free structure available, none show substantial alterations in their tertiary/quaternary structure upon carbohydrate binding. Legume lectins such as Lathyrus ochrus isolecitin I (22), pea lectin (23), Erythrina corallodendron lectin (24), winged-bean lectin (25), Griffonia simplicifolia lectin IV (26), Ulex europaeus lectin II (27), and peanut lectin (28) have both carbohydrate-bound and unbound structures reported in the literature. L. ochrus isolecitin I shows a slight displacement of residues 208–211 in the presence of ligand, but this movement varies with the packing envi-
ligand, the receptor dimer closes to change a number of the environment, and the r.m.s. deviation is less than 0.4 Å for backbone atoms of the two dimers (22). The pea lectin complex has been reported to experience a 1.1 Å shift of a loop (residues 216–218) toward the carbohydrate (23), whereas the other above mentioned legume lectins report no changes in the structures in the presence of ligand. Additionally, plant lectins *Urtica dioica* agglutinin (29), wheat germ agglutinin (30), mannos-specific bulb lectin from *Scilla campanulata* (31), and *Helianthus tuberosus* lectin (32) show only small changes upon ligand binding with reported r.m.s. deviations of less than 1 Å. Animal lectins follow the same trend set by the plant lectins of little to no change in structure upon ligand binding; as evidenced by the structures of the mannos-binding protein (33) and two galectin structures. Conger eel galectin and human galectin-7 structures are reported to have r.m.s. deviations of less than 1 Å between the bound and unbound forms of the protein (34, 35).

The structure of Asn<sup>81</sup>/STOP<sup>155</sup> solved in the absence of ligand provides insights into the functioning of the receptor. Unlike other lectins we have examined, this receptor appears to be unique in several structural aspects. In the absence of ligand, the receptor dimer closes to change a number of the intermonomer contacts. This raises the intriguing possibility that changes in the quaternary structure of the extracytoplasmic ligand-binding domains may be translated to the cytosolic region in the full-length receptor. This conformational change may in turn be relayed to an accessory adaptor protein(s), which could affect intracellular trafficking. Sandholzer et al. (36) have recently reported the results of a series of studies using chimeric receptors in which the cytoplasmic and/or transmembrane domains of the CD-MPR and IGFII/CI-MPR were exchanged. Analysis of the ability of the resulting chimeric receptors to internalize Man-6-P-containing ligands and to efficiently sort lysosomal enzymes provided evidence that MPR trafficking is influenced by the ligand binding region of the protein. Another structurally significant difference between the CD-MPR and other lectins resides in the large movement of residues within a loop. In the presence of ligand, loop D forms a side of the binding pocket, and residues Glu<sup>133</sup> and Arg<sup>135</sup> interact with the ligand. In the absence of ligand, the loop swings into a position which allows Glu<sup>133</sup>, Glu<sup>134</sup>, and Arg<sup>135</sup> to interact with residues of the binding pocket to form a hydrogen bond network that may serve to stabilize the pocket and keep the side chains of critical residues poised to bind ligand. However, the interaction of residues of loop D with the binding pocket does not appear to be a case of peptide mimicry as has been reported for concanavalin A (37) or peanut lectin (28), because the residues of loop D do not substitute for the mannos ring. In contrast, other lectins utilize tightly bound water molecules to serve as “place-holders” for the sugar hydroxyl groups (17).

Although the definitive analysis of the structural basis for the pH dependence of ligand binding must await a more complete structural analysis of the CD-MPR at various pH values, it is now possible to propose a plausible mechanism that facilitates ligand binding and unloading upon pH changes. The ligand binding affinity of the CD-MPR displays inflections at both high and low pH with a maximum at approximately pH 6.4 (38–40). The apparent pK<sub>a</sub> of the acidic side of the maximum (i.e. the low pH inflection point) is at pH ~5.2. Among the residues that are responsible for sugar binding is Glu<sup>133</sup>, which makes hydrogen bonds to both 2′-OH and 3′-OH of the terminal sugar moiety of the bound ligand. Glu<sup>133</sup> also makes a hydrogen bond with Tyr<sup>143</sup>, which in turn makes a hydrogen bond with Arg<sup>111</sup>, both of which are also essential residues for ligand binding. Although the pK<sub>a</sub> of the γ-carboxylate of glutamic acid is normally about 4.1, it is not unusual for the pK<sub>a</sub> value to shift by up to 3 pH units depending on its microenvironment (41). Therefore, it is reasonable to assume that the pH dependence of ligand binding at the acidic side of the pH maximum is at least partially attributable to Glu<sup>133</sup>. On the other hand, the apparent pK<sub>a</sub> of the basic side of the binding maximum is about 7.0. This corresponds to the pK<sub>a</sub> of the acid dissociation of the sugar phosphate moiety (42). This value is also close to the pK<sub>a</sub> of a histidine residue. Thus, it is not unreasonable to assume that His<sup>105</sup>, the only residue in which a side chain is involved in the binding of the phosphate moiety of the ligand, is accountable for the basic side of the maximum of the pH dependence. It is interesting to note that residues 102–105 (Tyr-Asp-Asn-His) are missing in the sequences of the two Man-6-P recognition sites of the IGF-II/CI-MPR (5), which, unlike the CD-MPR, binds the Man-6-P ligand at the cell surface where the pH is around 7.4 (38, 40). The combination of the two pH effects are responsible for the optimal binding of the CD-MPR to Man-6-P at a pH value around 6.5.

Overall, the CD-MPR appears to be structurally dynamic. It is possible that the structure we observe at pH 6.5 in the absence of ligand is an intermediate along the pathway taken...
by the receptor as it moves from the trans-Golgi network to the acidic late endosomal compartment. Therefore, future studies will be aimed at structural analyses of the receptor at various pH conditions. These additional structures, along with biochemical approaches, will confirm the above proposed mechanism by which the CD-MPR: 1) is able to bind lysosomal enzymes optimally in the Golgi (i.e. pH 6.5); 2) is able to release its ligand in the acidic environment (i.e. pH < 6.0) of late endosomal compartments; and 3) is unable to interact with lysosomal enzymes at the cell surface (i.e. pH 7.4). Furthermore, these structures will enable us to define a plausible sequence of structural changes during the transformation of the receptor molecule from the ligand-bound to ligand-free state (and vice versa) involving tertiary and quaternary structural changes such as loop flippings and changes at the receptor’s dimer interface.

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