The Three-dimensional Structure of Acarbose Bound to Glycogen Phosphorylase*

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Acarbose, a pseudotetrasaccharide with a conduritol ring at the nonreducing terminus, is a naturally occurring inhibitor of amylases. It is shown here to be an inhibitor of glycogen phosphorylase and to bind more tightly to the enzyme than the equivalent malto-oligosaccharide substrate.

X-ray crystallographic studies of the acarbose-phosphorylase a complex in the presence of glucose and caffeine reveal the structure of acarbose as bound to the storage site of phosphorylase. The acarbose binds in an orientation such that the conduritol ring makes no protein contacts. As with malto-oligosaccharides bound at this site, the observed conformation of acarbose is stabilized by O-2-O-3’ hydrogen bonding and is similar to, but not identical with, that predicted by hard-sphere exo-anomeric effect calculations and justified by ¹H nuclear magnetic resonance studies (Bock, K., and Pedersen, H. (1984) Carbohydr. Res. 132, 142–149). Intramolecular O2-O3’ hydrogen bonds appear to play an important role in stabilizing the conformation observed in these studies, even for those residues closely associated with the protein.

The pseudotetrasaccharide acarbose isolated from Actino-planes is a potent reversible inhibitor of many a-glucosidases and a-amylases (1). It is therefore of interest as a possible agent for controlling the uptake of glucose in the intestine and is being tested as a potential antidiabetic or antiobesity treatment (2–4).

Its inhibitory activity is ascribed to its unusual structure (Fig. 1), which comprises a hydroxymethyl conduritol residue a-(1-4)-linked to a 4-amino-4,6-dideoxyglucose which is in turn a-(1-4)-linked to maltose. Two structural features are considered to be responsible for its extremely high affinity for the active sites of many a-glucosidases (e.g. Kₐ = 10⁻⁴ M for Aspergillus niger glucoamylase (5)). First, the unsaturated cycloctiot moiety in its half-chain conformation is thought to act as a good “transition state analogue” of the putative oxocarbonium ion intermediate of glycodelase catalysis (5). Second, the presence of an exocyclic protonatable nitrogen atom at the glycosidic position has been shown previously (6) to enhance affinity in other glucosidase inhibitors and presumably plays a similar role in this case.

Glycogen phosphorylase catalyzes the degradation of glycogen to a-D-glucose-1-phosphate via sequential phosphorylysis of the a-(1-4)-linked glucose moieties. The structure of glycogen phosphorylase at 2.5 Å has been published (7), and a structure at 2.1 Å resolution in a refined state is available. Fragments of glycogen (maltpentaose and maltoheptaose), which act as good substrates, have been shown (8) to bind to the enzyme at an activator site known as the glycogen storage site, located some 15 Å from the active site on the exterior of the protein. In the presence of inhibitors that promote the inactive T-state conformation of the enzyme it is possible to bind oligosaccharides to the storage site of the T-state crystals of phosphorylase a essentially isomorphously, thus making it possible to determine accurately the structures of saccharides bound at this site through difference-Fourier analysis of x-ray diffraction data measured from crystalline phosphorylase-saccharide complexes. The study of maltoheptaose bound at the storage site (9, 10) showed it to bind in two subsites exhibiting a left-handed helical structure stabilized by O2-O3’ hydrogen-bonding interactions between adjacent glucose residues. This conformation was maintained for both residues in contact with the protein and for those extending away from the protein surface.

Since acarbose is an inhibitor of enzymes that hydrolyze amylase and glycogen, it also has potential as an inhibitor of glycogen phosphorylase. In this paper we present evidence that acarbose is indeed an inhibitor of glycogen phosphorylase, and we describe the x-ray crystallographically determined structure of acarbose bound to the glycogen storage site of this enzyme. Further, we present a comparison of this structure with that predicted for the inhibitor free in solution using the hard spheres exo-anomeric effect calculation and justified by ¹H nuclear magnetic resonance spectroscopy (11).

MATERIALS AND METHODS

All buffer chemicals and substrates were obtained from Sigma except for diithiothreitol, which was obtained from Bio-Rad. Acarbose was generously donated by Dr. E. Truscheit and Professor Dr. W. Frommer of Bayer AG West Germany.

Rabbit muscle phosphorylase b was prepared by the method of Fischer and Krebs (12) using diithiothreitol instead of cysteine and recrystallized at least three times before use. Phosphorylase a was prepared from phosphorylase b using phosphorylase kinase (EC 2.7.1.38) (13). Initial reaction rates in the direction of glycogenolysis were determined using the coupled assay procedure described previously (14) in a buffer containing sodium glycerophosphate (20 mM) and diithiothreitol (1 mM). An inhibition constant for acarbose was determined by measuring the rate of glycogenolysis at phosphate and maltoheptaose concentrations of 20 and 30 mM, respectively, and by use of eight different acarbose concentrations ranging from 0 to 200 mM. Data were plotted in the form 1/Fenzyme/Finhibited versus inhibitor.

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concentration and the slope of the straight line utilized to determine the $K_i$ value from the expression $K_i = K_m/slope(K_m + S)$ where $K_m$, the $K_i$ value of maltoheptaose under these conditions, independently determined to be 40 mM, and $S$ is the concentration of maltoheptaose used (30 mM).

Kinetic studies in the reverse direction, using glucose-1-phosphate as substrate, were performed by measuring phosphate released, as described previously (15). The rate of phosphate release was monitored at fixed concentrations of maltopentaose (10 mM) and glucose-1-phosphate (6 mM) in the presence of acarbose at two different concentrations (1 and 15 mM), both in the presence and absence of 5 mM phosphate and 5 mM fluorophosphate. Suitable controls were performed to determine the inhibition afforded by phosphate and fluorophosphate. The plots of these concentrations. No substrate activity could be detected for acarbose either in the direction of glycogen synthesis or glycogenolysis.

Crystals of phosphorylase $a$ in the glucose-inhibited conformation were kindly provided by Dr. N. B. Madsen and S. Shchonsky, Department of Biochemistry, University of Alberta. For the present study, the crystals were soaked in buffer containing 250 mM acarbose, 50 mM glucose, and 1 mM caffeine. The 12,600 strongest reflections to 2.5 Å were measured on a diffractometer, using 12 crystals. The average decay of intensity at the end of data collection was 26%. The scaling $R$ factor ($AF/F$) is 12.7% to glucose-inhibited phosphorylase $a$, and 10.8% to the maltoheptaose-phosphorylase $a$ complex also containing glucose and caffeine. The initial difference-Fourier for placement of the acarbose was calculated with phases either obtained from the refined structure of T-state phosphorylase $a$ or from the maltoheptaose-phosphorylase $a$ complex.

The primary structure of the acarbose molecule was modeled as described previously [9] for maltoheptaose except the conduritol ring, which was modeled from the half-chair monosaccharide D-glucono-(1-5)-lactone (16), to which appropriate substitutions were made. The bond angle at the bridging oxygen was set to 116.5° (9), the bridging nitrogen angle to 109.5°, and the N-C bond lengths to 1.48 Å.

The acarbose model was fit into the difference-Fourier map using the program FRODO (A. M. Jones) on an Evans and Sutherland PS300. The Hendrickson-Konertz restrained refinement program (17) of Rees and Lewis was used to refine the protein and saccharide. The starting coordinate set for the refinement was glucose-inhibited phosphorylase $a$, refined to 18% $R$-factor at 2.1 Å resolution and the model-built acarbose structure. The initial $R$-factor over the 12,600 data at 2.5 Å resolution was 23%. This refined in eight cycles of Hendrickson-Konertz refinement to 13.5%, with a root mean square deviation on bond lengths of 0.02 Å, and on bond angles of 3.8°.

Illustrations were prepared with the modeling programs INSIGHT (18) run on an Evans and Sutherland PS 300, and PLUTO (S. Motherwell, Cambridge, England).

RESULTS

Kinetic Studies—Acarbose was tested as a substrate for glycogen phosphorylase in place of glycogen, both in the direction of glycogen synthesis, and of glycogenolysis and found to be inactive in both cases. It was further tested as an inhibitor and found to be quite effective in the direction of glycogenolysis when assayed at essentially saturating (20 mM) phosphate concentrations against 3% maltoheptaose, yielding a $K_i$ (apparent) value of 26 mM. Acarbose was also tested at concentrations of 1 and 15 mM as an inhibitor of phosphorylase action in the direction of glycogen synthesis, but no inhibition could be detected, even in the presence of phosphophorylase (5 mM) or fluorophosphate (5 mM), which might have been required as obligate components of a ternary complex.

Structural Studies—The structure of the acarbose molecule fitted into the observed difference electron density is shown in Fig. 2, and its orientation is compared with that observed for maltoheptaose in Fig. 3. Coordinates for the bound acarbose are presented in Table I. The binding mode is very similar to that observed for the last 4 sugar residues of maltoheptaose at this site (9) (Fig. 3a), with the unsubstituted cyclitol ring A occupying site 7 of reference 9, and ring D occupying site 4 of reference 9. No electron density is observed at (maltoheptaose) site 3, although this site is occupied when maltotriose is bound to phosphorylase $a$ (8). Furthermore, acarbose does not bind either to the second activation site G1' to G4' observed previously (9). Nor does it bind to the active site, although such binding to the active site would not be expected in the presence of the two inhibitors caffeine and glucose (8).

The unsaturated cyclitol ring A at site 7 makes no protein contacts, and is less well-ordered than the remaining sugar residues. The corresponding residue in maltoheptaose is also relatively disordered at this site. However, contours calculated at a lower level (0.1 electrons/Å$^2$; i.e. twice $\sigma$ for the electron density) show clearly the orientation of the conduritol ring (Fig. 2b). Given the appearance of the electron density for the cyclitol, its relatively planar structure, and the constraint provided by the position of the well-ordered rings B-D, we estimate the errors in the conformation angles to be only a few degrees (Table I). The inter-sugar torsion angles at the N-linked cyclitol are different from those of the homopolymers of $\alpha$-D-glucose (9) (Table II). Nevertheless, the unsaturated cyclitol preserves the O2-O3' hydrogen bond found for maltoheptaose at this site. Our results on the conformation of acarbose are similar to those of Bock and Pedersen (11) determined by $^1$H NMR to the extent that C-6 of ring B is indeed near the vinylic hydrogen of the double bond on ring A. However, the ($\phi,\psi$) angles they report do not fit the electron density we observe.

Fig. 4 shows the superposition of maltoheptaose and acarbose. The two molecules superimpose best at site 4 (ring D) and least at site 7 (ring A). The acarbose molecule extends farther from the surface of the protein, and adopts a more shallow angle with ring B, as expected, given the half-chair conformation of the N-linked cyclitol.

Table III lists the interactions observed between acarbose and phosphorylase. The contacts at sites 4 and 5 (rings C and D) are identical to those observed for maltoheptaose (10). In comparing with (10) a change in numbering system necessitated by the discovery of an extra amino acid, isoleucine 308 in phosphorylase, must be taken into account. Ring B lacks the contact made by maltoheptaose involving O6, owing to the absence of this oxygen atom in acarbose. The conduritol ring A makes no protein contacts, either at this site or to neighboring phosphorylase molecules in the crystal lattice, as was also observed for maltoheptaose.

The temperature factor $B$ (Table II), a measure of the mean displacement for the ring, refined to values of 12, 18, and 15 for rings B-D, respectively. Such values are typical of well-ordered side chains for phosphorylase at this stage of refinement. The value of $B = 32$ for ring A is also a typical value for observable, but partially disordered, side chains.

DISCUSSION

Kinetic Studies—The absence of any substrate activity for acarbose in the glycogenolysis reaction is not surprising since
The Structure of the Acarbose-Phosphorylase Complex

**FIG. 2.** Acarbose fitted into difference electron density Fouier calculated with coefficients \((F_{\text{obs}} - F_{\text{precl}}) \exp (i\phi_{\text{precl}})\). *a,* entire acarbose molecule contoured at 0.16 electrons/Å³ in an orientation similar to that of Fig. 3. *b,* rings A and B contoured at 0.1 electrons/Å³ showing the fit of the partially ordered N-linked cyclitol. The root mean square electron density for the map is 0.06, negative contours have been omitted. Fig. 2a contains a well-ordered water molecule in addition to the saccharide.

This would require breakage of the relatively strong carbon-nitrogen bond. Its inactivity as a primer for glycogen synthesis presumably resides in its poor affinity in the required acceptor-binding mode and possibly an incorrect orientation and position of its 4-hydroxyl group when bound at the active site as a consequence of the partially planar ring structure.

It is an inhibitor of glycogenolysis \((K_{\text{inapp}}) = 26 \text{ mM}\), and binds more tightly than maltopentaose \((K_{\text{m}} = 40 \text{ mM})\) even though it has effectively one less sugar ring and lacks a 6-hydroxyl group at ring B. However, it is not a tight inhibitor when its affinity for phosphorylase is compared with that for jack-bean \(\alpha\)-amylase \((9.7 \text{ µM})\) or \(A. niger\) glucoamylase \((0.1 \text{ µM})\). Indeed the true meaning of its \(K_{\text{inapp}}\) value is unclear since the value measured may reflect contributions from binding at both the glycogen storage site and the active site.

The lack of inhibition in the reverse direction (glycogen synthesis) is possibly related to a need for a full ternary complex involving binding of both acarbose and phosphate to achieve significant interaction. A similar situation obtains with other nonphosphorylated "transition-state analogues" of phosphorylase such as \(\alpha\)-D-glucosyl fluoride \((20)\) and \(B\)-glucal
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FIG. 3. Oligosaccharides bound at the storage site of phosphorylase. An α-carbon diagram of the storage site (residues 397-437) with (a) maltoheptaose bound and labeled by site to conform with (9) and also labeled by residue to conform with Fig. 1 and Ref. 11. b, acarbose bound to the same site. Note that acarbose occupies the last four sites occupied by maltoheptaose.

(21) as substrates, and D-gluconolactone (22) as inhibitor, all of which are more effective in the presence of phosphate. Obviously, phosphorylase itself could not be added as a "cofactor" for inhibition studies in this direction, being a substrate for glycogenolysis, thus phosphite and fluorophosphate were utilized for inhibition studies in this direction, being a substrate for inhibition. Even under these conditions no inhibition could be detected. This may, however, be a consequence of the structural and electronic differences between phosphate and the analogues utilized. This apparent phosphate requirement for inhibitory action of acarbose may explain the previous claim (1) that acarbose is not an inhibitor of glycogen phosphorylase.

Conformation of the N-linked Cyclitol—The position and shape of the observed electron density shows that acarbose has a conformation similar to that found for maltoheptaose which is stabilized by a series of O2-O3' hydrogen bonds. The cyclitol moiety is partially ordered, even though no contacts are observed either within this protein-inhibitor complex or to neighboring protein molecules in the crystal structure. The presence of the O2-O3' hydrogen bond between the cyclitol and its neighboring sugar is presumably responsible for the observed order.

The conformation for the N-linked cyclitol reported previously (11) (Table II) is based on NMR measurement and hard spheres exo-anomeric calculations. The NMR data show that C-6 of ring B is close to the double bond of ring A; a fact which is also consistent with the structure presented here. However, the exact (φ,ψ) angles presented, which were predicted by hard spheres exo-anomeric calculation do not match our observation. This discrepancy may well be due to real

![Diagram of Oligosaccharides Bound at the Storage Site of Phosphorylase](image-url)
FIG. 4. Superposition of the structures of acarbose and maltoheptaose as observed at the storage site. a, acarbose alone. b, superposition. Acarbose is shown in thick lines, maltoheptaose in thin lines.
differences between the structures since binding of the inhibitor to the protein surface could indeed stabilize an alternate conformation. However, the conformation proposed is consistent with the NMR data and is inconsistent only with the hard spheres exo-anomeric calculations. Since the condu alternating ring contains no heteroatom, there will be no exo-anomeric effect to stabilize the conformation at this center, as noted (11), resulting in considerable uncertainty in the conformational predictions.

We have shown previously (9), that, when oligomers of alpha-D-glucose are bound to phosphorylase at the storage site, adjacent residues participate in an O2-O3' hydrogen bond. Inter-residue hydrogen bonds occur between glucosyl units in contact with the protein as well as between those which extend into the solvent-filled space between the protein molecules in the crystalline state. These studies extend such observations into the solvent-filled space between the protein molecules in the crystalline state. The crystallographic data for ring C (24, 25), and observations made on other disaccharide crystal structures (26) show that intramolecular hydrogen-bonding patterns are observed wherever possible in the crystalline state. These studies extend such observations and confirm that intramolecular hydrogen bonding is retained even in the highly solvated environment of the surface of a protein molecule.

**Acarbose Adopts a Single Binding Mode**—Acarbose adopts a single binding mode at the storage site of phosphorylase in which the N-linked cyclitol forms no protein contacts. No electron density is observed at maltoheptaose site 3, even though maltotriose shows occupancy of this site (8). In the conformation observed, the O-6 of ring C is present at site 5 and participates in the hydrogen-bonding network previously observed for maltoheptaose at this site (Table III). Ring B, which has no hydroxyl at C-6 and occupies site 6, thus loses only the single hydrogen bond which was observed for maltoheptaose at this site. Any binding mode placing ring B at site 5 would result in complete loss of the hydrogen-bonding network possible at this site. Thus, the acarbose binds in this single mode to maximize hydrogen bonding contacts with the protein.

This paper has, therefore, further demonstrated the feasibility of determination of a conformation of a small, noncrystalline molecule such as an oligosaccharide by determining the structure of its complex with a crystalline and structurally defined protein. This conformation may differ from those adopted free in solution but probably represents one of the conformations sampled in the unbound state. It is therefore a useful approach to determining such conformations in the absence of other techniques. In addition, we suggest that intramolecular hydrogen bonding can provide significant stabilization to the secondary structures of oligosaccharides and can therefore act as a powerful conformational determinant.

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