Comprehensive analysis of blood group antigen binding to classical and El Tor cholera toxin B-pentamers by NMR

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Introduction

Cholera is a life-threatening disease caused by the pathogenic bacterium Vibrio cholerae. The disease is caused by a secreted protein, the cholera toxin (CT), which consists of a heterohexamer formed by one toxic A-subunit (CTA) anchored in the middle of a ring formed by five B-subunits (CTB). The B-pentamer is responsible for targeting the toxin to human intestinal epithelial cells by binding to the GM1 ganglioside (Galβ3GalNAcβ4[NeuAcα3]Galβ3GalβCer) present on their surface. Binding of the toxin results in its internalization, and the release of the CTA subunit, which through a cascade of events ultimately leads to extreme diarrhea, and if untreated, death as a result of such rapid dehydration. The two major biotypes of cholera are Classical and El Tor, which produce toxins that typically differ only by two residues in their B-subunits at positions 18 and 47 (Y18 and Y47 in El Tor, H18 and T47 in classical CTB (cCTB)) (Sánchez and Holmgren 2008).

The severity of cholera symptoms, particularly for the El Tor biotype, has been reported to depend upon the patient’s blood group (Clemens et al. 1989; Harris et al. 2005, 2008). Blood type is, in part, determined by the ABH antigens, oligosaccharides expressed on cell surfaces, including those of the gastrointestinal tract. These three antigens define the blood groups A, B and O, respectively. They contain a common 2-O-fucosylgalactoside structure (as in H-tetrasaccharide 1, Figure 1), which is further substituted towards the non-reducing end by an α-galactosamine residue (in A-pentasaccharide 2, Figure 1) or an α-galactose (in B-pentasaccharide 3, Figure 1). Blood group O individuals, carrying the H-antigen, appear to be more at risk of developing severe cholera than those of other blood groups (Barua and Paguio 1977; Chaudhuri and De 1977; Glass et al. 1985; Swerdlow et al. 1994). This appears to be related to the toxin B-pentamer structure. Previous crystallographic studies revealed a secondary binding site in a CTB homolog, the human heat-labile enterotoxin from Escherichia coli (hLTB) (Holmner et al. 2007) as well as in a CTB/hLTB chimera (Holmner et al. 2004), which was able to interact with blood group oligosaccharides. These structural investigations showed the A type 2-antigen analog 2b (Figure 1) binding to the toxin B-pentamers in a region which includes residues 18 and 47 (Y18 and T47 in hLTB and in the CTB/hLTB chimera). In the crystal structures, the Tyr18 hydroxyl group was found to exhibit strong van der Waals interactions with the reducing end Glcβ of 2b and to coordinate a conserved water network at this site (Holmner et al. 2004). This suggested that the El Tor CTB (possessing Y18) could be capable of stronger interactions...
with the blood group antigens compared with cCTB (possessing H18), which would provide a possible explanation as to the stronger blood group dependence of El Tor cholera observed by Clemens et al. (1989). However, more recently, we have reported preliminary studies performed using surface plasmon resonance (SPR) and NMR spectroscopy showing that both El Tor and classical CTB-pentamers bind blood group determinants H and A with similar affinities, although with different kinetics (Heggelund et al. 2012). Concurrently, Turnbull and coworkers (Mandal et al. 2012) reported isothermal titration calorimetry (ITC) and NMR studies for the interaction of β-glycosides of H- and B-antigen analogs 1a and 3a, with El Tor CTB and hLTB, showing that the H-antigen analog binds to both El Tor CTB and hLTB. Surprisingly, the B-antigen analog 3a was found to bind only to hLTB but not to El Tor CTB. However, a single-point mutation at position 47 in El Tor CTB (I47T) restored the B-antigen-binding activity of the protein, which suggested a strong role for this residue in the interaction.

Here we report a comprehensive analysis using saturation transfer difference NMR (STD-NMR) and transferred NOESY (tr-NOESY) experiments (Dalvit 2009; Meyer and Peters 2003) to examine the interactions of cCTB and El Tor CTB with analogs of all three blood group determinants H-tetra, A-penta and B-penta (compounds 1b, 2b and 3b, respectively, Figure 1).

In all cases, the STD spectra showed clear interactions with both proteins and indicated that each oligosaccharide binds to the two different CTB-pentamers in a similar orientation. We observed some small but significant differences in the atomic details of the interaction of the three antigen analogs with either El Tor or cCTB, but the estimated $K_d$ values for the complexes are very similar. The tr-NOESY data support a binding conformation consistent with that observed in the X-ray structure of the A-antigen analog 2b bound to hLTB (Holmner et al. 2007). In addition, we have shown, through competition experiments, that the blood group H-tetrasaccharide 1b and the GM1 oligosaccharide (GM1-os, 4, Figure 1) bind at different sites on cCTB, as already suggested by Mandal et al. (2012) for El Tor CTB. Thus, this work offers a comprehensive frame for interpretation of the epidemiological analysis analysis linking cholera and blood type.

**Results**

The interaction of 1b–3b (Figure 1) with CTB was studied by STD-NMR and tr-NOESY. STD-NMR is one of the most used NMR methods to study the interactions between oligosaccharides and macromolecular receptors (Meyer and Peters 2003;
Bhunia et al. 2012). The technique helps identifying the epitope group of the ligands, revealing which moieties are closest to the receptor in the bound state. It is based on the transfer of magnetization from the protein to the bound ligand, which, by exchange, is released into solution where it is detected. The degree of saturation of individual ligand protons (expressed as absolute-STD percentage) reflects their proximity to the protein surface and can be used to describe the ligand–target interactions.

In addition to epitope mapping, $K_d$ values can be estimated from STD-NMR experiments. In fact, the STD intensity, which depends on the fraction of bound ligand, can be converted into the STD amplification factor (STD-AF), which is a function of the fraction of bound protein (Mayer and Meyer 1999, 2001). The evolution of the STD-AF along a ligand titration series enables the construction of a saturation curve, and the value of $K_d$ results from mathematical fitting of the experimental curve.

An NMR spectroscopic technique complementary to STD is tr-NOESY (Meyer et al. 1997; Haselhorst et al. 1999; Mayer and Meyer 2000; Post 2003). The observation of tr-NOEs relies on the different behavior of a small ligand molecule free in solution, rather than bound to a receptor protein. A ligand bound to a large molecular-weight protein behaves as part of the large molecule, rather than bound to a receptor protein. A ligand bound to a receptor protein can thus easily be distinguished by looking at the sign of the observed NOEs. These tr-NOEs reflect the bound conformation of the ligand, so that data can be inferred about the conformational equilibrium of the ligand during the binding event.

**Ligand interaction studies by saturation transfer difference and $^1H$ NMR**

The complete $^1H$ and $^{13}C$ NMR spectral assignments of free compounds $1b$–$3b$ are provided in Supplementary data, Table S1. All compounds are present as an equilibrium $\alpha/\beta$ anomic mixture of the reducing end glucose. The STD-NMR experiments were performed on soluble classical and El Tor CTB in the presence of antigen analogs $1b$–$3b$ in phosphate buffer at 298 K. STD spectra of the ligands in the absence of the CTB-pentamers did not show any signals. However, when the ligand complexes were analyzed, only signals resulting from the transfer of saturation from the protein receptor to the ligand protons were observed, thus permitting immediate mapping of the epitope. In all cases, clear binding was observed and binding constants could be determined from STD growth curves (Mayer and Peters 2003). Only one saturation time was selected for the data analysis, which allows only for a qualitative determination of binding epitopes. Indeed, the STD-NMR spectra showed rather low intensities that precluded a more quantitative approach using STD build-up curves, to avoid effects of different relaxation times (Mayer and James 2004) and rebinding effects (Angulo et al. 2008). However, the comparative nature of this study, both in terms of carbohydrate ligands and protein biotypes, supports the relevance of the qualitative approach used here.

The absolute STD values obtained for $1b$–$3b$ in the presence of cCTB and El Tor (ET CTB) are shown in Figure 2. STD values of overlapping signals were not included. In all cases, the highest STD signal was obtained for the anomic proton of the reducing-end glucose. This is most likely an artifact, due to interaction with water, and indeed these signals significantly change in intensity, depending on the use of water suppression sequences. Therefore, relative STD values were calculated for each of the molecules based on the second strongest signal. The relative STD values obtained with cCTB and El Tor CTB are shown in Figure 3. The relevant features of the interaction are discussed for each compound in the following sections.

**Blood group H tetrasaccharide Fucα2Galβ1[4/Fucα3]Glc 1b**

The STD spectrum of H-tetra $1b$ in the presence of cCTB is shown in Figure 4. Binding involves essentially the protons of the two fucose units, H1-Fuc(I) (5.2 ppm) and H1-Fuc(II) (5.3 ppm) (Figures 2A and 3). The signals of H2- and H4-Fuc(I) and H2- and H4-Fuc(II) also appear in the STD spectrum at 3.7 ppm, but cannot be quantified due to overlapping signals and are thus not included in Figure 2A.

The binding constant of H-tetra $1b$ to cCTB was calculated from a titration curve acquired by varying the H-tetrasaccharide concentration in the presence of the same amount of cCTB, which yielded a $K_d = 2.7 \pm 1.0$ mM.

For El Tor CTB, the interaction with $1b$ occurs primarily via H1-Fuc(I) (the most intense STD signal, 2.5 STD%), but also via H1-Fuc(II) (1.5 STD%) (Figures 2A and 3), hence the binding strength is reversed between the two fucose residues compared with cCTB. Binding further involves the H3 proton of the glucose unit (1.1 STD%), an interaction not observed in the H-tetra/cCTB complex. The interaction of El Tor CTB was previously investigated by ITC and NMR by Mandal et al. for a slightly different H-tetra analog $1a$ (Figure 1) containing GlcNAc rather than Glc at the reducing end and an alkyl linker (R) covalently attached to the anomeric position, yielding a $K_d$ value measured by ITC of $1.8 \pm 0.2$ mM (Mandal et al. 2012).

**Blood group H tetrasaccharide and GM1-os can bind to classical CTB simultaneously**

In order to exclude the possibility that the H-tetrasaccharide binds to the cCTB subunits at the same site as GM1-os (i.e. the primary binding site), competition studies were performed both by tr-NOESY and STD NMR. The NOESY spectrum of H-tetra in phosphate buffer shows positive NOE contacts (Figure 5A, red cross peaks). Addition of cCTB ($1b$/cCTB 20:1) to the solution results in negative NOE contacts in the tr-NOESY spectrum (Figure 5B, black cross peaks), indicating the event of binding and allowing the determination of the bound conformation of the ligand. GM1-os (4, 1:1 molar ratio with $1b$) was then added to the mixture, and tr-NOESY spectra were recorded again (Figure 5C). The binding affinity of GM1-os to cCTB was estimated as 43 nM by ITC (Tumbull et al. 2004), suggesting that GM1-os could easily displace the weaker H-tetrasaccharide ligand ($K_d$ in the mM range) if there were competition for the same binding site, as observed before for competitive CTB ligands (Bernardi et al. 2003). The H-tetra cross peaks are negative in Figure 5C, showing that $1b$ is not displaced from its binding site by GM1-os, and indicating that both compounds can interact simultaneously with CTB at their respective binding sites.
This is supported by the STD spectra acquired from the same sample and clearly showing the simultaneous presence of the Fuc anomeric signals of H-tetra 1b (5.2 and 5.3 ppm) and of the H3-NeuAc signal of GM1-os at 1.95 ppm (Figure 6).

A titration at different H-tetra concentrations (maintaining a fixed concentration of CTB and GM1-os), yielded a $K_d$ value of 3.0 mM, unchanged from the value measured for the interaction in the absence of GM1-os. This is further evidence that GM1 and blood group antigens bind to CTB at different sites and corroborate the observations of Mandal et al. (2012) for 1a/ El Tor CTB.

**Fig. 2.** Absolute STD values and calculated $K_d$ values for ABH antigen analogs. (A) H-tetra 1b with cCTB (blue bars) or El Tor CTB (ET) (gray bars). (B) A-penta 2b with cCTB (red bars) or ET (gray bars). (C) B-penta 3b with cCTB (green bars) or ET (gray bars). Note that the H5 Fuc(II) signal could not be analyzed in 3b, due to overlap. Residues Fuc(I), Fuc(II), Gal and Gal(II) are defined in Figure 3. Only data for non-overlapping signals are reported.


Preliminary results investigating the interaction of A-penta 2b with cCTB have been reported previously (Heggelund et al. 2012). In brief, the STD spectra of 2b acquired in the presence of cCTB (Figure 7A and B) indicate that the protons of the oligosaccharide that are more involved in interactions with the toxin are H1-GalNAc, H5-Fuc(II), H1-Fuc(II), H1-Fuc(I) and Ac-GalNAc (2.0, 1.3, 1.1, 1.1 and 1.0 absolute STD values, respectively) (Figure 2B). In addition, in the STD spectrum other signals belonging to the GalNAc, Gal, Fuc(I) and Fuc(II)
residues appear, although with weaker intensity. The relative STD values, grouped in four intensity ranges, are reported in Figure 3. The titration of A-penta in the presence of 43 μM cCTB yielded a $K_d$ of 5.2 ± 0.8 mM. This value is compatible with the value obtained previously by SPR (1.2 ± 0.3 mM) (Heggelund et al. 2012), especially given that overestimation of...
dissociation constants is a known feature of STD methods (Angulo et al. 2010; Angulo and Nieto 2011).

A-penta \(2b\) was also studied in the presence of El Tor CTB (Figure 7B and C). Absolute and relative STD values are shown in Figures 2B and 3. A comparison of the STD spectra acquired for both biotypes suggests that A-penta binds both proteins in a similar fashion, although with several important differences.

When comparing the interactions of A-penta \(2b\) with the toxin B-pentamers of the different biotypes, the STD signals are in general less intense for the \(2b\)/El Tor CTB complex compared with the cCTB complex (Figure 2B). Additionally, although the H1-GalNAc proton remains the most intense STD signal in both spectra, all other GalNAc proton signals are missing in the \(2b\)/El Tor CTB spectrum. Furthermore, the strong signal observed for H5-Fuc(II)/cCTB is missing in the \(2b\)/El Tor CTB spectrum. Despite these atomic-level differences, the \(K_d\) value for the A-penta/El Tor CTB complex is 6.4 ± 2.1 mM, very similar to the value for \(2b\)/cCTB.


The STD spectrum of B-penta \(3b\), acquired in the presence of cCTB (Figure 8A), shows interactions similar to those observed for the A-antigen analog \(2b\), involving principally the two fucose units, the blood group B-specific Gal(II), and the reducing end glucose residue (Figures 2C, 3 and 8A and B). A titration with increasing concentrations of B-penta in the presence of 43 \(\mu\)M cCTB yielded a dissociation constant \(K_d\) of 7.2 ± 1.6 mM.

When B-penta was studied in the presence of El Tor CTB, STD signals were also clearly observed (Figure 8C), and absolute values could be quantitatively calculated (Figure 2C). A \(K_d\) of 8.1 ± 2.3 mM was obtained, compared with cCTB. Only
Fig. 6. Simultaneous binding of H-tetra and GM1-os to cCTB confirmed by STD. (A) $^1$H NMR spectrum of H-tetra 1b (0.9 mM) in the presence of cCTB (43 μM). (B) $^1$H NMR spectrum of 1b (0.9 mM) and GM1-os 4 (0.9 mM) in the presence of cCTB (43 μM). The signals marked with an asterisk belong to GM1-os. (C) STD spectrum (obtained with 10,240 transients and 2.94 s of saturation time) corresponding to (B).

Fig. 7. Blood group A-pentasaccharide binding to the two CTB variants. (A) STD spectrum (obtained with 10,240 transients and 2.94 s of saturation time) of A-penta 2b (2.6 mM) and cCTB (43 μM) (60:1 ratio). (B) $^1$H NMR spectrum of 2b. (C) STD spectrum (10,240 transients and 2.94 s of saturation time) of A-penta 2b (1.7 mM) and El Tor CTB (34 μM) (50:1 ratio); the signals marked with an asterisk are artifacts.
minor differences were observed in the spectra for the two CTB variants, with the exception of H2-Glc, which gave a very strong signal for cCTB, but no signal for El Tor CTB, and H3-Fuc(II), which gave a medium intensity signal for cCTB, but no signal for El Tor CTB. Otherwise, most of the absolute and relative STD values are very similar, although generally less intense for El Tor CTB (Figures 2C and 3), except for H1-Gal(II) and H1-Fuc(II), where the opposite pattern was observed. The strongest interactions involve the two fucose units and the galactose residue Gal(II) characteristic of blood group B-antigens and, for cCTB, H2-Glc.

The observation made here that both classical and El Tor CTB bind to B-penta is consistent with previous preliminary experiments using surface plasmon resonance, where B-penta 3b was found to bind to El Tor CTB (Heggelund et al. 2012), although cCTB was not tested for binding in the previous study. These observations are conflicting with a recent report by Turnbull and coworkers, who found that the B-pentasaccharide glycoside 3a does not interact with El Tor CTB, except when Ile47 is replaced with Thr47 of the classical sequence (Mandal et al. 2012).

**Summary of interactions: Comparison of cholera biotypes**

All three blood group antigen analogs interact with both classical and El Tor CTB-pentamers, with dissociation constants in the millimolar range. Despite the two amino acid substitutions (H18Y and T47I; cCTB to El Tor CTB), the binding constants are equivalent within error limits for the two biotypes, with H-tetra 1b showing the strongest binding, followed by A-penta 2b, and B-penta 3b showing the weakest binding.

The blood group antigen-binding site is distinct from the GM1 binding site and presumably corresponds to the binding site identified for the A-analog 2b in the X-ray crystal structures of hLTB (Holmner et al. 2007) and the CTB/hLTB chimera (Holmner et al. 2004 and PDB ID: 2O2L, Holmner et al. 2007; Figure 9).

**Conformational studies by tr-NOESY**

To investigate the bound conformation of 1b–3b, tr-NOESY experiments were performed with both toxins. Details of these studies are described in Supplementary data. In brief, the tr-NOESY spectra obtained in the presence of either classical or El Tor CTB were similar to one another and to the spectra of the free sugars. The solution conformation of blood group antigens has been studied in careful detail (Yuriev et al. 2005 and references therein; see Supplementary data for additional references), and it can be described in all cases by one major conformation. This is maintained upon binding to either toxin. For B-penta 3b in complex with cCTB, only one additional NOE contact was observed (H5-Fuc(I)/ H2-Gal), indicating that in the bound form, Fucose (I) has a lower degree of mobility than in the free form and suggesting the selection of a more rigid conformation. For the A-antigen analog 2b, the conformation supported by tr-NOESY experiments corresponds to the one observed in the X-ray structures (PDB ID: 3EFX, Holmner et al. 2004 and PDB ID: 2O2L, Holmner et al. 2007; Figure 9).
The two fucose residues are involved in the binding of both biotypes for all three blood group antigens, with the H1 atoms playing a dominant role, whereas the central galactose residue only contributes to the binding of the A- and B-determinants (Figures 2 and 3). The GalNAc and Gal residues characteristic of A- and B-determinants, respectively, are involved in blood group antigen binding independent of the biotype. In each case, however, the intensity, number and exact positions of the STD contacts vary.

The atomic details of the variations are clearly visible for A-penta GalNAc, which shows multiple STD contacts with cCTB (H1-, H2-, H5-, Ac-GalNAc) and a single contact (H1-GalNAc) with El Tor CTB (Figure 2B). In the crystal structure of 2b in the CTB/hLTB chimera (Figure 9; PDB ID: 3EFX, Holmner et al. 2004), which like cCTB features a threonine residue at position 47, the GalNAc residue is positioned near Thr47 and binds to the protein mainly through water-mediated interactions, and through two H-bonds of the GalNAc N-acetyl nitrogen to Gly45 O and Thr47 OG1. The lower resolution structure of hLTB (T47) in complex with this ligand (PDB ID: 2O2L, Holmner et al. 2007) shows the same interactions. It is not clear how a substitution of Thr47 for Ile47 in El Tor CTB would affect ligand binding. The substitution of Thr47 for Ile in its preferred rotamer conformation (Figure 9) suggests that

Fig. 9. Modeled antigen interactions. (A) Close-up view of the A-pentasaccharide 2b bound to CTB, showing the relative STD intensity values for the interaction with cCTB (from Figure 3) as colored dots. The ligand and selected amino acid residues are given in stick representation, while the protein is shown as a surface representation (with different colors for the individual B-subunits). The figure was generated based on the crystal structures of cCTB (PDB ID: 3CHB, Merritt et al. 1998) and the ligand complex of a CTB/hLTB chimera (PDB ID: 3EFX, Holmner et al. 2004). The two residues differing between classical and El Tor CTB (18, 47) are shown in purple (classical) and green (El Tor), with Ile47 being introduced manually in its preferred rotamer conformation (insert: collage of CTB binding to the A-pentasaccharide (light gray) and GM1-os (turquoise)). (B) Stereo representation of A, including the water molecules of the A-penta complex (PDB ID: 3EFX, Holmner et al. 2004), and their H-bonding interactions. Note that the orientation of the blood group determinant is opposite to the conventional orientation shown in Figure 1, since CTB is displayed in its standard view, with the GM1 binding site facing down towards the cell surface.
small adaptations may be sufficient to accommodate the ligand; on the other hand, Mandal et al. (2012) observed no binding of this protein variant to a blood group B-determinant. The STD spectra of 2b further reveal small differences between the two biotypes in the region of Fuc(II) and, to some extent, Gal. Several contacts, most notably that of H5-Fuc(II), are only observed for one of the protein variants. On the other hand, Fuc(I) and Glc, which in the published crystal structures are in the proximity of residue 18 (Holmner et al. 2004, 2007), are not affected. In the CTB/hLTB chimera (PDB ID: 3EFX, Holmner et al. 2004), Tyr18 coordinates a water network (Figure 9B), which is of significant importance for glucose binding of 2b. In cCTB, Tyr18 is replaced by the smaller His18 which is unlikely to form a similar network. However, this does not necessarily prevent the interaction from occurring.

The absolute STD values measured for the fucose residues are essentially the same for A-penta 2b and B-penta 3b with either protein (H1-Fuc(I), H6-Fuc(I), H1-Fuc(II), H6-Fuc(II), Figure 2B and C). The signal of H5-Fuc(II), which is strong in the STD spectrum of 2b, could not be analyzed for B-penta 3b due to overlapping signals (at 4.71 ppm). Some differences between the analogs are associated with the different residues (GlcNAc and Gal) at the non-reducing end of each sugar and with the terminal Glc H2 proton. Another small difference concerns H3-Fuc(I). It is worth repeating that these differences do not lead to major changes in the measured \( K_d \) values, which are at the upper limit available to the technique.

**Discussion**

In the quest to understand the molecular mechanisms for the observed blood group dependence of cholera, a number of different methods have been used, including protein crystallography (Holmner et al. 2004, 2007), NMR (Heggelund et al. 2012; Mandal et al. 2012) and quantitative-binding studies by SPR (Heggelund et al. 2012) or ITC (Mandal et al. 2012). Both classical and El Tor CTB have been investigated, in addition to a CTB/hLTB chimera, which contains Tyr18, Thr47 and the CTB-specific residue Asn4 (Holmner et al. 2004). The tested ligands are analogs of the A, B and H blood group determinants A Lewis-y, B Lewis-y and Lewis-y. A and B Lewis-y represent the epitopes on the glycosphingolipids from the human small intestinal epithelium that tested binding positive in an initial study (Ångström et al. 2000) and are commonly found in the small intestinal mucosa, especially in secretors (Ravn and Dabelsteen 2000). In our current work, we expanded our investigation of the potential involvement of CTB in the cholera blood group dependence phenomenon using NMR, in particular STD and tr-NOESY. These are very versatile and sensitive methodologies that utilize NOE effects to investigate the interaction between protein and ligand. We confirmed that binding of the blood group oligosaccharides occurs at a site distinct from the primary GM1 binding site and showed that all three blood group antigen analogs (H-tetra 1b, A-penta 2b and B-penta 3b) bind to the El Tor CTB as well as to cCTB.

This is in contrast to the recent report by Turnbull and coworkers (Mandal et al. 2012), who observed, using STD and ITC, that only a \( \beta \)-glycoside of the H-tetrasaccharide 1a, but not the corresponding \( \beta \)-glycoside of the B-pentasaccharide 3a bound to CTB El Tor. This raises the question as to why two studies have produced such contrasting data for the interaction of the B-antigen interaction with CTB. Disregarding any potential technical problems with the STD experiments, the most likely cause for the difference is the different oligosaccharides investigated, as the recombinant constructs for El Tor CTB were identical (generously provided to us by T. Hirst, through the hands of B. Turnbull). The oligosaccharides used in our study are from human milk 1b-3b, which lack the N-acetyl group of GlcNAc and are used as anomic mixtures at the reducing end residue. This choice was dictated by the commercial availability of compounds in earlier experiments (Holmner et al. 2004, 2007). The Turnbull group employed synthetic analogs 1a and 3a (Figure 1), which contain the terminal N-acetyl group and in addition feature a 3-methoxycarbonylaminopropyl group (R) covalently attached to the anomic position of GlcNAc, locking the anomic carbon in the \( \beta \)-configuration. Non-binding could hence depend either on the additional N-acetyl group of GlcNAc or on the alky linkers attached to the anomic position of the reducing end sugar. The published crystal structures do not indicate any potential clash for the \( N \)-acetyl group, which in fact was mistakenly included in the original structural model of the CTB/hLTB chimera (PDB ID: 3EFX, Holmner et al. 2004). However, this structure featured Thr and not Ile at position 47 at the other end of the binding site (see Figure 9), and Turnbull and coworkers convincingly showed that they were able to restore the binding activity of the El Tor CTB for their B-analog by the substitution I47T (Thr being the classical residue) (Mandal et al. 2012). The CTB/hLTB chimera further showed significantly increased binding affinity to blood group A- and B-antigens in microtiter well assays compared with cCTB (Ångström et al. 2000), which features a histidine residue at position 18. These studies, however, were undertaken with glycosphingolipids rather than with blood group oligosaccharides. Together, these studies suggest that both Tyr18 and Thr47 correlate with enhanced binding, whereas His18 and Ile47 may correlate with weaker binding. The combinations His18/Thr47 and Tyr18/Ile47, as found in classical and El Tor CTB, respectively, may then compensate to yield a moderate effect, explaining why we obtained virtually identical binding constants for A- and B-penta, to either cCTB or El Tor CTB. V. cholerae strains that produce a toxin featuring both Tyr18 and Thr47 have also been reported, such as for some O139 Bengal strains circulating between 1999 and 2005 (Bhuiyan et al. 2009); however, to the best of our knowledge, they have not been analyzed with respect to blood group dependence.

When comparing the atomic details of binding interactions of the different blood group oligosaccharides to the two CTB variants, some differences were observed. For B-penta, the main difference in interaction intensity was observed at the reducing end of the oligosaccharide, which is expected to bind in the proximity of residue 18 of the protein in accordance with previous studies (Holmner et al. 2004, 2007). It is at the reducing end of the oligosaccharide, where the B-antigen analog investigated by us differs from the analog studied by Turnbull and coworkers (Mandal et al. 2012). For A-penta, the strongest differences were found at the non-reducing end, close to residue 47 at the interface to the neighboring toxin B-subunit.
While the structural changes caused by the amino acid substitutions are expected to be small, they may affect the ligand orientation, with consequences for ligand interactions at the other end of the binding site. Furthermore, the different binding kinetics of A- or B- compared with H-determinants (as seen by SPR; Heggelund et al. 2012) suggest that also adjustments of the subunits within the B-pentamer might occur, within a delicate balance of binding interactions.

In conclusion, the observed interaction of all three blood group antigen analogs tested here (H-tetra, A-penta and B-penta) with both classical and El Tor CTB provides evidence that also the natural blood group antigens can interact with CTB. Although a trend is clearly visible in the measured $K_d$ of H-tetra < $K_d$ of A-penta < $K_d$ of B-penta), the small difference in binding affinities for the three blood group determinants is unlikely to cause a large biological effect. We still contend that the blood group dependent association with cholera severity is likely to occur due to the difference in kinetics as previously reported (Heggelund et al. 2012). At present, we cannot explain why El Tor cholera should show a stronger blood group dependence than the classical disease, but we note that the data excluding classical cholera (Clemens et al. 1989) have a small sample size. Furthermore, El Tor strains of V. cholerae O1 in Bangladesh have, since 2001, been observed to produce the classical CT genotype (Nair et al. 2006), while blood group dependence is continuing to be reported (Harris et al. 2005, 2008), suggesting that there is no statistically significant difference in blood group dependence between CT genotypes. The secretor status of the individuals is more likely to be an important determining factor in cholera severity, as the presence of blood group antigens in the mucus layer can have a protective effect against cholera, in particular for blood group A and B individuals (Chaudhuri and DasAdhikary 1978; Arifuzzaman et al. 2011). A similar effect may underlie the protection conferred by fucosylated human milk oligosaccharides against diarrhea in breastfed infants (Newburg et al. 2004; Blank et al. 2012). Furthermore, with ~80% of the normal Indian population being secretor positive (Chaudhuri and DasAdhikary 1978), secretor status is likely to have strongly contributed to shaping the extreme blood group distribution in present Bangladesh. As the ancestral home of cholera and in addition a region endemic to malaria, which disproportionally affects blood group A individuals, this may explain why Bangladesh exhibits one of the highest incidences of the otherwise rare type B blood allele in the world.

Materials and methods

The blood group antigen analogs H-tetra, A-penta and B-penta were purchased from Elicityl (catalog number GLY066, GLY067 and GLY068, respectively).

Protein Production and sample preparation

Lyophilized cCTB was purchased from Sigma–Aldrich containing Tris buffer salts, NaCl, Na$_2$N$_3$ and Na$_2$EDTA. The powder was dissolved in D$_2$O, resulting in cCTB in 0.05 M Tris buffer, 0.2 M NaCl, 0.003 M Na$_2$N$_3$ and 0.001 M Na$_2$EDTA, pH 7.5. The signals of Tris and EDTA fall in the same region as the signals of the ligand, so the sample was ultrafiltered using a Millipore Amicon Ultra-4 centrifugal filter unit (MWCO 5000 Da), and the buffer was changed to phosphate buffer (20 mM, Na$_2$PO$_4$/Na$_3$HPO$_4$) at pH 7.5 in D$_2$O.

El Tor CTB was prepared as previously described (Heggelund et al. 2012). Briefly, the protein was overexpressed in Vibrio sp. 60, grown in LBS medium (LB medium with 15 g/l NaCl) at 30°C, 160 rpm, supplemented with 100 μl/mL ampicillin, until OD600 reached 0.2. After induction with 0.5 mM IPTG, the cultures were grown for additional 16–20 h. The CTB is naturally secreted into the growth medium, and after removal of bacteria by centrifugation, the supernatant was purified by affinity chromatography. The protein was captured by the immobilized β-galactose gel (Thermo Scientific) and eluted using a galactose gradient. After dialysis against 0.1 M MES pH 6.0, the protein was further purified by ion exchange chromatography (HiTrap™ SP XL, GE Healthcare) using an NaCl gradient. Finally, the purified CTB was dialyzed against a storage buffer (20 mM Tris–HCl, 200 mM NaCl, pH 7.5) and concentrated to 9.5 mg/mL. Prior to the NMR studies, the buffer was changed to phosphate buffer at pH 7.5 in D$_2$O, as described for cCTB.

NMR experiments

Each sample was prepared in a 3-mm NMR tube using different ligand concentrations (from 1 to 20 mM) in the presence of 43 or 46 μM cCTB, depending on the experiment, or 34 μM El Tor CTB, in 180 μL phosphate buffer (20 mM, Na$_2$PO$_4$/Na$_3$HPO$_4$) at pH 7.5 in D$_2$O.

The spectra were acquired with a Bruker Avance 600 MHz instrument at 298 K. For the complete assignment of the molecules and the conformational analysis, the following experiments were used: 1D, COSY, TOCSY with a mixing time of 60 ms, NOESY with mixing times of 200 and 700 ms, $^1$H,$^13$C HSQC ($J=145$ Hz). In the presence of an intense unwanted water signal at 4.7 ppm, solvent suppression was achieved by use of an excitation sculpting pulse sequence. The spectrum shows two set of signals (1:1 ratio) due to the presence of an equilibrium α/β anomeric mixture of the reducing end glucose.

The STD spectra were performed with an on-resonance irradiation frequency at ~0.05 or ~0.5 ppm, while 200 ppm was chosen as off-resonance frequency. The experiments were performed at different temperatures (283–298K), but the best results were obtained at 298 K.

Selective presaturation of the protein was achieved by a train of Gaussian shaped pulses, each of 49 ms in length. STD experiments were acquired with 0.98 or 2.94 s of total saturation times. The STD sequence was obtained from standard Bruker library and water suppression was achieved by using the WATERGATE 3-9-19 pulse sequence.

Supplementary data

Supplementary data for this article are available online at http://glycob.oxfordjournals.org/.
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Conflict of interest statement
None declared.

Abbreviations

References

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