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

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

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Running Title: NMR study of blood group antigen binding to cholera toxin

Supplementary data: Chemical shift assignment of blood group analogs 1b-3b; NOESY studies: conformational analysis; STD amplification factors and calculation of binding constants
Keywords: ABH blood group determinants / binding affinity / cholera blood group dependence / nuclear magnetic resonance spectroscopy (STD and tr-NOESY) / cholera toxin
ABSTRACT

Cholera is a diarrheal disease responsible for the deaths of thousands, possibly even hundreds of thousands of people every year, and its impact is predicted to further increase with climate change. It has been known since decades that blood group O individuals suffer more severe symptoms of cholera compared to individuals with other blood groups (A, B, AB). The observed blood group dependence is likely to be caused by the major virulence factor of Vibrio cholerae the cholera toxin. Here, we investigate the binding of ABH blood group determinants to both classical and El Tor cholera toxin B-pentamers using STD-NMR, and show that all three blood group determinants bind to both toxin variants. Although the details of the interactions differ, we see no large differences between the two toxin genotypes, and observe very similar binding constants. We also show that the blood group determinants bind to a site distinct from that of the primary receptor, GM1. Tr-NOESY data confirm that the conformations of the blood group determinants in complex with both toxin variants are similar to those of reported X-ray and solution structures. Taken together, this detailed analysis provides a framework for the interpretation of the epidemiological data linking the severity of cholera infection and individual’s blood group and brings us one step closer to understanding the molecular basis of cholera blood group dependence.
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]Galβ4GlcβCer) 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 I47 in El Tor, H18 and T47 in classical CTB) (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, Sack, et al. 1989, Harris, Khan, et al. 2005, Harris, LaRocque, et al. 2008). Blood type is, in part, determined by the ABH antigens, oligosaccharides expressed on cell surfaces, including those of the gastro-intestinal tract. These three antigens define the blood groups A, B and O, respectively. They contain a common 2-\(O\)-fucosyl-galactoside structure (as in H-tetrasaccharide 1, Figure 1), which is further substituted towards the non-reducing end by an \(\alpha\)-galactosamine residue (in A-pentasaccharide 2, Figure 1) or an \(\alpha\)-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, Holmgren, et al. 1985, Swerdlow, Mintz, 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, Askarieh, et al. 2007) as well as in a CTB/hLTB chimera (Holmner, Lebens, 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 Glc\(\beta\) of \(2b\), and to coordinate a conserved water network at this site (Holmner, Lebens, et al. 2004). This suggested that the El Tor CTB (possessing Y18) could be capable of stronger interactions with the blood group antigens compared to the classical CTB (possessing H18), which would provide a possible explanation as to the stronger blood group dependence of El Tor cholera observed by Clemens *et al* (Clemens, Sack, 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 cholera toxin B-pentamers bind blood group determinants H and A with similar affinities, although with different kinetics (Heggelund, Haugen, et al. 2012). Concurrently, Turnbull and coworkers (Mandal, Branson, et al. 2012) reported isothermal titration calorimetry (ITC) and NMR studies for the interaction of \(\beta\)-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 STD NMR and tr-NOESY experiments (Dalvit 2009, Meyer and Peters 2003) to examine the interactions of classical CTB 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 classical CTB, 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, Askarieh, 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 classical CTB, as already suggested by Mandal et al. for El Tor CTB (Mandal, Branson, et al. 2012). Thus, this work offers a comprehensive frame for interpretation of the epidemiological analysis linking CT infection and blood type.
RESULTS

The interaction of 1b-3b (Figure 1) with CTB was studied by STD-NMR and tr-NOESY. Saturation Transfer Difference NMR (STD–NMR) is one of the most used NMR methods to study the interactions between oligosaccharides and macromolecular receptors (Bhunia, Bhattacharjya, et al. 2012, Meyer and Peters 2003). 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, Mayer and Meyer 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 transferred NOESY (tr-NOESY) (Haselhorst, Espinosa, et al. 1999, Mayer and Meyer 2000, Meyer, Weimar, et al. 1997, 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, and adopts the corresponding NOE behavior, showing strong negative NOEs, so-called tr-NOEs. Binding of a ligand 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 STD and $^1$H-NMR**

The complete $^1$H- and $^{13}$C-NMR spectral assignments of free compounds 1b-3b are provided in the *Supplementary Information* (Table S1). All compounds are present as an equilibrium $\alpha/\beta$ anomeric 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 cholera toxin B-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 (see below) and binding constants could be determined from STD growth curves (Meyer 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, Díaz, 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 classical CTB (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 anomeric
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 classical CTB 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** Fuca2Galβ4[Fuca3]Glc 1b

The STD spectrum of H-tetra 1b in the presence of classical CTB 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) (Figure 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 classical CTB 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%) (Figure 2a and Figure 3), hence the binding strength is reversed between the two fucose residues compared to classical CTB. 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, Branson, 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 classical CTB subunits at the same site as GM1-os (i.e. the primary binding site), competition studies were performed both by tr-NOESY and STD experiments. The NOESY spectrum of H-tetra in phosphate buffer shows positive NOE contacts (Figure 5A, red cross peaks). Addition of classical CTB (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 (see below). 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 classical CTB was estimated as 43 nM by ITC (Turnbull, Precious, 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, Arosio, 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 performed on 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 corroborates the observations of Mandal et al. for 1a/El Tor CTB (Mandal, Branson, et al. 2012).


Preliminary results investigating the interaction of A-penta 2b with classical CTB have been reported previously (Heggelund, Haugen, et al. 2012). In brief, the STD spectra of 2b acquired in the presence of cCTB (Figure 7A,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, Haugen, et al. 2012), especially given that overestimation of dissociation constants is a known feature of STD methods (Angulo, Enríquez-Navas, et al. 2010, Angulo and Nieto 2011).

A-penta 2b was also studied in the presence of El Tor CTB (Figure 7B,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 to 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 the A-penta/El Tor CTB complex, is $6.4 \pm 2.1$ mM, very similar to the value for $2b$/cCTB.


The STD spectrum of B-penta $3b$, acquired in the presence of classical CTB (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 (Figure 2C, 3 and Fig. 8A,B). A titration with increasing concentrations of B-penta in the presence of 43 µM classical CTB yielded a dissociation constant $K_d$ of $7.2 \pm 1.6$ 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 $8.1 \pm 2.3$ mM was obtained, comparable to cCTB. Only 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 (Figure 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, Haugen, et al. 2012), although classical CTB 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, Branson, et al. 2012).

**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 the Supplementary Information. 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, Farrugia, et al. 2005 and references therein; see Supplementary Information for additional references) and it can be described in all cases by one major conformer. This is maintained upon binding to either toxin. For B-penta 3b in complex with classical CTB, 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, Lebens, et al. 2004 and PDB ID: 2O2L, Holmner, Askarieh, et al. 2007; Figure 9).

Summary of interactions - Comparison of cholera biotypes

All three blood group analogs interact with both classical and El Tor cholera toxin B-pentamers, with dissociation constants in the mM 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 showing the strongest binding, followed by A-penta, and B-penta 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 in the X-ray crystal structures of hLTB (Holmner, Askarieh, et al. 2007) and the CTB-LTB chimera (Holmner, Lebens, et al. 2004). 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, Lebens, 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, Askarieh, 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 small adaptations may be sufficient to accommodate the ligand; on the other hand, Mandal et al. observed no binding of this protein variant to a blood group B-determinant (Mandal, Branson, et al. 2012).

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, Askarieh, et al. 2007, Holmner, Lebens, et al. 2004), are not affected. In the CTB/hLTB chimera (PDB ID: 3EFX, Holmner, Lebens, et al. 2004), Y18 coordinates a water network (Figure 9B), which is of significant importance for glucose binding of 2b. In classical CTB, Y18 is replaced by the smaller H18 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,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 (GalNAc and Gal) at the non-reducing end of each sugar and with the
terminal Glc H2 proton. Another small difference concerns H3-Fuc(II). 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, Lebens, et al. 2004, Holmner, Askarieh, et al. 2007), NMR (Heggelund, Haugen, et al. 2012, Mandal, Branson, et al. 2012), and quantitative binding studies by SPR (Heggelund, Haugen, et al. 2012) or ITC (Mandal, Branson, et al. 2012). Both classical and El Tor CTB have been investigated, in addition to a CTB/LTB chimera, which contains Tyr18, Thr47 and the CTB-specific residue Asn4 (Holmner, Lebens, 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, Bäckström, 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 classical CTB.

This is in contrast to the recent report by Turnbull and coworkers (Mandal, Branson, 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, Lebens, et al. 2004; Holmner, Askarieh, et al. 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-methyloxycarbonylaminopropyl group (R) covalently attached to the anomic position of GlcNAc, locking the anomic carbon in the β-configuration. Non-binding could hence depend either on the additional N-acetyl group of GlcNAc or on the alkyl linker 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/LTB chimera (PDB ID: 3EFX, Holmner, Lebens, 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, Branson, et al. 2012). The CTB/LTB chimera further showed significantly increased binding affinity to blood group A and B antigens in microtiter well assays compared to classical CTB (Ångström, Bäckström, 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 classical CTB or El Tor CTB. \textit{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 to 2005 (Bhuiyan, Nusrin, 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, Lebens, et al. 2004, Holmner, Askarieh, et al. 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, Branson, 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 to H-determinants (as seen by SPR; Heggelund, Haugen, 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 $K_d$ ($K_d$ 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 dependence associated with cholera severity is likely to occur due to the difference in kinetics as previously reported (Heggelund, Haugen, 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, Sack, 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, Qadri, et al. 2006), while blood group dependence is continuing to be reported (Harris, Khan, et al. 2005, Harris, LaRocque, et al. 2008), suggesting that there is no statistically significant difference in blood group dependence between cholera toxin 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, Ahmed, et al. 2011). A similar effect may underlie the protection conferred by fucosylated human milk oligosaccharides against diarrhea in breastfed infants (Newburg, Ruiz-Palacios, et al. 2004; Blank, Dotz, et al. 2012). Furthermore, with approximately 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 (catalogue number GLY066, GLY067 and GLY068, respectively).

Protein Production and sample preparation

Lyophilized classical CTB was purchased from Sigma-Aldrich containing Tris buffer salts, NaCl, NaN₃ and Na₂EDTA. The powder was dissolved in D₂O, resulting in classical CTB in 0.05 M Tris buffer, 0.2 M NaCl, 0.003 M NaN₃ and 0.001 M Na₂EDTA, 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 5,000 Da) and the buffer was changed to phosphate buffer (20 mM, NaH₂PO₄/Na₂HPO₄) at pH 7.5 in D₂O.

El Tor CTB was prepared as previously described (Heggelund, Haugen, et al. 2012). Briefly, the protein was over-expressed in Vibrio sp. 60, grown in LBS medium (LB medium with 15g/l NaCl) 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 D-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 D₂O, as described for classical CTB.
NMR experiments

Each sample was prepared in a 3 mm NMR tube using different ligand concentrations (from 1 mM to 20 mM) in the presence of 43 µM or 46 µM c CTB, depending on the experiment, or 34 µM ET CTB, in 180 µl of phosphate buffer (20 mM, NaH$_2$PO$_4$/Na$_2$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, 1H,13C-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 $\alpha/\beta$ anomic mixture of the reducing end glucose.

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

Selective presaturation of the protein was achieved by a train of Gauss shaped pulses, each of 49 ms in length. STD experiments were acquired with 0.98s 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.

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LEGENDS TO FIGURES

Figure 1. Structures of the blood group antigen oligosaccharides 1-3 and the GM1 oligosaccharide 4 (GM1-os). Carbohydrate symbols follow the nomenclature of the Consortium for Functional Glycomics (Nomenclature committee, Consortium for Functional Glycomics, 2014); N-acetylenuraminic acid – purple diamond; D-galactose – yellow circle; N-acetylgalactosamine – yellow square; D-glucose – blue circle; N-acetylglucosamine – blue square; L-fucose – red triangle.

Figure 2. Absolute STD values and calculated $K_d$ values for ABH antigen analogs. (A) H-tetra 1b with classical CTB (cCTB) (blue bars) or El Tor CTB (ET) (grey bar). (B) A-penta 2b with cCTB (red bars) or ET (grey bar). (C) B-penta 3b with cCTB (green bars) or ET (grey bar). 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.

Figure 3. Relative STD values, grouped in four intensity ranges, for antigen analogs H-tetra (1b), A-penta (2b) and B-penta (3b), interacting with both toxins.

Figure 4. H tetrasaccharide binding to cCTB. (A) STD (10240 transients and 2.94s of saturation time) and (B) $^1$H-NMR spectrum of blood group H-tetrasaccharide 1b (2.6 mM) in the presence of classical CTB (43 µM) (1b/cCTB 60:1).

Figure 5. H-tetra and GM1-os interact with cCTB simultaneously. (A) NOESY spectrum of H-tetra 1b in phosphate buffer shows positive (red) cross peaks. (B) tr-
NOESY spectrum of H-tetra (0.9 mM) in the presence of cCTB, (43 µM); negative NOE contacts (black cross peaks) indicate binding. (C) tr-NOESY of H-tetra 1b + cCTB + GM1-os (1b/cCTB/GM1-os = 20:1:20) in buffer solution. The H-tetra cross peaks are negative, showing that the blood group H antigen analog is not displaced from its binding site. Negative cross peaks for GM1-os (yellow circles) indicate that both compounds, H-tetra and GM1-os, interact with CTB simultaneously.

**Figure 6.** Simultaneous binding of H-tetra and GM1-os to cCTB confirmed by STD. (A) 1H-NMR spectrum of H-tetra 1b (0.9 mM) in the presence of cCTB (43 µM). (B) 1H-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 10240 transients and 2.94s of saturation time) corresponding to (B).

**Figure 7.** Blood group A-pentasaccharide binding to the two CTB variants. (A) STD spectrum (obtained with 10240 transients and 2.94s of saturation time) of A-penta 2b (2.6 mM) and cCTB (43 µM) (60:1 ratio). (B) 1H-NMR spectrum of 2b. (C) STD spectrum (10240 transients and 2.94s of saturation time) of A-penta 2b (1.7 mM) and ET CTB (34 µM) (50:1 ratio); the signals marked with an asterisk are artifacts.

**Figure 8.** Blood group B-pentasaccharide binding to the two CTB variants. (A) STD spectrum (obtained with 10240 transients and 2.94s of saturation time) of B-penta 3b (2.0 mM) and cCTB (46 µM) (45:1 ratio). (B) 1H-NMR spectrum of 3b. (C) STD spectrum (10240 transients and 2.94s of saturation time) of B-penta 3b (1.7 mM) and ET CTB (34 µM) (50:1 ratio); the signals marked with an asterisk are artifacts.
**Figure 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 classical CTB (PDB ID: 3CHB, Merritt, Kuhn, et al. 1998) and the ligand complex of a CTB/LTB chimera (PDB ID: 3EFX, Holmner, Lebens, et al. 2004). The two residues differing between classical and El Tor CTB 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 grey) and GM1-os (turquoise)). (B) Stereo representation of A, including the water molecules of the A-penta complex (PDB ID: 3EFX, Holmner, Lebens, et al. 2004), and their H-boon 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.
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