Circulating gut-associated antigens of Schistosoma mansoni : biological, immunological, and molecular aspects

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Chapter 8

The immunologically reactive part of immunopurified circulating anodic antigen from *Schistosoma mansoni* is a threonine-linked polysaccharide consisting of →6)-[β-D-Glc*p*A-(1→3)]-β-D-Gal*p*NAc-(1→ repeating units

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Abstract

The gut-associated excretory antigen CAA (circulating anodic antigen) from adult *Schistosoma mansoni* worms was isolated by immunoaffinity chromatography. Amino acid analysis following alkaline borohydride treatment indicated that CAA is a glycoprotein, O-glycosylated at Thr. The primary structure of the released O-glycan moiety was investigated by one- and two-dimensional, homo- and heteronuclear ^{1}H and ^{13}C NMR spectroscopy. It was found that the major carbohydrate chains have a novel polysaccharide structure, consisting of a branched disaccharide repeating unit, containing 2-acetamido-2-deoxy-β-D-galactopyranose (β-D-Gal*p*NAc) and β-D-gluco-pyranuronic acid (β-D-Glc*p*A):

$$[\rightarrow 6)\text{-}\beta\text{-D-Gal}p\text{NAc-}(1\rightarrow]_n$$
$$\begin{matrix} 3 \\ \uparrow \\ 1 \end{matrix}$$
$$\beta\text{-D-Glc}p\text{A}$$

The major antigenic character of CAA arises from this novel polysaccharide, which was shown to be an absolutely specific diagnostic marker in schistosomiasis. The cross-reactivity of CAA with anti-CCA (circulating cathodic antigen) monoclonal antibodies is caused by the presence of a small amount of O-linked CCA-poly-Lewis x carbohydrate chains on the CAA protein chain.

Introduction

Schistosoma mansoni is a parasitic blood fluke residing in the portal and mesenteric veins of humans and various mammalian species. Although exposed to a cellular and humoral immune response, the worms persist in the host for 3–5 years [1], and in exceptional cases 40 years after infection viable eggs were found to be excreted [2]. Antigen analysis plays an essential role in elucidating the immunological and immunopathological interactions between *Schistosoma mansoni* and its host. Despite the apparent importance of the glycoconjugate glycans in these immune interactions [11,14,23,29], so far characterization of their primary structure has been hampered because of the availability of only limited amounts of parasite antigens.

Most of the research on antigens of the (developing) worm has been focused on tegument antigens [21,22,36]. However, in the humoral immune response of the host an early and strong reactivity is also observed against a number of schistosome gut–associated antigens [6,13,26,34,35]. Such antigens are regularly released into the circulation of the host by the schistosome, upon regurgitating the undigested contents of the gut. For the purpose of immunodiagnosis of active schistosomiasis in sero–epidemiology, the detection of the schistosome–specific gut–associated excretory antigens, circulating cathodic antigen (CCA) and circulating anodic antigen (CAA), is increasingly used [10,44].

CAA displays an extreme stability as can be illustrated by its detection in 5000 year old mummies [24]. The antigen is highly immunogenic and its production by the parasite has been assumed to play a role in the protection of the schistosome gut [25]. Moreover, an interaction of CAA with the first complement component C1q has been reported, possibly interfering with the binding of C1q to the C1q–receptor [43]. This interaction may result in a blocking of the cellular immune effector mechanisms, which are usually activated through the C1q receptor, present on *e.g.* monocytes, neutrophils and platelets. Since the schistosome is living in the bloodstream of the host and regularly ingests whole blood for feeding, these cells are found within the gut and may cause damage to the parasite's gut.

The observed stability towards protein–denaturing agents, and the reduction of the antigenicity after periodate treatment has previously indicated that the immunoreactive portion is located in the glycoconjugate glycans of CAA [4,5,26,29]. Preliminary biochemical characterizations performed on CAA indicated a proteoglycan–like antigen (named GASP, gut–associated proteoglycan [28], later found to be identical with CAA [27]), rich in *N*–acetylgalactosamine (GalNAc) and glucuronic acid (GlcA). In this paper, the

primary structures of the immunologically reactive O-linked carbohydrate chains of CAA are presented.

Materials and Methods

Isolation of antigens

Adult *S. mansoni* worms (Puerto Rico strain) were collected from golden hamsters by perfusion of the hepatic portal system with a balanced salt solution, 7 weeks after infection with 1500 cercariae. A trichloroacetic acid (TCA)-soluble (7.5% w/v) fraction of homogenized adult worm antigen (AWA-TCA) was prepared as described [12], and used as a reference preparation. The AWA-TCA preparation contains 2.5% CAA, as determined using the immunopurified preparation discussed in this paper, and 3% CCA, using an immunopurified CCA-preparation [42].

A combined CAA-/CCA-containing fraction was prepared from washed and lyophilized worms (8 g) as described [42]. CCA was separated from CAA in a series of consecutive steps, finally resulting in a CCA-containing supernatant and a CAA-containing pellet [42]. The pellet was washed twice with water and then suspended in 0.1 M Tris, pH 7.6, containing 7 M urea and 0.15 M NaCl. Nonsoluble material was precipitated by centrifugation for 10 min at 20 000 × g, and the precipitate was washed five times with the urea/Tris buffer. The combined CAA-containing supernatants were pressure dialyzed against 0.1 M Tris, pH 7.6, containing 0.15 M NaCl in an Amicon ultrafiltration cell, using a PM10 filter. CAA was further purified on a Protein-A-based immunoaffinity column [37], using murine McAb 51-4G5-A (IgG3, CAA-specific [13]) as capture antibody. Bound CAA was eluted with 75 mM Hepes/NaOH buffer, pH 7.2, containing 25% (w/v) ethylene glycol and 3.0 M $MgCl_2$ [41]. The CAA solution was dialyzed under pressure against water, and finally desalted and filtered by chromatography on a column (2.6 x 35 cm) of Bio-Gel P-2 (Bio-Rad) eluted with water.

During the isolation, the purity of the antigen was checked by enzyme-linked immunosorbent assay (ELISA, see below) and expressed as percentage of AWA-TCA.

ELISA for antigen detection

The ELISA was performed as described [11]. Briefly, the antigen was immobilized onto McAb 120-1B10-A-coated ELISA-plates (Maxisorp Nunc, Denmark) and detected using alkaline phosphatase-labeled McAb 120-1B10-A. After color development, using *p*-nitrophenylphosphate as a substrate, the absorbance was measured at 405 nm. The CAA concentration was read against a standard curve of AWA-TCA. For direct antigen detection, different antigen preparations (AWA-TCA, intact- and alkaline borohydride-

treated purified CAA) in phosphate-buffered saline were coated onto the ELISA-plate and detected with the alkaline phosphatase-labeled McAb 120-1B10-A, after which color development was established as described above.

Monosaccharide analysis

Monosaccharide analysis, using trimethylsilylated (methyl ester) methyl glycosides, was carried out by gas-liquid chromatography (GLC) [19] and verified by GLC followed by mass spectrometry (GLC-MS), using a Fisons Instruments system, including an MD800 quadrupole mass analyzer, operating in the electron impact mode. The absolute configuration of GlcA and GalNAc was determined by GLC of the trimethylsilylated (-)-2-butyl ester and trimethylsilylated re-*N*-acetylated (-)-2-butyl glycosides, respectively [15].

Liberation and isolation of the carbohydrate chains

A solution of 11 mg CAA in 5 ml 0.5 M NaOH, containing 1 M $NaBH_4$, was incubated for 16 h at room temperature under nitrogen. Then, the solution was adjusted to pH 6.0 with formic acid and transferred to a column (2.6 x 35 cm) of Bio-Gel P-2 (200-400 mesh, Bio-Rad), which was eluted with 25 mM NH_4HCO_3 at a flow rate of 60 ml/h. The absorbance of the effluent was monitored at 206 nm, and the two collected fractions eluting at and after the position of the void volume (V_o), respectively, were lyophilized. The fraction eluting after the V_o was acidified to pH 5.0 with 0.25 M formic acid and then applied to a column (3.5 x 1 cm) of Dowex 50W-X8, H^+ form (100-200 mesh, Fluka). The column was eluted with 30 ml 0.01 M formic acid, and the eluate was lyophilized. Boric acid was removed by co-evaporation with methanol under reduced pressure.

Amino acid analysis

Samples of 150 μg material were hydrolyzed with 6.0 M HCl for 22 h at 110°C under nitrogen. Amino acid analyses were performed on an LKB 4151 Alpha Plus amino acid analyzer, using a five-buffer lithium citrate system [8].

Chondroitinase ABC digestion

AWA-TCA (2 mg) was dissolved in 0.1 ml 40 mM Tris/HCl, pH 8.0, containing 50 mM sodium acetate and 0.05% (w/v) BSA, and 0.1 U of chondroitinase ABC (Sigma) was added [40]. Parallel digestions were carried out with 0.5 ml (20 mg/ml) of chondroitin-4-sulfate and chondroitin-6-sulfate, which were used as positive controls. At several time-intervals, 2-μl aliquots were taken and diluted with 250 μl 10 mM HCl, after which the absorbance was measured at 232 nm (method to monitor the progress of the digestion).

In vitro $^{35}SO_4$ labeling test

Seven-week old *S. mansoni* worms were obtained after perfusion of golden hamsters and washed several times with sterile RPMI-1640 medium. Ten male and ten female worms, and ten male/female worm pairs were incubated in duplicate for 5 days at 37°C in the presence of 0, 20 or 40 μCi of $^{35}SO_4^{2-}$ (sodium salt; Amersham Corp.). Culture supernatants were taken and tested for CAA at several dilutions in the standard CAA-ELISA (see above), using individual wells (Greiner, Alphen a/d Rijn, The Netherlands) instead of 96-well ELISA-plates. After color development as described above (ELISA) and measurement of the absorbance at 405 nm, the individual wells were put into scintillation vials, mixed with 3 ml of scintillation liquid (Liquid Scintillation Cocktail Ultima Gold, Packard Instruments) and counted for radioactivity in an LKB Wallac 1219 Rackbeta Liquid Scintillation Counter.

Anion-exchange chromatography on Mono Q

Fractionation according to charge of intact or alkaline borohydride treated CAA was carried out on a Mono Q HR 5/5 anion-exchange column in an LKB HPLC system, at a flow rate of 60 ml/h. A stepwise gradient from 0 to 1 to 2 M NaCl in 10 ml 20 mM Tris/HCl, pH 7.6, was applied, holding each step for 3 ml. The runs were monitored at 214 nm and the fractions were tested in CAA- (see above) and CCA- [9] specific antigen-capture ELISAs. The reactivities of the different fractions in these assays were expressed as percentages of a standard of 1 μg of purified CAA or CCA, respectively.

^1H and ^{13}C NMR spectroscopy

Bio-Gel P-2 fractions were repeatedly exchanged in 99.8% ^2H_2O (MSD Isotopes) at p^{2}H 7 with intermediate lyophilization. Finally, the samples were extensively mixed in 99.96% ^2H_2O, and after centrifugation for 5 min at 12 000 × g, the supernatants were taken for NMR analysis. Prior to ^1H NMR spectroscopy in ^1H_2O, the Bio-Gel P-2 V_o fraction was dissolved in 450 μl 20 mM K_2HPO_4, containing 92% (v/v) ^1H_2O, 8% (v/v) ^2H_2O, and 0.02% sodium azide; the pH was adjusted to 5.7 with 0.5 M HCl [17]. The 500- and 600-MHz ^1H NMR spectra were recorded on Bruker AMX-500 and AMXT-600 spectrometers (Bijvoet Center, Department of NMR spectroscopy, Utrecht University), at a probe temperature of 300 K. 75-MHz ^{13}C NMR spectra were recorded on a Bruker AC-300 spectrometer (Department of Organic Chemistry, Utrecht University), at a probe temperature of 300 K. Chemical shifts are expressed in ppm relative to internal acetone (in ^2H_2O at 300 K: ^1H, δ 2.225; ^{13}C, δ 31.55).

The one-dimensional (1D) ^1H NMR spectra were recorded as described [18,45]. In the case of two-dimensional (2D) NMR experiments of the Bio-Gel P-2 V_o fraction, data sets of 512 x 2048 points were acquired at 500 MHz, or otherwise as indicated, using software supplied by Bruker. The $^1HO^2H/^1H_2O$ signal was presaturated for 1 s during the relaxation delay. Phase-sensitive handling of the data in the f_1 dimension was carried

out using the time-proportional phase increment method. The time domain data of the 2D experiments were zero-filled to 1024 x 2048 data matrices prior to multiplication with a squared-bell function, phase shifted by $\pi/3$. Except in the case of one 2D homonuclear Hartmann-Hahn (HOHAHA) experiment, all 2D spectra were recorded of samples dissolved in ^2H_2O.

The HOHAHA spectrum of the Bio-Gel P-2 V_o fraction in ^2H_2O was recorded using an MLEV-17 mixing sequence of 120 ms. The spin-lock fieldstrength corresponded to a 90° ^1H pulse-width of 29.0 μs, and the data matrix represented a spectral width of 4505 Hz in each dimension. For the 2D HOHAHA spectrum of the Bio-Gel P-2 V_o fraction in ^1H_2O at 295 K, an MLEV-17 mixing sequence of 120 ms with a spin-lock fieldstrength corresponding to a 90° ^1H pulse-width of 27.8 μs was used. In this case the spectral width was 4000 Hz in each dimension, and a number of 307 x 2048 data points were recorded.

The 2D spectrum obtained from a nuclear Overhauser enhancement spectroscopy (NOESY) experiment was recorded with a mixing time of 75 ms. This relatively short mixing time was chosen to restrict as far as possible spindiffusion in view of the expected relatively long rotational correlation times (τ_c). The data set represented a spectral width of 4032 Hz in each dimension. Double quantum filtered ^1H-^1H 2D scalar shift-correlated spectroscopy (COSY) was carried out using a spectral width of 4032 Hz in each dimension.

The 75-MHz 1D ^{13}C NMR spectrum was recorded using noise ^1H-decoupling. The 75-MHz 1D attached proton test ^{13}C NMR spectrum was acquired using a spin-echo *J*-modulated pulse sequence, t_1-90°-t_2-180°-t_2-acq. Broadband ^1H-decoupling was used throughout the sequence, except during the t_2 period. The t_1-delay period was set to 3 s. Zero and double quantum coherence ^1H-detected single bond ^1H-^{13}C-heteronuclear spectroscopy (HMQC) at 600 MHz was performed using a delay time Δ (1/(2*J*)) of 3 ms, and a GARP decoupling sequence at the carbon frequency during the acquisition. The spectral window was 30 000 Hz in the f_1 dimension (^{13}C) and 6024 Hz in the f_2 dimension (^1H).

Results

Starting from 8 g dried *S. mansoni* wormpairs, the immunoaffinity-based isolation procedure yielded 11 mg of purified CAA, which is 90% of the total amount detectable by ELISA in the starting preparation. Monosaccharide analysis of immunopurified CAA revealed the presence of GalNAc and GlcA in a molar ratio of 1.0:1.0. Additionally, Fuc, Gal, GlcNAc, Xyl and Glc were found in molar ratios $\leq$ 0.2, as compared to GalNAc or GlcA. The carbohydrate content of the CAA preparation was estimated to be 30% by mass. The absence of Man in the monosaccharide analysis indicates that in CAA no N-linked carbohydrate chains occur.

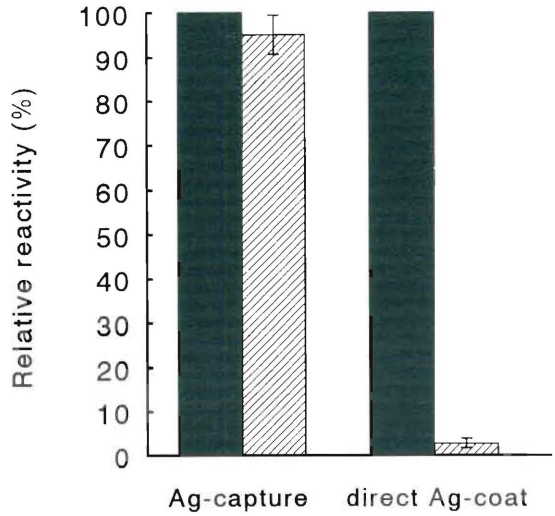

Figure 1. Detection of CAA-specific immunogenicity in AWA-TCA before (closed bars) and after (shaded bars) alkaline borohydride treatment, using both the antigen-capture ELISA and the direct antigen-coated ELISA. The former shows that the immunoreactivity of the antigenic determinants is not affected by the alkaline borohydride treatment, while the latter demonstrates that the determinants are O-linked immunogenic carbohydrates, which in a released form fail to bind to the microtitration plate.

Using an AWA-TCA preparation, which is obtained by a TCA precipitation of an adult worm homogenate and contains both CAA and CCA, the immunologically reactive part could be released from CAA by alkaline borohydride treatment (Fig. 1). Amino acid analyses of immunopurified CAA following the same treatment showed that Thr was partially converted into α-aminobutyric acid for about 25%, demonstrating the occurrence of O-linked carbohydrate chains attached to Thr in the protein backbone of CAA. Therefore, the alkaline sensitivity of the epitope indicates that the CAA-specific immunogenicity is located in Thr-bound O-glycans. The carbohydrate moiety, released by alkaline borohydride treatment of the immunopurified CAA, comprised GalNAc, GlcA, Fuc, Gal, GlcNAc, Xyl and Glc in molar ratios identical to that of intact CAA. For the analysis of the primary structures, the released carbohydrate chains were purified by gel permeation chromatography on Bio-Gel P-2. ^{1}H NMR spectroscopy and monosaccharide analysis of the collected fractions, showed the presence of carbohydrate material exclusively in the void volume (V_o) fraction, which is further referred to as CAA-***P***. The absence of carbohydrate material in the Bio-Gel P-2 fraction, eluting after the V_o, revealed that no small (neutral) O-linked saccharides are attached to CAA. Fraction CAA-***P*** contained GalNAc and GlcA in a molar ratio of 1.0:1.0, whereas Fuc, Gal, GlcNAc and Glc were detected in molar ratios $\leq$ 0.1, as compared to GalNAc or GlcA. GLC-MS analysis suggested the additional presence of GlcNAc-ol. Determination of the

absolute configuration of GlcA and GalNAc revealed the presence of exclusively D-enantiomers. Amino acid analysis of fraction CAA-*P* showed only trace amounts of amino acids (totally less than 1.5% (w/w)).

Since GalNAc and GlcA were detected as major constituent monosaccharides and in equal amounts, initial experiments were carried out to investigate the possibility of structural similarities of the glycans in fraction CAA-*P* with chondroitin sulfate (CS). In contrast to CS, incubation of the AWA-TCA preparation with chondroitinase ABC did not lead to the formation of unsaturated carbohydrate material. Furthermore, *in vitro* incubations of adult worms with $Na_2{}^{35}SO_4$ for 5 days did not give rise to ($^{35}SO_4$)-labeled CAA, indicating that the carbohydrate chains of CAA are not substituted with sulfate groups, since CAA could readily be detected in the culture supernatants. Summarizing both experiments, a CS-like structure was excluded.

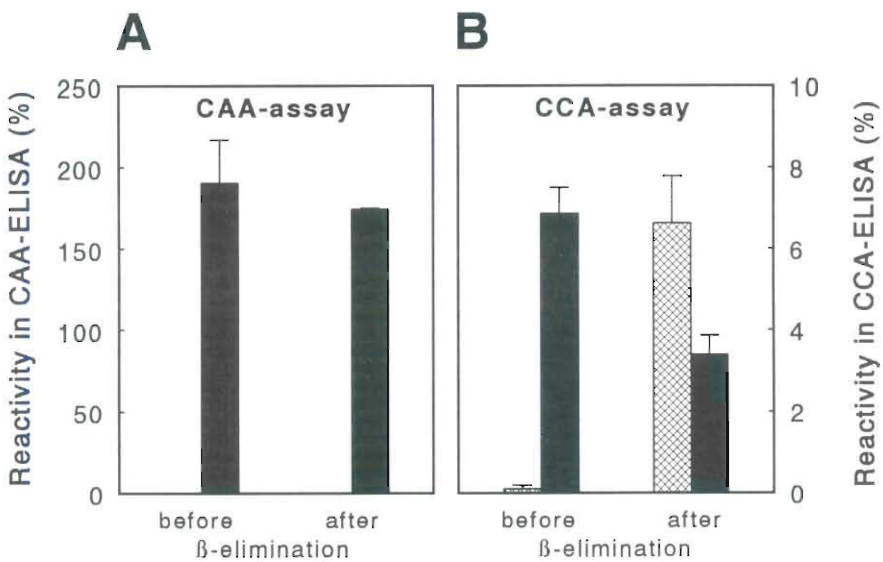

Figure 2. Detection of (A) CAA and (B) CCA in neutral (shaded bars) and negatively charged (closed bars) Mono Q fractions of native and alkaline borohydride-treated immunopurified CAA. The reactivities of the different fractions in these assays were expressed as percentages of a standard of 1 μg (100%) immunopurified CAA or CCA, respectively.

In order to reveal the origin of the minor Fuc, Gal and GlcNAc residues, the presence of CCA-specific neutral O-linked poly-Lewis x carbohydrate chains [42] on the polypeptide backbone of CAA was explored. For this purpose, anion-exchange chromatography on Mono Q before and after alkaline borohydride treatment of immunopurified CAA was performed, using CAA- and CCA-specific ELISAs as monitoring systems (Fig. 2). Fractionation of native CAA

gave rise to a single negatively charged fraction, eluting at 1 M NaCl and reacting positively in both CAA- and CCA-specific ELISAs. However, fractionation of the alkaline borohydride-treated CAA resulted in a major negatively charged CAA-ELISA-positive fraction and a very minor neutral CCA-ELISA-positive fraction. The very minor negatively charged CCA-ELISA-positive fraction probably reflects a not completely passed β-elimination reaction. Therefore, it can be concluded that CCA- and CAA-ELISA-positive O-linked carbohydrate chains were originally attached to the polypeptide backbone of CAA. These results also indicate that not the protein part but the GlcA residues in the carbohydrate part account predominantly for the negative charge of CAA. From the coelution with colominic acid standards (poly-(2→8)-linked α-Neu5Ac) on Mono Q, it is suggested that the carbohydrate chains contain more than 30 GlcA residues.

Further support for the attachment of CCA-ELISA-positive O-glycans to the protein chain in CAA was given by the NMR-analysis of CAA-***P***. Close inspection of the 1D ^{1}H NMR spectrum of CAA-***P*** shows two subspectra, corresponding with the presence of a major and a minor component. The structural reporter group ^{1}H NMR signals of the minor component reflect the presence of a polysaccharide having →3)-β-Gal-(1→4)-[α-Fuc-(1→3)]-β-GlcNAc-(1→ repeating units, because the values of the Fuc H-1 (δ 5.120), Fuc H-5 (δ 4.805), Fuc CH_3 (δ 1.145) and GlcNAc H-1 (δ 4.704) signals match those of the Lewis x repeating units in the internal position of the O-linked polysaccharide chains derived from CCA (Fuc H-1, δ 5.118; Fuc H-5, δ 4.805; Fuc CH_3, δ 1.145; GlcNAc H-1, 4.704) [42]. From the signal intensities of the poly-Lewis x-containing carbohydrate chains relative to those of the major carbohydrate component of fraction CAA-***P*** (further referred to as CAA-***P****), in

Table 1. ^{1}H NMR chemical shifts of the protons of the constituent monosaccharides of the polysaccharide alditols (CAA-*P**), having repeating →6)-[β-Glc*p*A-(1→3)]-β-Gal*p*NAc-(1→ units, derived from *S. mansoni* circulating anodic antigen. Chemical shifts are given in ppm relative to internal acetone (δ 2.225) in ^2H_2O (300 K, p^{2}H 7) and in ^1H_2O (NH: 295 K, pH 5.7) [45].

Residue	Chemical shift for proton								
	H-1	H-2	H-3	H-4	H-5	H-6	H-6'	NAc	NH
GlcA	4.560	3.331	3.497	4.035	4.112				
GalNAc	4.505	3.510	3.716	3.881	3.856	3.969[a]	3.781[a]	2.039	8.24[b]

[a] Since the values of $^3J_{H-6,H-6}$ and $^3J_{H-6,H-6'}$ could not be determined, the proton signal appearing at upfield position is called H-6', and the proton signal appearing at downfield position is called H-6.

[b] Value measured by 2D HOHAHA spectroscopy.

combination with the ELISA data, it was estimated that the amount of CCA-specific glycans attached to the protein chain of CAA is less than 5% of the total carbohydrate content of immunopurified CAA. The coexistence of a minor amount of poly-Lewis x carbohydrate chains, in addition to GlcA/GalNAc-containing polysaccharides (see below), on the CAA protein chain, may explain the generally observed cross-reactivity of anti-CCA McAbs with CAA.

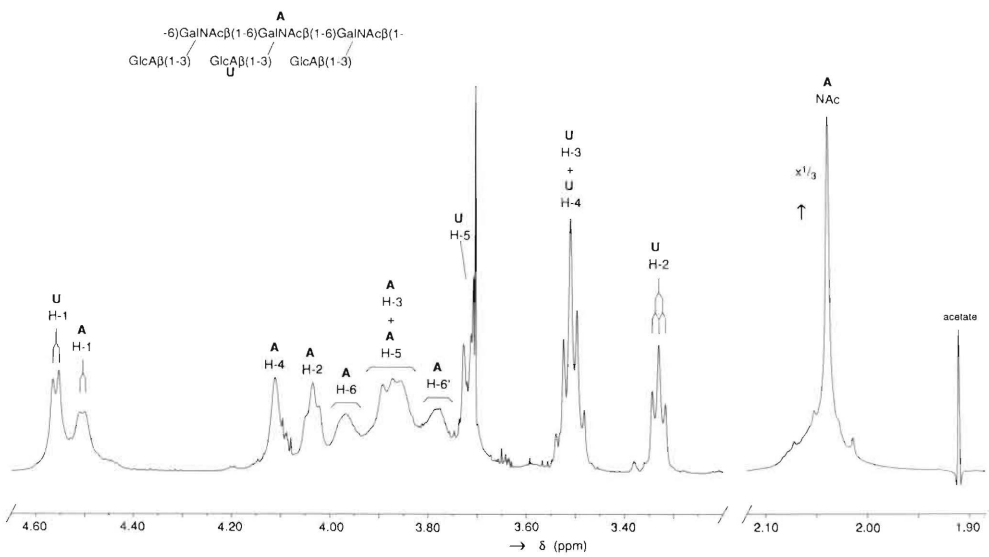

Figure 3. Resolution-enhanced 500-MHz 1D ^{1}H NMR spectrum of fraction CAA-*P* in ^2H_2O at p^{2}H 7 and 300 K.

The structure of the major component, CAA-***P****, was investigated by ^{1}H (Fig. 3) and ^{13}C (Fig. 4) NMR spectroscopy, using 1D and 2D, homo- and heteronuclear techniques. The ^{1}H and ^{13}C NMR spectral data are given in Tables 1 and 2, respectively. The H-1 signals of the prevalent monosaccharide residues, GlcA and GalNAc, can be assigned on the basis of their spin-coupling systems in the 2D HOHAHA spectra recorded in ^2H_2O or in ^1H_2O. From the absence of a N^{1}H-signal, the H-1 track at δ 4.560 can be assigned to that of GlcA. Starting at H-1, the ^{1}H NMR signals of GlcA H-2,3,4,5 could be assigned by inspection of the 2D COSY and HOHAHA spectra, recorded in ^1H_2O. Following the single amide proton signal track at δ 8.24 (^1H_2O) in the 2D HOHAHA spectrum, recorded in ^1H_2O, the GalNAc H-1 signal can be traced, which formed the starting point for the identification of GalNAc H-2,3,4 in the COSY spectrum (^2H_2O). The ^{1}H chemical shifts and coupling constants of GlcA H-1 (δ 4.560,

$^3J_{H-1,H-2}$ 7 Hz) and GalNAc H–1 (δ 4.505, $^3J_{H-1,H-2}$ 6 Hz) are indicative of residues in the pyranose ring form, having β–configuration. In order to identify the set of GalNAc H–5,6,6′ signals, a 1D attached proton test ^{13}C NMR spectrum (not shown) was recorded, which revealed the position of GalNAc C–6 at δ 71.5. Inspection of the 2D HMQC spectrum (Fig. 5) revealed the ^1H–resonance positions of GalNAc H–6 (δ 3.969) and GalNAc H–6′ (δ 3.781), respectively, on the GalNAc C–6 track. The GalNAc H–5 resonance was found by identification of the remaining GalNAc C/H–5 cross–peak after the assignment of the GalNAc C/H–1,2,3,4,6(6′) and GlcA C/H–1,2,3,4,5 cross–peaks in the HMQC spectrum. It should be noted that the carbonyl–group signals at δ 176.0 and δ 177.1 were tentatively assigned to GalNAc NAc and GlcA C–6, respectively, by comparing these values with those of reference β–D–Gal*p*NAc–(1→6)–β–D–Gal*p*NAc [7] and β–D–Glc*p*A [16] (Table 2). The 1D ^1H NMR subspectra for GlcA and GalNAc are virtually equally intense, indicating, together with the monosaccharide analysis data, the presence of a repeating unit consisting of one GalNAc and one GlcA residue.

Table 2. ^{13}C NMR chemical shifts of the carbons of the constituent monosaccharides of the polysaccharide alditols (CAA–*P), having repeating →6)–[β–Glc*p*A–(1→3)]–β–Gal*p*NAc–(1→ units, derived from *S. mansoni* circulating anodic antigen, together with those of reference β–Glc*p*A [16] and β–Gal*p*NAc–(1→6)–β–Gal*p*NAc [7].** Chemical shifts are given in ppm relative to internal acetone (δ 31.55) in ^2H_2O at 300 K and p^{2}H 7.

Residue	carbon atom	CAA–*P**	β–Glc*p*A[a]	β–Gal*p*NAc–(1→6)–β–Gal*p*NAc	
GlcA	C–1	105.6	96.70		
	C–2	74.2	74.74		
	C–3	76.6	76.30		
	C–4	73.2	72.42		
	C–5	77.6	76.76		
	C–6	177.1	177.32		
GalNAc	C–1	103.2		103.3[b]	96.6[c]
	C–2	52.5		53.6	55.0
	C–3	81.6		72.2	72.2
	C–4	69.2		69.0	69.0
	C–5	74.5		76.3	75.1
	C–6	71.5		62.2	69.0
	CH_3	23.9		23.5	23.5
	C=O	176.0		175.9	175.9

[a] Values measured at pH 6.5.
[b] Values stemming from the non–reducing GalNAc residue of the disaccharide.
[c] Values stemming from the reducing GalNAc residue of the disaccharide.

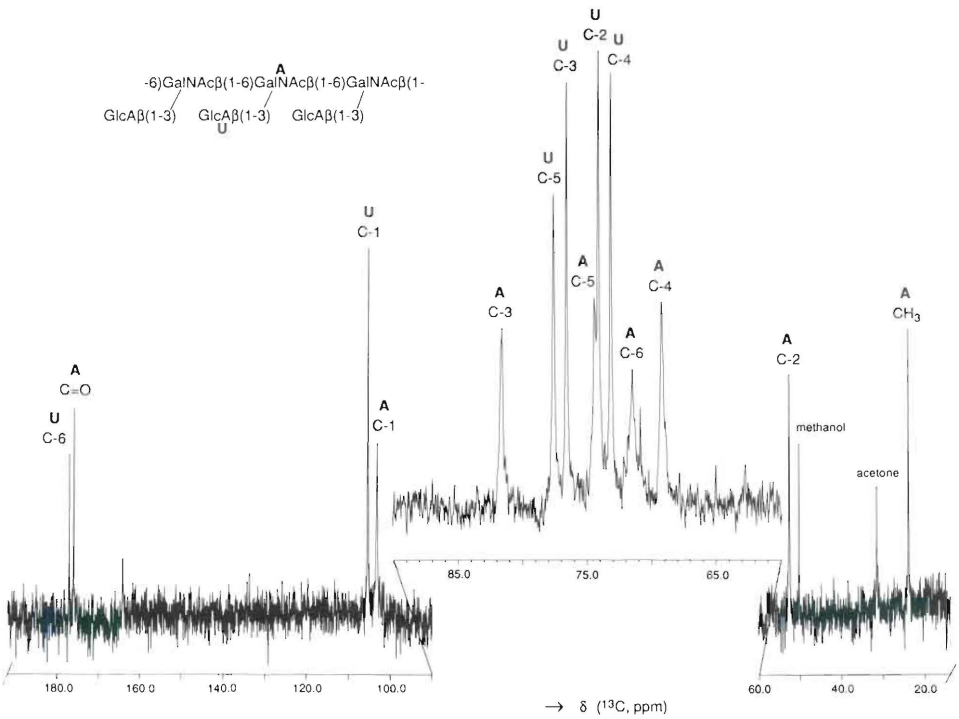

Figure 4. 75-MHz 1D noise ^{1}H-decoupled ^{13}C NMR spectrum of fraction CAA-*P* in ^2H_2O at p^{2}H 7 and 300 K.

To establish the substitution patterns of GlcA and GalNAc, respectively, the ^{13}C NMR chemical shift data of the skeleton carbon atoms were used. The values of GlcA C-2,3,4,5,6 (CAA-***P****) are similar to those of reference β-D-Glc*p*A, whereas GlcA C-1 (CAA-***P****) is shifted downfield ($\Delta\delta$ +8.9) relative to reference β-D-Glc*p*A, typical for a terminal non-reducing monosaccharide residue. The GalNAc C-1 (CAA-***P****) signal matches that of the non-reducing β-D-Gal*p*NAc, and the GalNAc C-5,6 signals (CAA-***P****) are comparable with those of the reducing β-D-Gal*p*NAc residue in reference β-D-Gal*p*NAc-(1→6)- β-D-Gal*p*NAc [7], showing that β-GalNAc (CAA-***P****) occurs as a →6)-β-GalNAc-(1→ structural element. Comparison of the GalNAc C-2,3,4 (CAA-***P****) data with those of the reducing β-D-Gal*p*NAc residue in reference β-D-Gal*p*NAc-(1→6)-β-D-Gal*p*NAc, revealed a downfield shift of $\Delta\delta$ +9.4 for GalNAc C-3, and relatively small upfield shifts for GalNAc C-2 and GalNAc C-4, pointing out that GalNAc is not only glycosylated at C-6, but also at the C-3 position.

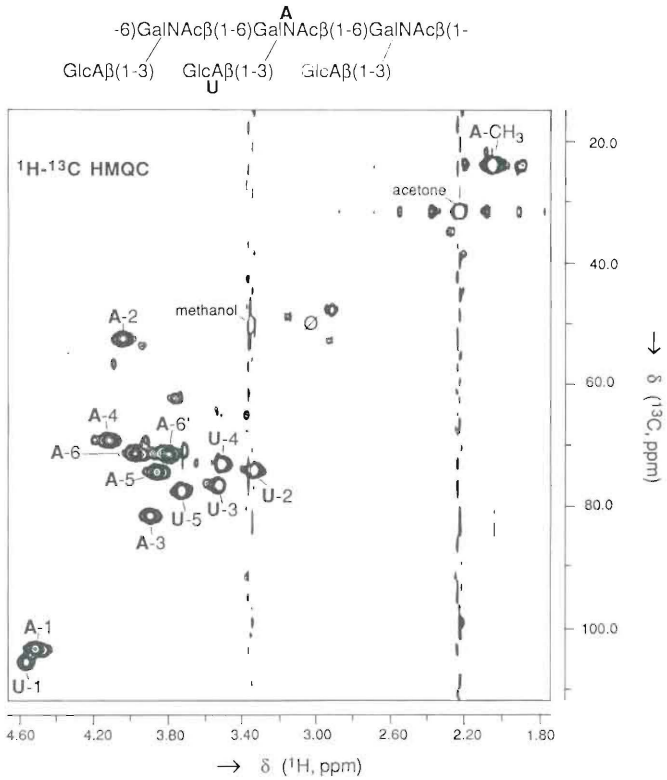

Figure 5. 600-MHz 2D HMQC spectrum of fraction CAA-*P* recorded in ^2H_2O at p^{2}H 7 and 300 K. A letter-number combination near the *cross-peak* refers to a monosaccharide residue (**A**, GalNAc; **U**, GlcA) and its proton (1-6/6'), which shows a one-band ^{13}C-^1H coupling with the corresponding C-atom.

Furthermore, the 2D NOESY spectrum of CAA-***P**** (Fig. 6) revealed the complete structure of the repeating unit. Interresidual nuclear Overhauser enhancement (n.O.e.) contacts are observed between GlcA H-1 and GalNAc H-3 (strong), and between GlcA H-1 and GalNAc H-4 (weak), demonstrating that β-Glc*p*A has to be the substituent at the C-3 position of β-Gal*p*NAc. The GalNAc H-1 track shows, besides the intraresidual GalNAc H-1,3 and H-1,5 n.O.e. cross-peaks, additional cross-peaks with the remaining H-atoms of GalNAc. Those between GalNAc H-1 and GalNAc H-4, between GalNAc H-1 and GalNAc H-6, and between GalNAc H-1 and GalNAc H-6' have to be explained as interresidual contacts, and demonstrate that β-Gal*p*NAc is (1→6)-linked to an adjacent

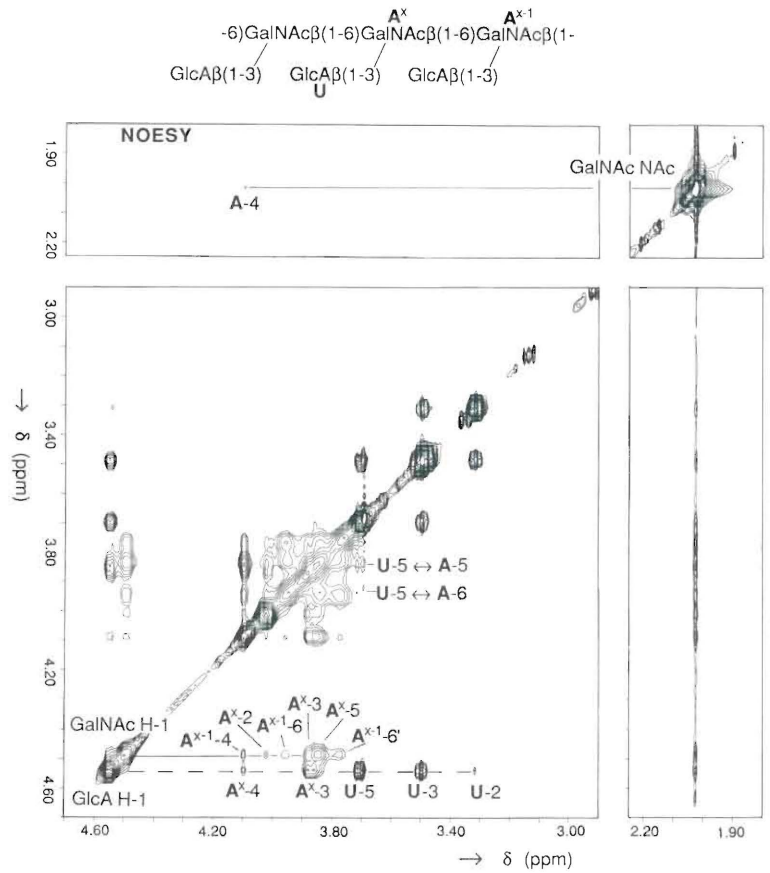

Figure 6. 500-MHz 2D NOESY spectrum of fraction CAA-*P* recorded in ^2H_2O at p^{2}H 7 and 300 K, with a mixing-time of 75 ms. *Dashed* (---, GlcA) and *solid* (——, GalNAc) *lines* are drawn to show the inter- and intraresidual magnetic dipole interactions of (*from top to bottom*) GalNAc NAc, GalNAc H-1 and GlcA H-1, respectively. A letter-number combination near the *cross-peak* refers to a monosaccharide residue (**A**, GalNAc; **U**, GlcA) and its proton (1-6/6'), which shows a nuclear Overhauser enhancement (n.O.e.) contact with the proton of the monosaccharide indicated at the corresponding *diagonal peak*. In addition, the interresidual n.O.e. contacts of GlcA H-5 are indicated. The superscript *x-1* refers to the position of the adjacent repeating unit on the reducing side of the observed repeating unit (*x*). Since it remains to be clarified if the U-5↔A-5 and U-5↔A-6 n.O.e. cross-peaks are between GlcA and GalNAc residues within one repeating unit or from two distinct repeating units, superscripts are not indicated.

β-Gal*p*NAc residue. The Gal*p*NAc-backbone is apparently completely substituted with (1→3)-linked β-Glc*p*A residues, since exclusively cross-peaks stemming from 3,6-substituted GalNAc were detected. In conclusion, the polysaccharide has the following branched disaccharide as repeating unit:

$$[\rightarrow 6)\text{-}\beta\text{-D-Gal}p\text{NAc-}(1\rightarrow]_n$$
$$\begin{array}{c} 3 \\ \uparrow \\ 1 \end{array}$$
$$\beta\text{-D-Glc}p\text{A}$$

Discussion

Several studies have appeared dealing with various glycoprotein and glycolipid fractions from *S. mansoni* at different stages of the development of the blood fluke. Analysis of proteolytic digests of glycoprotein extracts from adult male schistosomes and 48-h-old schistosomula show the presence of O- as well as N-linked carbohydrate chains [30,31]. The O-linked carbohydrate chains comprise clusters of mainly terminal O-linked GlcNAc residues, whereas oligomannose, *N*-acetyllactosamine, β-GalNAc-(1→4)-β-GlcNAc, and possibly hybrid type structures have been found as N-glycans [32,33]. Carbohydrate chains of the β-GalNAc-(1→4)-β-GlcNAc-type contain typically terminal β-GalNAc residues [33], which are detected in β-GalNAc-(1→4)-β-GlcNAc and β-GalNAc-(1→4)-[α-Fuc-(1→3)]-β-GlcNAc elements [38]. At least four repeating →3)-β-Gal-(1→4)-[α-Fuc-(1→3)]-β-GlcNAc-(1→ (Lewis x) units have been found as part of di-, tri-, tri'- or tetraantennary N-glycans [39]. So far, sulfate groups and sialic acid residues have not been detected in the N,O-carbohydrate chains [31,33]. Repeating units of →2)-Fuc-(1→4)-[Fuc-(1→3)]-GlcNAc-(1→ have been identified in immunogenic glycosphingolipids from *S. mansoni* eggs [20].

Recently, we demonstrated that the immunologically reactive O-linked polysaccharides of immunopurified gut-associated excretory antigen CCA from *S. mansoni* have repeating Lewis x units [42].

In this paper, the purification of the gut-associated excretory antigen CAA by ammonium sulfate-precipitation and McAb-based immunoaffinity chromatography is reported. In contrast to the usual TCA treatment of homogenized schistosome worms, the 40% ammonium sulfate treatment produced, after work-up of the supernatant, a CAA fraction that was not soluble in water. After dissolving this preparation in 7 M urea, followed by immunoaffinity chromatography and work-up, CAA showed finally a good

solubility in water. Besides 7 M urea, also high salt concentrations or detergents, like Tween-20 and octyl α-D-glucopyranoside, improved the CAA solubility (data not shown). Such features may indicate that before excretion, CAA exists as a membrane-bound antigen in the adult worm, but further investigation is needed to establish the physiological expression of CAA.

Immunopurified CAA was used to elucidate the primary structure of the antigenic carbohydrate chains, which are apparently exclusively Thr-linked. The major polysaccharides CAA-***P**** (>95%) have repeating →6)-[β-D-Glc*p*A-(1→3)]-β-D-Gal*p*NAc-(1→ units, which are probably connected to the protein via an as yet unknown core saccharide, like in proteoglycans [3], with GlcNAc at the reducing end. A more detailed study of the linkage will be undertaken. The carbohydrate chains, which can be considered as a novel type of O-linked carbohydrate chains in glycoproteins, are involved in the primary immunoreactivity of CAA. The unique polysaccharide may explain the 100%-specificity of the CAA-based assays for the diagnosis of schistosomiasis in the patient's urine and/or serum [11]. The detection of small amounts (<5%) of the CCA-specific O-linked polysaccharides, having Lewis x as repeating unit, which were simultaneously released with the GlcA/GalNAc-containing polysaccharide from the CAA-protein chain, accounts for the cross-reactivity of anti-CCA McAbs with intact CAA.

In principle, isolation of a glycoprotein via immunoaffinity chromatography using a monoclonal antibody, which reacts specifically with a carbohydrate epitope of that glycoprotein, can lead to a preparation contaminated with glycoproteins bearing the same epitope. For this reason the immunopurified CAA preparation was subjected to SDS-PAGE. Invariably, the preparations gave rise to a smear upon SDS-PAGE. Because CAA contains 30% carbohydrate, which is relatively high, and furthermore the carbohydrate is heavily charged, the outcome of SDS-PAGE was interpreted in terms of microheterogeneity of the carbohydrate chains, in conjunction with effects due to the high density of negative charge. Furthermore, in the Edman sequence analysis even after 11 steps no amino acids could be detected, suggesting that the protein can not be degraded due to blocking of the N-terminus and indicating that the protein moiety behaves, as if it were homogeneous. It seems unlikely that any contamination with other proteins would be with compounds that all have a blocked N-terminus. However, strictly speaking the data mentioned above do not prove the chemical purity of the polypeptide backbone, which means that CAA can represent a collection of glycoproteins all having the same immunologically reactive carbohydrate part, and further investigations with respect to this aspect will be necessary.

As indicated under "Results", the degree of polymerization of CAA-***P**** is probably more than 30. It is tempting to suggest a higher ordered structure for

such relatively long carbohydrate chains in CAA. Close inspection of the NOESY spectrum of CAA-P^* (*cf.* Fig. 6) revealed the presence of cross-peaks between GlcA H-5 and GalNAc H-5 (weak), between GlcA H-5 and GalNAc H-6′ (very weak), and between GalNAc H-4 and GalNAc NAc (weak). It is likely that they stem from interactions between residues of different repeating units. Furthermore, the differences in "line shape" as observed in the 1D ^1H and ^{13}C NMR spectra for the set of resonances arising from GlcA (relatively sharp) and that arising from GalNAc (relatively broad) indicate clear differences in flexibility between the two residues. These findings, in terms of three-dimensional structure, are currently under investigation. In this polysaccharide, the GlcA residues may enwrap the GalNAc backbone thereby giving rise to a negatively charged surface.

The detection of CAA by ELISA has been shown to be a valuable approach in the diagnosis of active *Schistosoma* infections. Furthermore, the unique structure of the carbohydrate epitopes involved in immunorecognition may be exploited to study specific humoral immune responses. Besides giving additional diagnostic information, these responses will also affect the immune clearance of the antigen and consequently influence the relation between CAA levels detected in the serum and number of worms harbored by the host. The latter relationship is highly important in the epidemiology of schistosomiasis, *e.g.* in parasite transmission dynamics, in development of pathology, or in development of vaccines. To facilitate these specific studies, research on the chemical synthesis of fragments of the novel GalNAc/GlcA polymer is in progress.

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