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Affinity and kinetic analysis of the bovine plasma C-type lectin collectin-43 (CL-43) interacting with mannan

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Abstract Collectins are C-type lectins which have been implied to play an important role in the innate immune defence against microorganisms. The critical discriminatory event in the opsonization of microorganisms by collectins is the interaction of the C-type lectin domain with microbial carbohydrates. Surface plasmon resonance measurements allow for quantitative real-time measurements of binding interaction between immobilized carbohydrate and unlabelled lectin in solution. Binding analysis were carried out with purified collectin-43 (CL-43) which structurally is the simplest collectin consisting of only three polypeptides each terminating in a C-type lectin domain. The target was immobilized yeast mannan. The molecular mass of native CL-43 was determinated by mass spectroscopy to 99.8 kDa. The dissociation rate (k_{diss}) of the C-type lectin-carbohydrate binding was fast ($1.19–1.36\times10^{-2}$ second^{-1}), and the association rate (k_{ass}) was $4.37–5.07\times10^{5}$ M^{-1} second^{-1}. The equilibrium constant for dissociation (K_d) was $2.68–2.72\times10^{-8}$ M.

Key words: Collectin-43; C-type lectin; Affinity; Kinetics; BIAcore

1. Introduction

Collectin-43 (CL-43) is a member of the collectin family of proteins that play a role in the innate immune defense against microorganisms [1–3]. Five collectins have been identified: the pulmonary surfactant proteins SP-A, and SP-D; the plasma lectins, conglutinin, mannan-binding lectin (MBL), and CL-43.

The collectins have been shown to bind to the surface of various micro-organisms and particles, and the critical discriminatory event in this process is the interaction of the C-type lectin domain of the collectins and microbial carbohydrates [4–9]. One collectin, MBL, can enhance opsonization by activation of the classical pathway of complement in a C1q- and antibody-independent way [10–13]. Conglutinin opsonizes through binding to iC3b deposited onto the surface of micro-organisms [14]. The collagenous regions of the collectins are ligands for the collectin receptor on phagocytes and other cells [15].

The collectins are oligomeric lectins built of subunits composed of three identical polypeptides. Each polypeptide chain consists of a short N-terminal sequence, followed by a collagen-like regions, a short α-helical sequence, and the carbohydrate recognition domain (CRD). The collagen-like region of three polypeptides forms a stalk-like structure which is followed by a α-helical coiled-coil neck region and three independent folding globular CRDs [16]. The crystal structure of MBL CRD showed that asparagine and glutamine residues interact with the equatorial 3 and 4 hydroxyl groups of mannose as well as with the calcium ion involved in ligand binding through coordination bonds. Binding studies have shown that the collectins generally bind carbohydrates with equatorial 3 and 4 hydroxyl groups such as mannose and glucose, but also L-fucose which contains equatorial 2 and 3 hydroxyl groups that can be superimposed on the 3 and 4 hydroxyl group of mannose [16]. The five residues involved in the carbohydrate and calcium interaction, as judged from the crystal structure, are conserved between the collectins. Nevertheless, the relative affinities for monosaccharides, as judged by inhibition of binding to mannan, vary considerably [17].

The cruciform collectins, conglutinin and SP-D, are composed of four trimeric subunits and the serpiform collectins MBL and SP-A of six subunits, while CL-43 is seen only as structures corresponding to a single subunit [18]. CL-43 thus seems ideally suited for kinetic analysis of interactions with carbohydrates by surface plasmon resonance.

2. Materials and methods

2.1. Buffers, reagents and equipment

BIAcore system, sensor scip CM5, surfactant P20 and amine coupling kit containing N-hydroxysuccinimide (NHS), N-ethyl-N′(dimethylaminopropyl)carbodiimide (EDC) and 1 M ethanolamine-hydrochloride (pH 8.5) were obtained from Pharmacia Biosensor AB, Uppsala, Sweden. Mannan was purified from *Saccharomyces cerevisiae* according to [19]. Tris-buffered saline (TBS): 140 mM NaCl, 10 mM Tris-HCl, 2 mM NaN_3 (pH 7.2). TBS/Tw: TBS containing 0.05% (v/v) Tween 20 (polyoxyethylene sorbitan monolaurate, Merck-Schuchardt, FRG). HBS (10 mM HEPES, 0.15 M NaCl, 0.05% Surfactant P20) (pH 7.4). HBS-EDTA: HBS containing 10 mM EDTA. HBS-Ca^{2+}: HBS containing 5 mM $CaCl_2$. Buffer A: 10 mM Tris, 10 mM EDTA, 0.05% (v/v) Emulphogene (BC 720, Sigma, St. Louis, MO, USA) (pH 7.5).

Rabbit anti-bovine IgG (heavy and light chains) (Code Z249, Dakopatts A/S, Kamstrup, Denmark) was coupled to CNBr-activated Sepharose 4B (Pharmacia, Sweden) at a concentration of 2.5 mg antibody per ml gel.

2.2. SDS-PAGE

Electrophoresis was performed on 4–20% (w/v) polyacrylamide gradient gels with the discontinuous buffers described in [20]. Samples were reduced by heating at 100°C for 3 min in 40 mM dithiothreitol, 1.5% (w/v) SDS, 5% (v/v) glycerol, 0.1 M Tris (pH 8.0), and carboxamidated by the addition of iodoacetamide to a concentration of 90 mM. Unreduced samples were heated in sample buffer with 90 mM iodoacetamide. Protein bands were detected by Coomassie blue staining.

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2.3. Purification of CL-43

Purification of CL-43 was modified from the previously described method [17]. The purification was carried out on a computer-monitored FPLC system (FPLCdirector Version 1.3, Pharmacia). The euglobulins from one liter of bovine serum were precipitated with 4% (w/v) of PEG 6000 by incubation on ice for 1 h. After centrifugation (2000×*g*, 15 min), the precipitate was washed with 600 ml of 6% (w/v) PEG 6000 in TBS containing 5 mM $CaCl_2$. After further centrifugation the precipitate was dissolved in 100 ml TBS containing 5 mM $CaCl_2$ and passed through a column containing 100 ml of mannan-Sepharose. The mannan-Sepharose column was washed extensively (until $E_{280} < 0.005$), and bound proteins were eluted with TBS containing 2 mM GlcNAc, and then with 50 mM mannose. Fractions containing CL-43 were then passed through a column containing 15 ml rabbit anti-bovine-Ig-Sepharose in order to remove contaminating anti-mannan antibodies. The effluent containing CL-43 was pooled, diluted 10 times in buffer A and applied to a Resource Q column (1 ml prepacked column, Pharmacia) and eluted with a gradient from 0 to 100 mM NaCl in buffer A. CL-43 was eluted at 20 mM NaCl. CL-43 containing fractions were then passed through a 7.5-ml TSK-mannose column. After wash the column was eluted with linear mannose gradients first from 0–6 mM mannose and then from 6–100 mM mannose. CL-43 eluted at 3 mM mannose. CL-43 was further purified by gel permeation chromatography on a Superose 6 column (1.6×58 cm, Pharmacia). The fractions containing CL-43 were identified by SDS-PAGE, and the concentration of CL-43 in the purified preparation was estimated by quantitative amino acid analysis.

2.4. Mass spectrometry

The exact molecular mass of native CL-43 was obtained by matrix-assisted laser desorption mass spectrometry (MALDI-MS). Approx. 100 pmol of intact CL-43 in 0.1 M ammoniumbicarbonate were analyzed by MALDI-MS.

2.5. Preparation of sensor surfaces

Immobilization of mannan to sensor chip CM5 was performed in the biosensor system as described previously [21]. A continuous flow of 5 μl/min was maintained. Between reagent injections the flow was maintained with HBS. The carboxylated dextran matrix was activated by injecting of 35 μl of a freshly made mixture of 70 μl 100 mM NHS and 70 μl 400 mM EDC. Next, 35 μl of mannan (700 μg/ml in 10 mM formic acid, pH 3.4) was injected followed by 35 μl ethanolamine to block the remaining NHS-ester groups. After conditioning with 0.1 M glycine-HCl (pH 2.4), HBS-EDTA, HBS-Ca^{2+}, the sensor surface was ready to use. The immobilization level was 3500–4000 refractory units (RU).

As a control recombinant CD4 (a gift from Neil Barkley) was immobilized to the carboxylated dextran matrix instead of mannan.

2.6. Interaction of covalently immobilized yeast mannan with CL-43

Purified CL-43 at various concentrations in the range of 0.62–5.0 μg/ml (6.2 nM–50 nM) (concentration with regard to structural unit composed of three polypeptide chains with a molecular mass of 99.8 kDa) in HBS-Ca^{2+} was injected and allowed to react with immobilized mannan. A flow rate of 5 μl/min was maintained and 25 μl of CL-43 solution was injected. The analytical cycle was followed by a 1-min injection of HBS-EDTA which regenerated the surface completely. During each regeneration less than 0.1% of RU was lost.

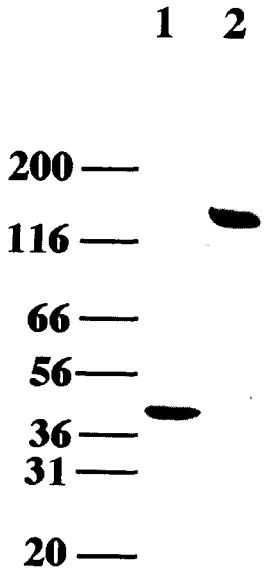

Fig. 1. SDS-PAGE of reduced (lane 1) and non-reduced (lane 2) CL-43 on a 4–20% (w/v) gradient gel stained by Coomassie blue.

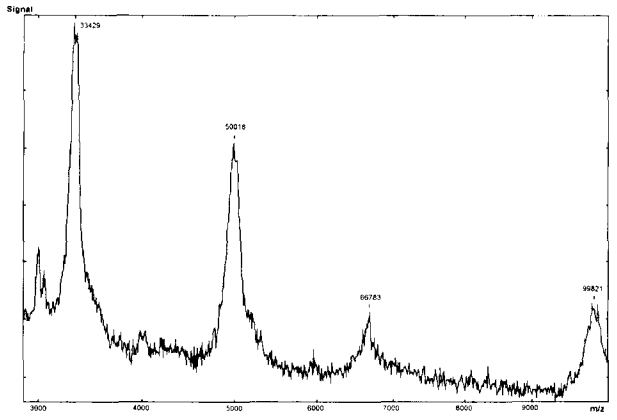

Fig. 2. Mass spectroscopy of native CL-43. Approx. 100 pmol of intact CL-43 were analyzed by assisted laser desorption mass spectrometry. The spectrum shows the singly, doubly and triply charged ions of the intact triple-chain molecule. The small peak at m/z 66783 most likely represents a small amount of two-chain CL-43 present in the preparation.

3. Results and discussion

CL-43 was purified by PEG precipitation, sugar affinity chromatography using step-wise elution with GlcNAc to remove conglutinin and gradient elution with mannose to separate CL-43 from bovine MBP. Anti-carbohydrate antibodies were removed by affinity chromatography. CL-43 was further purified by ion-exchange chromatography and size separation chromatography. The purified CL-43 is shown in Fig. 1.

The cDNA of CL-43 encodes a polypeptide of 301 amino acid residues, with a molecular mass of the single polypeptide of 31.5 kDa as estimated from the sequence [22]. The apparent size of the CL-43 polypeptide chain as judged by SDS-PAGE is 43 kDa, and the covalent oligomer seen under non-reducing conditions is 120 kDa [17]. This is in agreement with the estimate of 119–138 kDa seen by sucrose-density gradient centrifugation and gel chromatography under non-denaturing conditions [18]. No oligomers of the structural unit were seen by gel permeation chromatography or sucrose-density gradient centrifugation, consistent with the observation of only single units of three peptides by electron microscopy [18].

In order to obtain the exact molecular mass of CL-43, we analyzed the native molecule by mass spectroscopy (Fig. 2). The molecular mass of intact CL-43 was 99.8 Da. The difference between the calculated mass of 94.5 kDa (based on cDNA) and the mass of 99.8 kDa found by mass spectroscopy must primarily be due to hydroxylations of prolines and ly-

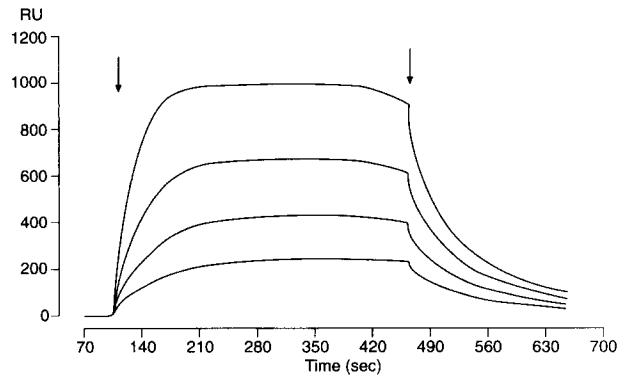

Fig. 3. Kinetic binding curves (sensorgrams) of CL-43 binding to a biosensor surface coupled with mannan. Four different binding experiments were done at the concentrations of 50 nM, 25 nM, 12.5 nM, 6.2 nM. The binding curves are expressed as resonance units (RU) as a function of time. The first arrow indicates the initiation of the injection of CL-43, and the second indicates the beginning of the buffer washout.

sines and to O-linked carbohydrates, since no potential N-hydroxylation site is found in the CL-43 sequence. The molecular mass of 99.8 kDa was used for the calculation of the molar concentration of CL-43.

The surface plasmon response during the analytical cycle of CL-43 injection, binding to and dissociation from mannan is shown in Fig. 3, with different CL-43 concentrations presented as overlay plots.

The kinetic analysis using a 'one-receptor model' revealed a moderate to fast association rate (k_{ass}) $4.37–5.07\times10^{5}$ M^{-1} s^{-1}, and a very rapid dissociation rate (k_{diss}) ($1.19–1.36\times10^{-2}$ s^{-1}) leading to a calculated value for the dissociation constant (K_d) of $2.68–2.72\times10^{-8}$ M. No binding was seen when CL-43 was passed over a CD4 matrix (data not shown).

The rapid off-rate could indicate that the interaction of one subunit with three CRD would have insufficient time to trigger an effector-mediated response. Multiple binding of several CL-43 molecules to repetitive carbohydrate moieties on the surface of the microorganism may compensate for this rapid off-rate. For the closely related collectins SP-D and conglutinin a decreased effective off-rate could be achieved by multivalent binding of the up to 12 CRD in a single molecule, and the dissociation constant (K_d) of the binding between human MBL and ^{125}I-mannan has been estimated to be 2.3×10^{-9} [23]. In this case up to 18 CRDs are involved in the binding.

So far CL-43 has only been shown to bind to the acapsular form of *Cryptococcus neoformans* [9], but the sugar binding profile of the molecule indicates, that CL-43, like the other collectins, will bind to carbohydrate moieties on the surface of a variety of microorganisms, after which the binding to the collectin receptor could mediate the binding and internalization of the CL-43 opsonized microorganisms.

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