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Failure of glucose-binding lectins Con A and Lentil Lectin to identify glycation of haemoglobin

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Abstract

We have studied the interaction of Concanavalin A and Lentil Lectin with glycohaemoglobin by a nephelometric lectin–glycogen/dextran precipitation system and monitored the inhibitory effect of glycohaemoglobin on the precipitation. Although inhibitory effects were clearly demonstrated using simple sugars and transferrin, no effect was observed by glycohaemoglobin in relevant concentrations. This is compared to affinity chromatography, binding studies using gel filtration and electrophoresis, and affinity studies using Concanavalin A immobilised on magnetisable polymer particles. Lack of interaction between glycohaemoglobin and lectins is discussed in view of steric constraints and reduced availability of the glycated residues and the stereochemical form of the glycated 1-amino-1-deoxy-fructosyl residues in glycohaemoglobin.

Keywords: Glycohaemoglobin; Con A; Lentil Lectin; Glycohaemoglobin–lectin binding

1. Introduction

The lectins Concanavalin A (Con A) and Lentil Lectin (*Lens culinaris* Haemagglutinin) possess the ability to react with glycoproteins with specificity for D-glucose and D-mannose residues, in particular those present in *N*-linked protein glycans. The glycated haemoglobin variant HbA_{1c} and glycohaemoglobin in general, is the single most important parameter in long-time monitoring of *diabetes mellitus* [1]. The glycation process resulting in ketoamine-linked glucose to amino-terminal ends and ϵ-amino groups of lysine residues of the α- and β-chains of haemoglobin (Hb), has a reaction

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rate depending upon the mean blood glucose concentration during the life-span of the erythrocyte [2]. A high degree of site selectivity is seen in post-translational non-enzymatic glycation. In human haemoglobin the *N*-terminal valine residue of the β-chain is the most available glycation site, resulting in the glycation variant known as HbA_{1c} [2]. The in vivo formation of glycated lysine residues are most favourable taking place at Lys-66 (β), Lys-61 (α), and Lys-17 (β) with the same order of prevalence [3]. Irrespective of glycation site, it is generally accepted that the end product after Amadori rearrangement of the labile aldimine-Schiff base exists as a ring stabilised form of ketoamine linked hexose [3–6].

The carbohydrate-recognising properties of lectins [7] resulting in binding of carbohydrates, polysaccharides, and glycoproteins, and the multitude of other interesting and unusual chemical and biological properties exhibited by lectins [8–11], has made them very useful in analytical biochemistry. The plant lectins *Lens Culinaris* Haemagglutinin and Con A both show specificity for D-glucose, D-mannose, *N*-acetyl-D-glucosamine and sterically related residues, although the former appears to distinguish less sharply between the glucose and mannose residues than does Con A [7,12,13]. Okada et al. [14] showed that binding of ^{125}I-labelled Con A to erythrocytes from diabetic patients was significantly higher than the corresponding binding to erythrocytes from normal subjects, indicating an increased Con A binding to glycated proteins or other glycated structures in the cell membrane of diabetics.

The aim of the present study was to investigate lectin binding to glycohaemoglobin. In order to verify such interaction, various analytical methods were used to obtain proper binding conditions. Lectin–glycohaemoglobin interactions have been studied by competitive inhibition of a Con A–dextran/glycogen precipitation system [7] using glycohaemoglobin as a potential competing agent for the binding sites of Con A, affinity chromatography on immobilised Con A and Lentil Lectin, binding to Con A immobilised on magnetisable polymer particles, size-exclusion chromatography, and electrophoresis.

2. Experimental

Materials.—Con A, Dextran (B1355S), Con A-Sepharose and Lentil Lectin-Sepharose were purchased from Pharmacia Biotech Norden AB, Sollentuna, Sweden. Glycogen, methyl-α-D-mannopyranoside (α-mmp) and D-glucose were purchased from Sigma Chemical Co., St. Louis, USA. Amine functionalised, 0.5–1.5 μm sized, magnetisable polymer particles (BioMag M4100) were obtained from Advanced Magnetics Inc., Cambridge, MA, USA and hydroxy-functionalised particles (Dynospheres XP-6006, nominal size: 2.8 μm) from Dyno Particles AS, Lillestrøm, Norway. Human transferrin (apoprotein, Sigma Chemical Co.) was used as a positive lectin-binding glycoprotein control in the binding studies.

Haemoglobin samples with 4.5–15% HbA_{1c} were purified from freshly collected blood samples as described elsewhere [15]. Additionally, a "non-glycated" haemoglobin sample (< 0.2% GHb) and two samples of highly purified glycohaemoglobin (one

containing the bound glycated Hb fraction eluted from boronic acid affinity columns and one containing purified HbA_{1c} evaluated to be of $>90\%$ purity by ion-exchange chromatography) were used.

Inhibition studies.—Con A–dextran / glycogen precipitin reaction. A Con A–Dextran (1355S) and Con A–glycogen precipitin reaction with nephelometric reading (675 nm) was developed to study lectin–glycohaemoglobin interaction. As a potential competitive inhibitor glycohaemoglobin was added (final concentration up to 1 mg haemoglobin/mL with various degree of glycation). Precipitation was followed by time studies and end point nephelometric readings using a thermostatted cell holder. Precipitin inhibition experiments were performed using 20 mM triethanolamine, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$, pH 7.4 (TEA buffer) and all working reagents were filtered (0.4 μm) before use. Experimental details are included in the figure legends. D-Glucose, α-mmp and human transferrin were used as positive controls to verify the binding specificity of the system.

Lectin–glycohaemoglobin affinity interaction. Non-chromatographic experiments using Lectin-Sepharose were performed after an initial wash and equilibration of the gel matrices in 0.02 M Tris–HCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, pH 7.4, containing 0.1–0.5 M NaCl (AC buffer). A 3 mL haemoglobin solution (0.085 to 2.5 mg haemoglobin/mL) was added to 200 μL gel suspension (50%, v/v). The mixture was gently shaken and incubated at room temperature for 20 min. After low speed centrifugation the amount of protein remaining in the supernatant was determined and the binding to the affinity matrix calculated. α-Mmp (0.1 M) was used to block specific interactions.

Affinity chromatography was performed using columns (1.0×5.0 cm) of Con A-Sepharose and Lentil Lectin-Sepharose equilibrated with AC buffer (flow rate 0.2 mL/min, detection at both 280 nm and at 405 nm). After injection of sample (0.1–1 mL, 2 mg/mL) and subsequent washing with ten bed volumes of buffer, the bound protein fraction was eluted using a linear gradient of buffer containing α-mmp (50–200 mM).

Con A immobilised on particles. Immobilisation of Con A on polymer particles was carried out using glutaraldehyde-activated [16] (BioMag M4100) and 1,1'-carbonyldiimidazole-activated [17] (Dynospheres XP-6006) particles. Particle suspensions were made up to a concentration of approximately 20 mg/mL in 0.1 M Tris–HCl, 0.1 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, pH 7.4 before use.

To test glycohaemoglobin binding, Con A/particle–haemoglobin suspensions were made by varying the particle/haemoglobin ratios. Mixtures were incubated at room temperature for up to 60 min before being separated. Protein binding was estimated either directly by spectrophotometric reading of the particle-treated protein solutions, or by assaying the peroxidase activity of haemoglobin bound to the separated particles after washing in assay buffer.

Size-exclusion chromatography.—Mixtures of Con A (1 mg/mL) and highly purified glycohaemoglobin (1–5 mg/mL) were incubated for 30 min before chromatography on a Bio-Gel P-200 column (Bio-Rad Laboratories, USA) (1.5×40 cm) using TEA or AC buffer as eluant (flow rate 0.1 mL/min). A 200 μL sample was injected and the eluant monitored at 280 nm and 405 nm.

Electrophoretic studies.—Polyacrylamide gel electrophoresis studies of Con A–glycohaemoglobin interaction were performed using pre-cast polyacrylamide gel plates (PhastSystem, Pharmacia, Sweden) with gradient 8–25 (native PAGE 8–25). Separations were performed using buffer strips containing either 0.20 mM Tris–HCl, pH 7.5 or 0.88 M L-Alanine, 0.25 M Tris, pH 8.8. Mixtures of glycohaemoglobin samples and Con A were analysed after incubation for up to 60 min at room temperature.

Protein concentration.—The concentration of Con A was determined based on an absorption coefficient ($E^{1\%}_{1\,cm}$) of 13.7 at pH 7.4 (ref. [18]). Haemoglobin concentration was determined by the method of Drabkins using "Mercotest" (E. Merck, Darmstadt, Germany). Other protein concentrations were determined using the BCA method [19].

3. Results

Inhibition of Con A–polysaccharide precipitin reaction.—Fig. 1 illustrates a typical nephelometric signal profile of the precipitin reaction obtained using Con A and dextran or Con A and glycogen. Generally the Con A–Dextran (1355S) system gave more than twice the signal strength of a comparable Con A–glycogen system. Nevertheless, the reproducibility of the precipitin readings was favourable using the Con A–glycogen system (CV, $SD \times 100/\bar{x} < 5\%$), mostly due to the high background readings using dextran. The final aim was to develop a Con A–polysaccharide precipitation method

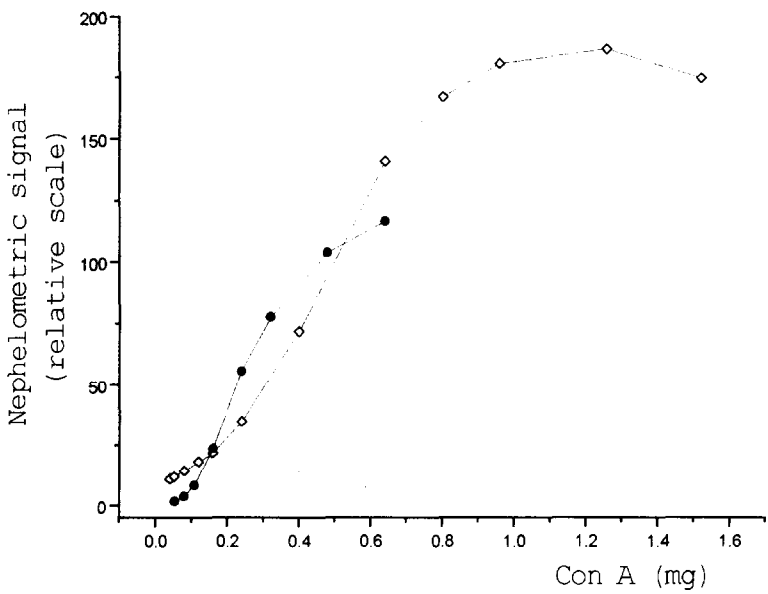

Fig. 1. Precipitin reaction of Con A with Dextran 1355S and glycogen in TEA buffer, pH 7.4, 22 °C. To a fixed concentration of Dextran 1355S (80 μg, ◇) or glycogen (160 μg, ●) increasing concentrations of Con A were added (total volume 2 mL). The nephelometric signal was recorded at 675 nm, 1 min after mixing the reagents.

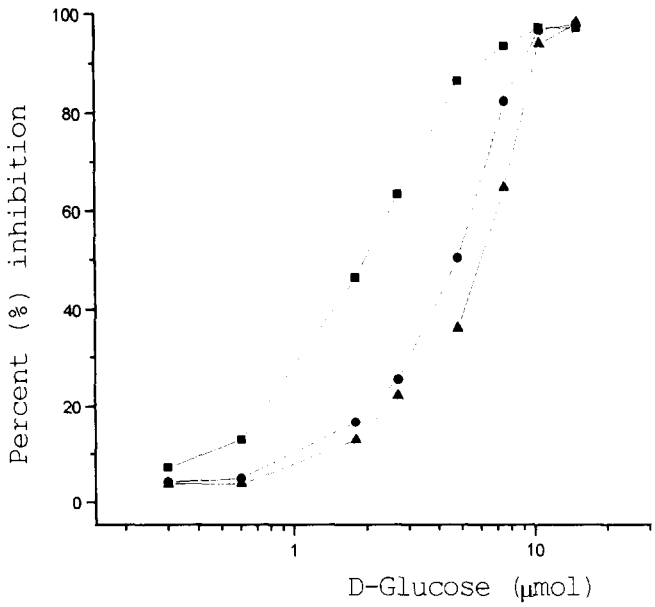

Fig. 2. Inhibition of Con A–glycogen precipitin reaction by D-glucose in TEA buffer, pH 7.4, 22 °C. Solutions containing Con A (80 μg) and increasing amounts of D-glucose were incubated for 5 min before adding glycogen (40 μg) — total volume 2 mL. The nephelometric signal was recorded at 675 nm at different intervals after mixing the reagents (reading after 1 min: ■, 3 min: ●, 5 min: ▲). Percent inhibition was calculated relative to no glucose added.

sensitive enough to detect small amounts of competing compounds occupying the binding sites of Con A. Initially the competitive effect of both D-glucose and α-mmp were studied. However, due to the high sensitivity obtained and the following high dilutions necessary using α-mmp, the system was further optimised using D-glucose. The specificity of the Con A–glycogen precipitin reaction and the effect of glucose as a competitive inhibitor is depicted in Fig. 2. Almost complete inhibition was obtained using glucose at a concentration of 3 mM. For comparison similar experiments using transferrin (final cons. 20 μg/mL) resulted in significant inhibitory effects. Another important factor to realise regarding the inhibitory effect of glucose was the strong dependence on the recording interval after mixing of the reagents. Using Con A–dextran in a reaction with 83 μm/mL Con A, 10.5 μg/mL Dextran (1355S) and recording of the nephelometric signal 1 min after mixing, a glucose concentration of 2 mM was necessary to give 50% inhibition. Dialysis and subsequent lyophilisation of the glycogen used resulted in increased reproducibility of the nephelometric readings. Both the Con A–Dextran (1355S) and Con A–glycogen system showed the inhibitory effect of glucose to be twice as high at 11 °C compared to 37 °C (reading 1 min after mixing the reactants). For the sake of convenience, the temperature was maintained constant at room temperature (22 °C) in most of the experiments.

Glycohaemoglobin inhibition studies.—Fig. 3 shows the results achieved adding glycohaemoglobin (GHb < 0.2–95%) as a competing agent to the Con A–glycogen

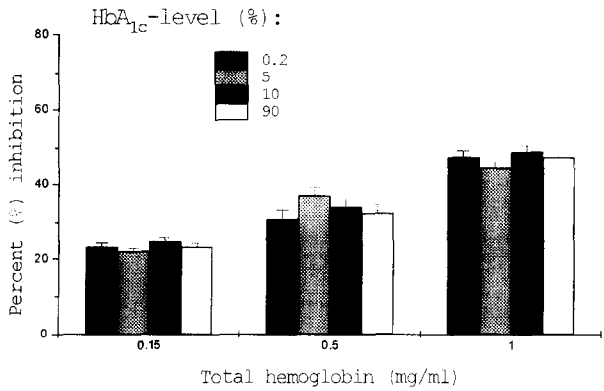

Fig. 3. Inhibition of Con A–glycogen precipitin reaction by glycohaemoglobin (HbA_{1c}) in TEA buffer, pH 7.4, 22 °C. Inhibition shown as the percentage inhibition of the nephelometric signal relative to the signal obtained with no protein added (signal set to 100%). Solutions containing Con A (80 μg) and increasing concentrations of haemoglobin with varying degree of glycation were incubated for 15 min at 22 °C before glycogen (40 μg) was added — total volume 2 mL. The nephelometric signal was recorded at 675 nm, 5 min after glycogen was added (the mean value of three replicates is presented).

precipitin system. Although reduced precipitation was observed due to the protein added (also observed using albumin at similar concentration), no significant inhibitory effect of glycation was observed even for the highly glycated samples. Under similar conditions transferrin (20 μg/mL) resulted in significant inhibitory effects on the precipitin reaction. Varying the incubation time of Con A and Hb (up to 1 h) before adding dextran or glycogen to start the precipitin reaction did not alter the results. Similarly, variation of temperature and time intervals before recording the nephelometric signal did not show any significant effect.

Glycohaemoglobin–lectin affinity studies.—Even in the presence of HbA_{1c} levels above 90% no glycation-specific binding of haemoglobin was detected using Lectin-Sepharose columns, nor could any glycohaemoglobin-specific binding be detected using Con A immobilised on particles. Although a slight binding of Hb was detected in some of the experimental set-ups, this binding was always "unspecific/non-sugar specific" and could not be blocked using glucose or α-mmp. Varying the ionic strength of the buffer (AC buffer with 0–0.5 M NaCl) or changing the pH, using 0.05 M sodium acetate, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.15 M NaCl, pH 5.0 as buffer, did not alter the results.

The experiments performed using gel filtration and electrophoresis only confirmed the negative results already observed using the before mentioned analytical methods.

Denaturation of Hb by 6 M guanidinium chloride followed by dialysis in assay buffer prior to the inhibition and chromatographic studies did not alter the results.

4. Discussion

None of the methods used in this study could verify a binding between glycated haemoglobin and the lectins Con A and Lentil Lectin. Some of the methods used have

limitations and constraints regarding their ability to verify weak interactions, however, from an overall assessment it is reasonable to conclude that the studied interaction, if it exists, must be of low affinity.

The protomers of the Con A tetramer contain two metal binding sites, one carbohydrate binding site and the other a hydrophobic cavity [7,20]. Thus, lectin–protein interaction can involve both specific carbohydrate binding, non-specific electrostatic and hydrophobic interactions [21,22] as well as be influenced by steric factors. As a result, both pH and temperature can be of importance for the specific binding to lectins. However, changing these parameters did not effect the specific lectin–glycohaemoglobin binding. Although showing marked differences in binding of glycopeptides, glycoproteins, and polysaccharides [7,12,23,24], Con A and Lentil Lectin display similar carbohydrate binding specificity. The minimal structural features required for binding to Con A are, according to Goldstein and Hayes [7], unmodified hydroxyl groups of the D-arabino configuration at C-3, C-4, and C-5 of the 1,5-anhydrohexitol ring systems, or C-2, C-3, and C-4 of the 1,4-anhydropentitol ring systems. The stable glycated ketoamine adduct of haemoglobin 1-amino-1-deoxy-fructose, present either at the *N*-terminal valine residue of the β-chain (HbA_{1c}) or at ε-amino groups of lysine residues, can be presented in ring-stabilised furanose or pyranose forms. Since Con A and Lentil Lectin both bind D-fructose, certain D-fructose derivatives and D-fructofuranosyl end groups [23–25], the basic structural requirements for binding are expected to be present in the furanose stabilised form of glycohaemoglobin. When no binding was found, this may be attributed to either specific stereochemical factors related to the conformation and structure of the glycated residue or micro-environmental factors relating to the accessibility of the glycated residues.

Stereochemistry of the glycated residue as initial requirement for lectin binding.— Stabilised furanose and pyranose structures have earlier been presented to illustrate the configuration of HbA_{1c} [3–6]. Both Fischer and Winterhalter [5] and Keil et al. [6], although the latter not agreeing with the experimental evidence presented by the former, have advocated the β-D-pyranose configuration of HbA_{1c}. The lectins used possess anomeric specificity favouring the α-anomers in their binding of carbohydrates [7,12,24]. This may be of importance discussing the results, however, as long as other major binding requirements are fulfilled, this specificity usually does not restrict binding of β-anomers. In general, lectins seems to tolerate very little variation at C-3 of the sugars they bind. Due to the C-3 hydroxyl group it seems obvious that the pyranose form of glycohaemoglobin deviates from the requirements necessary for Con A binding [7]. Taking this into consideration and assuming that the glycated residues of glycohaemoglobin in fact are present in the 1-deoxy-β-D-fructopyranosyl configuration, this may explain why no binding was observed.

Steric constraints.—Although denaturation was applied in some experiments to unfold haemoglobin, there might still be some variation in exposure to lectin binding due to unique conformational aspects at the different sites of glycation. Restricted access to the glycated residues of glycohaemoglobin can therefore not be excluded. Yet, some accessibility of the glycated residues are to be expected since the highly glycated haemoglobin samples used in this study most probably were glycated at various sites along the polypeptide chain [3]. Although not fully comparable with lectin binding,

immunoaffinity chromatography using monoclonal antibodies raised against glycohaemoglobin has been demonstrated to bind glycohaemoglobin even without using denaturing agents [26]. Such antibody glycohaemoglobin binding may therefore serve as an illustration of the accessibility of glycated residues in glycohaemoglobin. Taking this into consideration, restricted availability and steric constraints may be a part of, even if not the only explanation of, the results achieved.

This study can not fully be taken as evidence for the stereochemical form of the glycated residues in glycohaemoglobin. However, the presence of the residues in the 1-deoxy-(β)-D-fructopyranosyl conformation serve as a reasonable explanation of the results observed.

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