

Interaction of glycophorin A with lectins as measured by surface plasmon resonance (SPR)[⊙]

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Glycophorin A (GPA), the major sialoglycoprotein of the human erythrocyte membrane, was isolated from erythrocytes of healthy individuals of blood groups A, B and O using phenol-water extraction of erythrocyte membranes. Interaction of individual GPA samples with three lectins (*Psathyrella velutina* lectin, PVL; *Triticum vulgare* lectin, WGA and *Sambucus nigra* I agglutinin SNA-I) was analyzed using a BIAcore™ biosensor equipped with a surface plasmon resonance (SPR) detector. The experiments showed no substantial differences in the interaction between native and desialylated GPA samples originating from erythrocytes of either blood group and each of the lectins. Desialylated samples reacted weaker than the native ones with all three lectins. PVL reacted about 50-fold more strongly than WGA which, similar to PVL, recognizes GlcNAc and Neu5Ac residues. SNA-I lectin, recognizing α 2-6 linked Neu5Ac residues, showed relatively weak reaction with native and only residual reaction with desialylated GPA samples. The data obtained show that SPR is a valuable method to determine interaction of glycoproteins with lectins, which potentially can be used to detect differences in the carbohydrate moiety of individual glycoprotein samples.

The BIAcore™ instrument is a biosensor designed to investigate interactions between different molecules (biospecific interaction ana-

lysis); the instrument makes it possible to analyze these interactions in real time. The study of biomolecular interactions is of basic impor-

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Abbreviations: GlcNAc, *N*-acetylglucosamine; GPA, human glycophorin A; GPA-A, GPA-B, GPA-O; glycophorin isolated from blood group A, B and O erythrocytes, respectively; Neu5Ac, *N*-acetylneuraminic acid; PVL, *Psathyrella velutina* lectin; RA, rheumatoid arthritis; RU, resonance units; SNA-I, *Sambucus nigra* I agglutinin; SPR, surface plasmon resonance; WGA, *Triticum vulgare* agglutinin.

tance in searching and understanding the processes of molecular recognition and biological function of molecules. The procedure of analysis is as follows: one of the analyzed substances, called the *ligand*, is immobilized in the working channel of a specially designed sensor chip; the other substance, called the *analyte*, is in a solution which, during the experiment, is in contact with the ligand immobilized on the sensor chip. This physical contact enables the interaction between the two analyzed substances to take place. BIAcore instrument has a flow cell to deliver a constant supply of the analyte to the sensor surface during the association phase; during the dissociation phase the flow system is essential for removing free analyte released from the working surface. The mode of detection, used in the BIAcore biosensor, is the surface plasmon resonance (SPR) technique. SPR is a phenomenon which occurs between incoming photons and the electrons on the surface of a thin gold film coated onto a glass support on the sensor chip. At a specific wavelength and a specific angle of incident light the energy is transferred to the electrons in the gold surface causing the reflected light to disappear. This angle of non-reflectance, called the *resonance angle*, changes in response to the change of the refractive index in the vicinity of the gold surface. A signal from the detector, measured in resonance units (RU), is generated when two interacting molecules form a complex. The signal is proportional to the change in mass (for proteins 1 RU is equal to approx. 1 pg/mm²) (Stenberg *et al.*, 1991). Using the BIAcore instrument interactions between various molecules can be measured, e.g., antigen and antibody, ligand and its receptor, enzyme and its substrate, lectin and glycoprotein. Therefore, one can study the binding of nucleic acids, lipid micelles, peptides, drugs and other small molecules. The technology is valuable in a wide range of applications, from basic research to drug discovery and development, monoclonal antibody characterization, infectious disease research, etc.

The BIAcore system measures the change in surface concentration of the analyzed substance down to 1 pg/mm² and can monitor these changes on a time scale of 0.1 s. Moreover, the measurements allow, for typical molecular binding, accurate kinetic calculations in the range of 10³–10⁷ M⁻¹s⁻¹ for association rate constant (k_a) and 10⁻⁶–10⁻¹ s⁻¹ for dissociation rate constant (k_d), together with concomitant association constant K_A (10⁴–10¹² M⁻¹) and dissociation constant K_D (10⁻⁴–10⁻¹² M). An important advantage of this method is the lack of labeling requirements, which means simplicity and uniformity of different applications. The use of the BIAcore biosensor in biochemical and biomedical sciences is growing extensively (Rich & Myszk, 2000). Not only soluble substances can be analyzed using this methodology, but also larger particles like whole cells, e.g. erythrocytes (Quinn *et al.*, 1997).

In this work we applied a BIAcore-1000 biosensor to investigate the interaction of glycophorin A (GPA), native and desialylated, with three different lectins. Glycophorin A is the major sialoglycoprotein of the human erythrocyte membrane (Lisowska, 2001). This glycoprotein contains two types of glycans, i.e. one N-glycan and about 15 O-glycans per molecule; it has been used in numerous immunochemical and biochemical structural investigations, e.g. (Krotkiewski *et al.*, 1993; 1997; 1999; Podbielska & Krotkiewski, 2000). GPA contains bisecting *N*-acetylglucosamine in the N-linked glycan (Yoshima *et al.*, 1980). Most of the O-glycans of GPA contain two Neu5Ac residues (Lisowska *et al.*, 1980). Together with two sialic acid residues from the N-glycan they represent receptors, which can be recognized by appropriate lectin. The analyses performed in this work regarded glycophorin samples derived from individual human donors of defined ABO blood group. We used the following lectins: *Psathyrella velutina* lectin (PVL), *Triticum vulgare* lectin (wheat germ agglutinin, WGA) and *Sambucus nigra* lectin (SNA-I). The first two exhibit the same specificity, i.e.

they recognize GlcNAc and Neu5Ac residues. The third lectin reacts with sialic acid bound α 2-6 to Gal/GalNAc which means it is sensitive to desialylation of the reactant. The aim of this study was to establish immobilization and regeneration procedures, sensitivity of GPA interaction with the chosen lectins, influence, if any, of the blood group on GPA-lectin interaction and to check the reproducibility of the results.

MATERIALS AND METHODS

Materials. *Psathyrella velutina* lectin (PVL) was from WAKO (Germany), *Triticum vulgare* lectin (WGA) from Pharmacia (Sweden) and *Sambucus nigra* lectin (SNA-I) from Sigma (U.S.A.). BIAcore sensor chips CM5 were from Biacore AB (Uppsala, Sweden). Two chemicals used for the sensor chip activation procedure: EDC (*N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride) and NHS (*N*-hydroxysuccinimide) were from Sigma (U.S.A.); ethanolamine was from Merck (Germany). All solutions were prepared using high quality water from a Milli Q apparatus (Millipore), working in a reversed-osmose mode (electrical resistance \geq 16 M Ω). The binding buffer (HBS, 10 mM HEPES/NaOH, pH 7.4, 0.15 M NaCl, 3.4 mM EDTA, 0.05% Tween 20) was prepared according to the manufacturer's prescription.

Instrumentation. To measure interactions between glycophorin and the lectins a BIAcore-1000 instrument was used (Pharmacia Biosensor AB, Uppsala, Sweden). The technological fluctuations of the base line were \pm 5 resonance units (RU). The sensor chips CM5 with carboxymethylated dextran matrix were used throughout all experiments. The kinetic data from individual binding experiments were determined using the BIAevaluation software (Pharmacia, Sweden). Instrument compartment with the sensor chip was thermostated at 20°C during all experiments. All the solutions were filtered

(0.22 μ m, Millipore) and degassed before use.

Human glycophorin A. Glycophorin was isolated from the membranes of outdated human erythrocytes of individual subjects with blood groups A, B and O by phenol-water extraction, as described previously (Lisowska *et al.*, 1987). The yield of crude GPA preparation, obtained from one individual blood sample (300 ml), was about 50–60 mg. For comparative purposes three crude glycophorin preparations: GPA-A, GPA-B and GPA-O were purified by gel filtration on a Sephadex G-200 column in a buffer containing 1% SDS (Podbielska & Krotkiewski, 2000). After removing the bulk of SDS from the purified samples by dialysis against 50% ethanol and then water the samples were treated with Bio-Beads SM-2 adsorbent (BioRad) to remove residual detergent. The content of SDS in the final purified GPA samples was determined using a colorimetric method (Hayashi, 1975). The recovery of glycophorin after purification was about 60% of the starting material.

Desialylation. Desialylation of glycophorin samples (1–2 mg) was performed in 0.05 M sulfuric acid at 60°C for 4 h. After neutralization with NaOH the samples were dialyzed against distilled water. The residual content of Neu5Ac in the desialylated GPA samples was determined by a colorimetric method (Jourdain *et al.*, 1971). It was lower than 4.0% of the protein content, which is in agreement with our previous experiments which showed that desialylated GPA never lacks sialic acid completely.

Protein determination. Protein was determined on a micro scale (96-well polystyrene plates) using a colorimetric method with bicinchoninic acid (BCA) (Smith *et al.*, 1985). Bovine serum albumin (BSA) was used as a standard (Sigma, U.S.A.).

Immobilization of the ligand on the sensor surface. The immobilization was performed on CM5 sensor chips, according to the manufacturer's description based on published data (Johnsson *et al.*, 1991). Briefly, the

procedure was as follows: step no. 1 – *preconcentration*. Aliquots of 0.01 M sodium acetate buffer of different pH values (pH 4.0, 4.5, 5.0, 5.5, 6.0) were mixed with sample solutions, each containing lectin (5–20 μg). These solutions were then used in binding experiments in 2-min pulses. The buffer, for which the binding was the highest was chosen for the immobilization procedure. Step no. 2 – *activation of the carboxymethylated dextran surface*. This was done using EDC/NHS system, i.e. 50 μl of 0.04 M NHS solution was mixed with 75 μl of 0.16 M EDC; the final solution was pumped across the working channel (5 $\mu\text{l}/\text{min}$ for 7 min), followed by HBS buffer. After this step the baseline should rise by *ca* 150–200 RU. Step no. 3 – *ligand immobilization*. Lectin sample (5–20 μg) was dissolved in 0.01 M sodium acetate buffer with the previously determined optimum pH; this solution was pumped across the channel (5 $\mu\text{l}/\text{min}$) in several pulses, together lasting from 2 to 7 min. The rise of the baseline at this step of the procedure was between 2500–8000 RU. Step no. 4 – *deactivation*. The residual NHS esters, generated on the working channel surface, were destroyed with 1 M ethanolamine, pH 8.5, for 12 min. Step no. 5 – *washing*. The final washing of the channel was performed using 0.5 M NaCl for 10 min. Between each of the steps the working channel was washed with HBS buffer.

Binding studies. For the binding experiments the following durations, solution compositions and flow rates were used: 1° for PVL and WGA lectin: initial HBS flow – 5 $\mu\text{l}/\text{min}$, association – 2 $\mu\text{l}/\text{min}$ for 10 min, dissociation – 20 $\mu\text{l}/\text{min}$ of HBS for 3 min, regeneration – 20 $\mu\text{l}/\text{min}$ of 0.2 M GlcNAc in HBS for 10 min, final HBS flow – 20 $\mu\text{l}/\text{min}$ for 5 min, 2° for SNA lectin: initial HBS flow – 5 $\mu\text{l}/\text{min}$, association – 2 $\mu\text{l}/\text{min}$ for 10 min, dissociation – 60 $\mu\text{l}/\text{min}$ of HBS for 7 min, regeneration – 60 $\mu\text{l}/\text{min}$ of 0.5 M lactose in 0.1 M HCl for 10 min, final HBS flow rate – 20 $\mu\text{l}/\text{min}$ for 5 min.

Kinetic parameters. The equilibrium dissociation constant K_D was calculated based on the binding data, using the BIAevaluation version 3 program (Biacore AB, Uppsala, Sweden).

RESULTS AND DISCUSSION

Binding experiments

We measured interaction of three lectins with human glycophorin A. Two lectins – *P. velutina* lectin (PVL) and *T. vulgaris* agglutinin (WGA) have similar specificity, i.e. they recognize two carbohydrate epitopes: GlcNAc and Neu5Ac residues. The third lectin, *S. nigra* (SNA-I), recognizes only one of those epitopes, i.e. Neu5Ac. Therefore, for desialylated glycophorin we could expect diminished binding with PVL and WGA, and close to zero binding with SNA-I.

Psathyrella velutina lectin (PVL)

The multi-ligand lectin from the fruiting body of *P. velutina* mushroom (PVL) is known to react best with terminal, non-reducing *N*-acetylglucosamine and *N*-acetylneuraminic acid (Neu5Ac) residues (Ueda *et al.*, 1999a; 1999b). It was also found that the differential binding of oligosaccharides to PVL is due both to the number as well as the linkage of the non-reducing terminal carbohydrate residues, distinguishable by the lectin (Ueda *et al.*, 1999a). For example, the sequence GlcNAc β 1-2Man α 1... reacts with PVL more strongly than GlcNAc linked β 1-4 or β 1-6 to mannose (Endo *et al.*, 1992). Accordingly, PVL has been applied to detect glycosylation abnormality in rheumatoid arthritis (RA) IgG (Tsuchiya *et al.*, 1993). In IgG of RA patients there is a substantial lack of galactose residues at the non-reducing ends of Asn-297-linked N-glycans. The absence of these Gal residues exposes terminal,

non-reducing GlcNAc residues which can be detected using appropriate reagent, e.g. a lectin.

During the pre-concentration procedure performed for PV lectin, the optimum pH at which the lectin gave the highest SPR response, was 5.4. After immobilizing the lectin onto the working channel the SPR baseline rose by 7340 RU. This is a relatively big change of the SPR signal which, according to the literature, may be a result of the known tendency of PVL to aggregate (Kochibe & Matta, 1989). If this is the case then the layer of this lectin, created during immobilization, is not monomolecular but thicker, giving more carbohydrate-binding sites. Figure 1 presents an example sensorgram of the bind-

ing from the SPR detector, which is considered as "binding". The binding of different glycoprotein samples to PVL is presented in Fig. 2; this interaction was measured for both native and desialylated glycoprotein samples. The binding of GPA to PV lectin was relatively strong (about 1200 RU); the glycoprotein concentration in the analyzed solution was 0.083 μ M (0.5 μ g protein/200 μ l HBS). As can be seen, the interaction of immobilized PVL with different native glycoprotein samples is similar, regardless of the blood group of the erythrocytes which were the source of these glycoprotein samples. Slightly lower binding was observed for GPA-A samples, as compared with GPA-B and GPA-O. All desialylated samples showed clearly lower binding, as com-

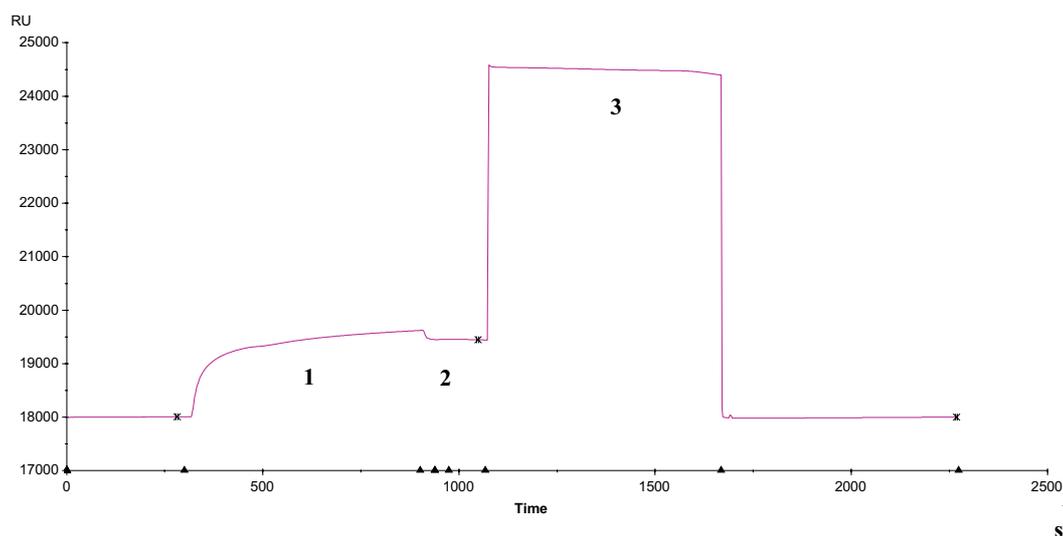


Figure 1. Example sensorgram showing the interaction between immobilized PVL lectin and human glycoprotein.

GPA concentration was 0.5 μ g protein/200 μ l of HBS buffer. The experiment consisted of the following steps: **1**, association, 10 min; **2**, dissociation, 3 min; **3**, regeneration of the channel, 10 min. Sensorgrams showing interaction of glycoprotein samples with two other immobilized lectins, WGA and SNA-I, were entirely the same.

ing experiment obtained for human glycoprotein as the analyte and immobilized PV lectin. The sensorgram exhibits three typical experimental time intervals: association, dissociation and regeneration of the working channel. The dissociation removes non-specifically bound molecules of the analyte; at the end of this time interval the instrument automatically measures the signal

pared with the parent ones, Fig. 2-II. This agrees with the described specificity of PVL. Estimation of sialic acid in the desialylated samples revealed the presence of 2–4% sialic acid relative to protein content (not shown). Therefore, the residual presence of Neu5Ac in the desialylated samples together with the presence of bi-secting GlcNAc residues in N-glycans explain the interaction of desia-

lylated GPA samples with PVL. As shown in Fig. 2, purified samples of GPA-A, GPA-B and GPA-O exhibit very similar binding, which is lower than that of crude GPA preparations. It is probable, therefore, that the crude glycophorin preparations may include some amount of carbohydrate-containing impurities (e.g., glycolipids), interacting with PV lectin and making the SPR response of the sample slightly higher. One should also keep in mind that the glycophorin samples analyzed here originated from erythrocytes of individual persons and there always is a possibility that differences in the interaction of such individual glycophorin preparations with a lectin may reflect differences in glycosylation of these glycophorin samples, based on native microheterogeneity or the health status of the donor. The latter has been described, i.e. human glycophorin A was shown to be underglycosylated in CDG patients (Krotkiewska *et al.*, 1999; Zdebska *et al.*, 2001).

Triticum vulgare lectin (wheat-germ agglutinin, WGA)

This lectin is a dimer of two identical 18 kDa subunits, each consisting of four homologous domains of 43 amino acids each. WGA specifically recognizes GlcNAc and Neu5Ac. The binding interactions, observed in crystal complexes with WGA (Wright & Kellogg, 1996) are consistent with the presence of two high affinity and two low affinity, thermodynamically independent sites per subunit, which means eight independent carbohydrate binding sites per one lectin molecule. From the results of affinity chromatography on WGA-agarose it may be suggested that there are different binding sites for GlcNAc and Neu5Ac in a WGA molecule (Gallagher *et al.*, 1985).

During the pre-concentration procedure performed for WGA lectin the optimum pH of sodium acetate buffer was 4.5. This buffer was used, therefore, for the immobilization of the lectin in the working channel, which resulted

in an SPR baseline rise by 4180 RU. The results of binding, measured for different GPA samples and immobilized WGA lectin, are shown in the Fig. 3. A characteristic feature of this interaction is clearly a weaker SPR response, compared with GPA-PVL interaction (Fig. 2). In the binding experiments with WGA, glycophorin samples had the concentration $0.83 \mu\text{M}$ ($2.5 \mu\text{g}$ protein/ $100 \mu\text{l}$ HBS), which was 10 times more than in the experiments with PVL. For the native, crude glycophorin preparations the highest reaction was for GPA-A, and a little weaker for GPA-B and GPA-O. The corresponding desialylated preparations gave lower SPR responses, which were comparable to GPA binding to PVL (Fig. 2). Three purified, native preparations, one of each blood group, exhibited almost the same binding, around 170 RU. These

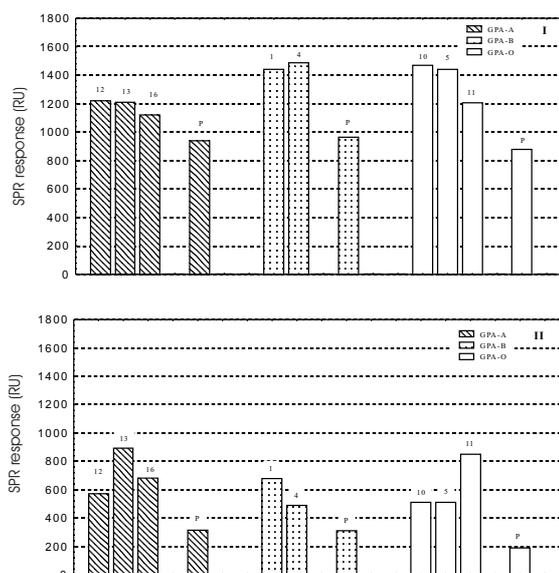


Figure 2. Results of GPA binding to immobilized PV lectin.

Glycophorin samples originated from individual subjects indicated by numbers over the bars. Concentration of all glycophorin samples, native and desialylated, was $0.5 \mu\text{g}$ protein/ $200 \mu\text{l}$ of HBS buffer ($0.083 \mu\text{M}$). The bars represent mean value of two measurements, which always differed by less than 5%. In the control experiment the binding of BSA was negligible (less than 10%). I, native glycoprotein; II, desialylated glycoprotein; p, purified sample.

values were higher than for crude preparations, except for one GPA-A preparation. After desialylation these three samples showed lower reactivity, similar to desialylated crude glycophorin samples. Similarly to PVL, Fig. 2, desialylated glycophorin samples still reacted with WGA. According to the lectin specificity this binding probably resulted from the fact that WGA, like PVL, exhibits double specificity (GlcNAc and Neu5Ac) and desialylated GPA still contained sugar residues (i.e. the bisecting GlcNAc in N-glycans and residual sialic acid) recognizable by the lectin.

Sambucus nigra lectin I (SNA-I)

SNA-I lectin is isolated from a partially purified bark extract of elderberry tree; it exhibits specificity towards Neu5Ac α (2-6)Gal/ GalNAc sequence (Van Damme *et al.*, 1996). Both types of receptors exist in human glycophorin, i.e. Neu5Ac α 2-6Gal β 1... exist as non-reducing termini in N-glycan and Neu5Ac α 2-6GalNAc sequence is present in most O-glycans. Therefore, human glycophorin can react with SNA-I.

During the pre-concentration procedure performed for SNA-I lectin the optimum pH of sodium acetate buffer was 4.0. This buffer was used, therefore, for the immobilization of the lectin onto the working channel; after the procedure was completed the SPR baseline rose by 2650 RU. During binding experiments the concentration of the glycophorin solutions was maintained at 0.83 μ M (2.5 μ g protein/100 μ l HBS), similarly like for WGA. The results of binding are presented in Fig. 4. As can be seen, the magnitudes of the reactions are comparable for the samples from different blood groups; these bindings are a little lower than with WGA lectin (Fig. 3). For desialylated GPA samples the reaction is very low, in some cases close to zero. This is in agreement with the described specificity of SNA-I lectin: if the efficiency of the desialylation procedure is close to 100%, the reaction of glycophorin with SNA-I drops close to zero.

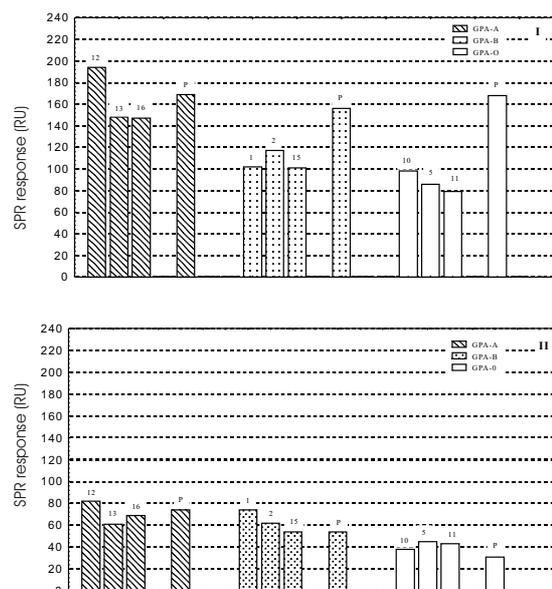


Figure 3. Results of GPA binding to immobilized WGA lectin.

Glycophorin samples originated from individual subjects. Concentration of all glycophorin samples, native and desialylated, was 2.5 μ g protein/100 μ l of HBS buffer (0.83 μ M). For further explanations see legend to Fig. 2.

Kinetic parameters

We have calculated dissociation constants K_D , which indicate the concentration of the analyte (lectin in this case) in the equilibrium state. If the K_D value is higher, the binding of the analyte to the immobilized ligand is weaker and *vice versa*. We performed this calculation by fitting each measurement data to a single site interaction model, which is a common procedure used in this type of experiments (Myszka *et al.*, 1998). The results are summarized in Table 1. As can be seen, different K_D values were obtained for different lectins. For WGA the calculated K_D values were the highest. They exhibited a regular tendency, i.e. for the native GPA samples K_D values were lower than for the desialylated ones, for all three blood groups. The same tendency can be seen for the interaction of glycophorin with PVL, although in this case K_D values

Table 1. Equilibrium dissociation constants K_D , calculated for complexes of three types of GPA with different lectins

Glycophorin	WGA	PVL	SNA-I
GPA-A -native	175 nM	4.3 nM	38 nM
-desialylated	300 nM	5.0 nM	1.6 nM
GPA-B -native	26 nM	n.d.	42 nM
-desialylated	150 nM	8.4 nM	0.4 nM
GPA-O -native	270 nM	3.9 nM	41 nM
-desialylated	475 nM	12.3 nM	2 nM

n.d., not determined (in this case K_D could not be calculated).

were more than 10 times lower (in comparison with WGA). This result indicates that interaction of glycophorin with PVL is stronger and more specific than with WGA. A reciprocal tendency of K_D values was observed in the case of SNA-I lectin. Although the absolute binding of GPA was lower for desialylated samples, see Fig. 4, the K_D values were very low (0.4–2 nM). To the latter result two explanations seem to be applicable: first – the binding of desialylated GPA to SNA-I is so weak that the equilibrium concentration of the analyte cannot be higher than about 2 nM; second – during desialylation of GPA the majority of the sugar epitopes recognizable by SNA-I lectin were lost, but some new epitopes appeared giving a strong binding of the lectin. We plan to explain this phenomenon in further experiments, using other GPA-binding lectins.

CONCLUSIONS

Based on the results obtained in this investigation one can conclude that the biosensor BIAcore, with SPR as a detection method, is a powerful method for investigating the interaction between lectins and glycoproteins. Measurement of the interaction between glycophorin and lectin may serve to detect even small changes in the carbohydrate moiety of this glycoprotein. The results showed that there is a small difference in binding between crude and purified glycophorin sam-

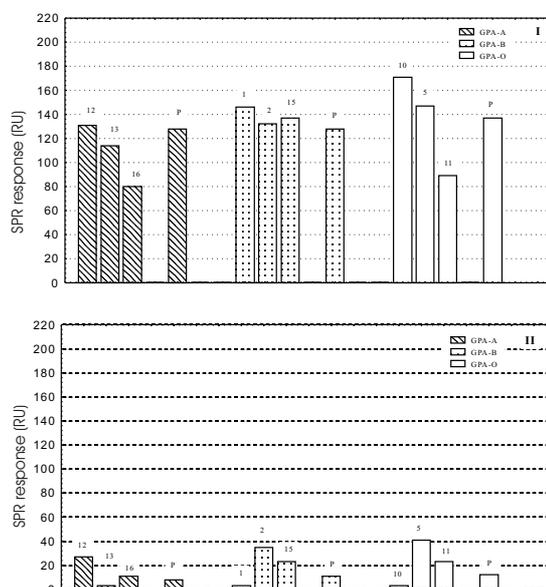


Figure 4. Results of GPA binding to immobilized SNA-I lectin.

Glycophorin samples originated from individual subjects. Concentration of all glycophorin samples, native and desialylated, was 2.5 μg protein/100 μl of HBS buffer (0.83 μM). For further explanations see legend to Fig. 2.

ples. It means that there is a possibility to analyze glycophorin preparations derived from small (e.g., 1 ml) blood samples, not suitable for standard purification. We plan to apply this finding to the analysis of GPA samples derived from patients with defined diseases. Finally, we noticed during the experiments that lectin immobilized in a single working channel could serve to perform over 200 binding measurements.

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