Applications of the ProteOn™ GLH Sensor Chip: Interactions Between Proteins and Small Molecules

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Introduction

The ProteOn GLH sensor chip is one of several types of sensor chips available for use with the ProteOn XPR36 protein interaction array system (Figure 1). The chip is designed for protein-small molecule and protein-protein interaction studies in which highest sensitivity is of primary concern.

The GLH sensor chip, similar to other general amine coupling ProteOn sensor chips (GLC and GLM), utilizes a proprietary surface chemistry enabling easy activation of carboxylic groups by EDAC and *N*-hydroxysulfosuccinimide (sulfo-NHS). This activation provides efficient binding of proteins via their amine groups and ensures high ligand activity in many biological applications (see bulletin 5404).

Of the available ProteOn sensor chips, the GLH chip offers the highest ligand binding capacity, making it optimal for the study of protein-small molecule interactions. This higher capacity is attained through the structure of its surface binding layer, comprising a unique formula of modified polysaccharides. Higher binding capacity, together with efficient preservation of the protein's biological activity, ensures high analytical response upon binding of the analyte to the ligand — a key advantage when measuring the response of small molecule compounds.

Immobilization of Proteins With Different pI Values

In this report, we describe the use of the ProteOn GLH sensor chip with the ProteOn XPR36 system. To examine the high binding capacity and the versatility of the GLH chip, immobilization levels of 11 different proteins with a wide range of isoelectric point (pI) values were evaluated.

Small Molecule Interactions

Interactions between bound proteins and small molecules (MW <1,000) were tested using three biological models: 1) carbonic anhydrase II (CAII) and small molecule inhibitors, 2) a monoclonal antibody specific to the dinitrophenyl (DNP) group and DNP-labeled amino acids, and 3) human serum albumin and the drug digitoxin.

CAII Small Molecule Inhibitors

The family of CA proteins is a group of metalloenzymes that catalyze the conversion of carbon dioxide to bicarbonate and protons. Some CA inhibitors are active ingredients in drugs that treat diseases such as glaucoma or epilepsy. Kinetic studies of the interaction between CAII and its inhibitors appear in the literature (Myszka 2004, Myszka et al. 2003). The interaction of CAII with ten different inhibitors was studied using the ProteOn GLH sensor chip, and the results demonstrate higher analytical response in comparison to published data using conventional chip surfaces.

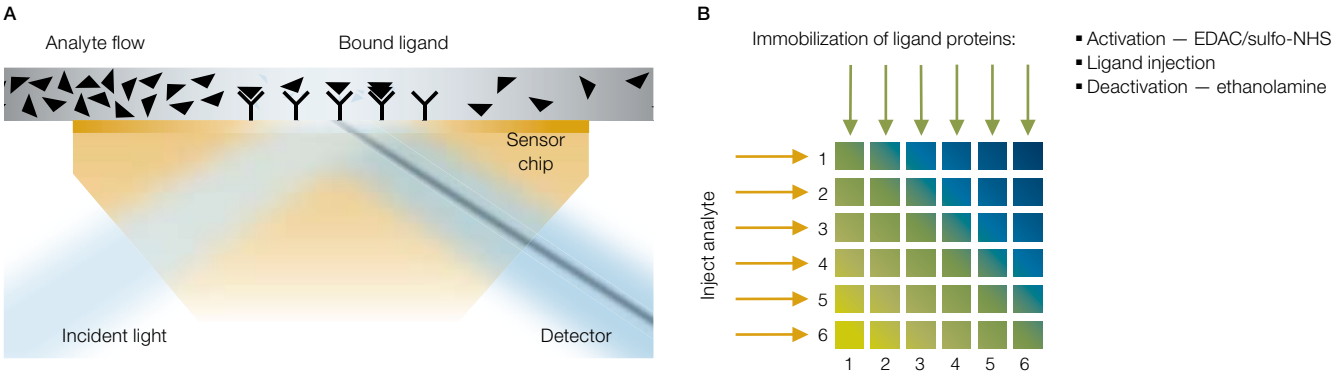

Fig. 1. Schematic illustration of ProteOn XPR36 protein interaction array system technology. A, detection of ligand to analyte interaction; **B**, general experimental procedure for the parallel and simultaneous immobilization of up to six ligand proteins on the sensor chip and the simultaneous flow of small molecules as analytes for the protein-small molecule interaction kinetic studies. EDAC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride.

A Monoclonal Antibody (mAb) Specific to the DNP Group and Three Types of DNP-Labeled Amino Acids

The labeling of peptides, proteins, and other biomolecules with DNP groups and the subsequent use of antibodies to bind DNP is widely used as a detection method in research and diagnostic applications (for example, Jasani et al. 1992). The binding of three DNP-labeled amino acids to anti-DNP mAb was studied to illustrate the ability of the GLH chip to measure the binding of small analytes to large proteins such as antibodies.

Human Serum Albumin (HSA) and the Drug Digitoxin

HSA is the most abundant protein in plasma, and serves as the primary drug transporter in blood. Binding to plasma proteins is a key parameter in evaluating candidate lead compounds. In this study, we examined the binding of the small molecule drug digitoxin to HSA, to investigate the capabilities of the GLH chip in such applications.

Multi-Chip Study of the CAII/CBS Interaction

Additionally, the ligand activity and analytical response were further evaluated by a multi-chip study of the interaction of CAII with one of its inhibitors, 4-carboxybenzenesulfonamide (CBS). CAII was immobilized at different densities and exposed to six concentrations of CBS. Analysis of the results revealed that CAII ligand activity was more than 80% and thus yielded exceptionally high analyte signals.

Methods

Instrument and Reagents

Experiments were performed using the ProteOn XPR36 protein interaction array system with ProteOn GLH sensor chips (Bio-Rad Laboratories, Inc.). ProteOn PBS/Tween running buffer (phosphate buffered saline, pH 7.4 with 0.005% Tween 20) was used. In certain cases, 3% or 10% dimethyl sulfoxide (DMSO) was added to enable dissolution of the organic analytes. For immobilization of proteins, ProteOn reagents and buffers were used as described in Bronner et al. 2006. The ProteOn amine coupling reagents were EDAC, sulfo-NHS, and 1 M ethanolamine hydrochloride, pH 8.5. The ProteOn immobilization buffers were 10 mM sodium acetate solutions, pH 4.0, 4.5, 5.0, or 5.5; manual pH adjustment with 1 N HCl or NaOH was used to generate other pH values. All proteins and small molecule analytes were purchased from Sigma-Aldrich Co. All experiments were performed at 25°C.

Immobilization of Proteins on Chip — General Procedure

All experiments performed in this report started with the multi-channel module (MCM) of the ProteOn system in the vertical position (Figure 1B). Up to six different ligand proteins were immobilized simultaneously onto one chip with each protein in a separate channel. Only three injections were required to complete this immobilization process as described below. The flow rate was set to 30 µl/min, and the chosen channels were activated simultaneously by injecting 200 µl of a freshly mixed solution of EDAC and Sulfo-NHS. The concentrations of these activators were 200 mM and 50 mM, respectively, unless noted otherwise. Immediately after activation, 180 µl of ligand protein solution was injected into each of the activated channels. Details of immobilization conditions are shown in Table 1. For deactivation, 150 µl of ethanolamine hydrochloride (1 M, pH 8.5) was injected into the relevant channels simultaneously. The final amount of immobilized protein was measured in response units (RU) relative to the baseline signal before activation.

Immobilization of Proteins With Different pI Values

Eleven proteins with pI of 3.0 to 9.3 (Table 1) were immobilized onto the GLH surface, using the above general procedure. Owing to the simultaneous availability of six channels in the ProteOn system, only two GLH chips were required to complete this experiment.

Small Molecule Interactions

Using the above general procedure, four proteins — CAII, anti-DNP antibody, HSA, and rabbit IgG were immobilized to the surface of one GLH chip. The specific conditions used for each protein are indicated in Table 1. The channel containing rabbit IgG was used as a reference in the interaction studies.

Multi-Chip Study of the CAII/CBS Interaction

Seven GLH chips were used to perform this study. On each chip, CAII was immobilized in five channels, and rabbit IgG was bound to the sixth channel as a reference. This design was used for all chips, with CAII solution at 125 µg/ml in 10 mM acetate buffer, pH 5.0 and rabbit IgG of 50 µg/ml in 10 mM acetate buffer, pH 4.5. The amount of CAII bound to each vertical channel was varied by diluting the standard activation solution (EDAC 200 mM, Sulfo-NHS 50 mM) up to 30 times with purified water thus creating a varied density surface.

Table 1. Results of immobilization of 11 proteins with various pI values onto ProteOn GLH chips.

Protein	pI	MW	Immobilization Conditions*	Final Amount of Bound Ligand, RU
Pepsin	3.0	34,700	800 µg/ml, pH 2.7	2,470
Ovalbumin	4.5	43,500	400 µg/ml, pH 4.0	6,800
Soybean trypsin inhibitor	4.5	20,000	400 µg/ml, pH 4.0	21,200
Protein A	5.1	41,000	300 µg/ml, pH 4.5	18,800
HSA	5.1	66,000	50 µg/ml, pH 5.0	22,000
CAII	5.9	29,000	125 µg/ml, pH 5.0	21,200
NeutrAvidin	6.3	60,000	50 µg/ml, pH 4.5	22,350
Myoglobin	6.9–7.4	17,000	400 µg/ml, pH 6.0	12,200
Polyclonal rabbit IgG	6.0–8.0	150,000	25 µg/ml, pH 4.5	22,200
Aldolase	8.2–8.6	161,000	100 µg/ml, pH 6.0	14,850
Ribonuclease A	9.3	13,700	400 µg/ml, pH 6.0	11,300

* In 10 mM sodium acetate solution at the indicated pH.

Small Molecule Interactions — General Procedure

After ligand immobilization, the MCM of the ProteOn system was rotated by 90° to the horizontal position. For the CAII studies, a surface conditioning treatment was performed first (see details below). Analyte was injected simultaneously into the six horizontal channels, using a different concentration in each channel (Figure 1B). The interactions of each ligand immobilized in the vertical channels with six analyte concentrations were obtained in one single injection (One-shot Kinetics™ technology, see Bronner et al. 2006 for a description).

CAII/Small Molecule Inhibitors

Surface conditioning for CAII and its inhibitors was performed by a short injection of 50 mM NaOH (30 µl at flow rate of 30 µl/min) into all horizontal channels. The running buffer was then changed to PBST with 3% DMSO, and the flow rate was set to 100 µl/min. The same buffer was used to dissolve and dilute the analytes. Ten different analytes with molecular weights ranging from 95 to 341 Da were used on the same GLH chip (Table 2). For each analyte, six concentrations were prepared by a threefold dilution series, and 100 µl of each of these solutions was injected. The highest concentration used for each compound is listed in Table 2. All analytes were allowed to fully dissociate from the surface before the next analyte was injected.

Multi-Chip Study of the CAII/CBS Interaction

Surface conditioning was performed as described above. The flow rate was set to 100 µl/min. PBST was used as sample buffer. Six concentrations of CBS were prepared by a threefold dilution series starting from 20 µM, and 100 µl of each solution was injected in parallel. Owing to the unique multiplexing abilities of the ProteOn XPR36 system, a single injection of analyte yielded five sets of full kinetic analyses, each containing six sensorgrams relating to different surface densities of ligands.

Anti-DNP mAb/DNP-Labeled AA Interactions

The flow rate was set to 100 µl/min for anti-DNP mAb antibody and DNP-labeled amino acids. For each analyte, six concentrations were prepared by a threefold dilution series starting from 1 µM, and 100 µl of each of these solutions was injected. PBST was used as the running buffer and diluent. Three different DNP-labeled analytes were used in this experiment (Table 3). All analytes were allowed to fully dissociate from the surface before the next analyte was injected.

HSA/Digitoxin Interaction

The running buffer was changed to PBST with 10% DMSO, and the flow rate was set to 100 µl/min for HSA and digitoxin. The same buffer was used to dissolve and dilute the analyte. Six concentrations of digitoxin were prepared by a twofold dilution series starting from 500 µM, and 100 µl of each of these solutions was injected.

Sensorgram Acquisition and Data Analysis

In each of the kinetic studies, the interactions of six analyte concentrations with up to five immobilized ligands and one reference protein were monitored in parallel. The data were analyzed with the ProteOn Manager™ 2.0 software.

Values derived from the interaction spots containing immobilized reference protein (rabbit IgG) were used for background subtraction. Although the ProteOn XPR36 system enables the use of unmodified spots or interspots as references, in cases of very high ligand density, it is recommended to use spots with a reference protein where conditions are more similar to the active spots.

Each set of six reference-subtracted sensorgrams was fitted globally to curves describing a homogeneous 1:1 biomolecular reaction model. Global kinetic rate constants (k_a and k_d) were derived for each reaction, and the equilibrium dissociation constant, K_D, was calculated using the equation $K_D = k_d / k_a$. The R_{max} values, the maximal analyte signals at saturation of the active binding sites of the ligand, were also calculated from this analysis.

For the CAII/methylsulfonamide interaction, kinetic analysis could not be obtained due to fast reactions and low signals (Figure 3). Determination of K_D was done using the equilibrium response for each of the six analyte concentrations. These equilibrium response levels (R_{eq}) were then fitted to a simple bimolecular equilibrium model at 50% saturation response.

Table 2. Results of the interactions of CAII (MW 29,000) with ten different inhibitors.

Analyte	MW	Highest Concentration Used, µM	k_a, $M^{-1}sec^{-1}$	k_d, sec^{-1}	K_D, M	R_{max}, RU
Sulpiride	341	250	2.52×10^3	0.26	1.0×10^{-4}	188
Sulfanilamide	172	50	2.40×10^4	0.12	4.8×10^{-6}	112
Furosemide	331	50	5.15×10^4	0.04	7.1×10^{-7}	180
CBS	201	50	2.83×10^4	0.03	1.2×10^{-6}	105
Dansylamide	250	10	1.33×10^5	0.09	6.5×10^{-7}	105
1,3-benzene-disulfonamide	236	10	1.11×10^5	0.09	8.1×10^{-7}	99
Benzenesulfonamide	157	50	1.17×10^5	0.12	1.0×10^{-6}	114
7-fluoro-2,1, 3-benzoxadiazole-4-sulfonamide	217	2	4.64×10^5	0.01	2.8×10^{-8}	82
Acetazolamide	222	2	9.28×10^5	0.02	2.6×10^{-8}	99
Methylsulfonamide	95	2,500	—	—	3.2×10^{-4}	22

Table 3. Results of the interactions of anti-DNP mAb (150 kD) with three DNP-labeled amino acids.

Analyte	MW	k_a, $M^{-1}sec^{-1}$	k_b, sec^{-1}	K_D, M	R_{max}, RU
DNP-glycine	241	1.99×10^6	0.095	4.77×10^{-8}	36
DNP-valine	283	1.24×10^6	0.098	7.90×10^{-8}	41
DNP-tryptophan	370	7.14×10^5	0.251	3.52×10^{-7}	75

Results and Discussion

Immobilization of Proteins With Different pI Values

Proteins with various pI values were immobilized onto the ProteOn GLH chips. The results are illustrated in Figure 2 and summarized in Table 1. Figure 2 compares the immobilization levels of the GLH chip to the ProteOn GLM chip, and to published results for a series of proteins immobilized under similar conditions (Johnsson et al. 1991). The GLH chip, used with EDAC/sulfo-NHS activation, is capable of immobilizing high levels of proteins with a wide range of pI values. Using ProteOn GLH chips, it is possible to effectively bind proteins with very low pI values such as pepsin, which is difficult based on methods reported in the literature.

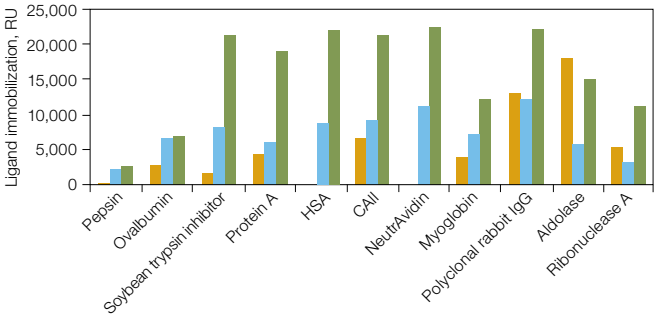

Fig. 2. Comparison of ligand immobilization levels for various proteins between previously published values using conventional surface ( ) chips, and results obtained on GLM ( ) and GLH ( ) chips. Data for conventional surfaces with HSA and NeutrAvidin were not reported in the literature. RU, response units.

CAII Small Molecule Inhibitors

CAII protein was immobilized at a level of 20,000 RU, and the binding of ten small molecule inhibitors was studied. The data for the kinetic analysis are shown in Figure 3, and the results are summarized in Table 2. While the k_a and k_d values are in agreement with data published in the literature, the maximal analytical response was found to be at least four times higher in all cases than shown in similar studies using a conventional sensor chip (Myszka 2004).

Monoclonal Antibody and DNP-Labeled Amino Acids

The binding of three DNP-labeled amino acids (DNP-glycine, DNP-valine, and DNP-tryptophan) was studied to illustrate the ability of the ProteOn GLH sensor chip to measure the binding of small analytes to large ligands (Table 3, Figure 4). The amount of immobilized anti-DNP antibody was 18,550 RU, with more than 50% of the total binding sites being active. In the case of DNP-glycine, the molecular weight ratio of ligand to analyte was greater than 300 (assuming two available ligand binding sites per ligand molecule), and binding of such analytes could be readily detected and measured.

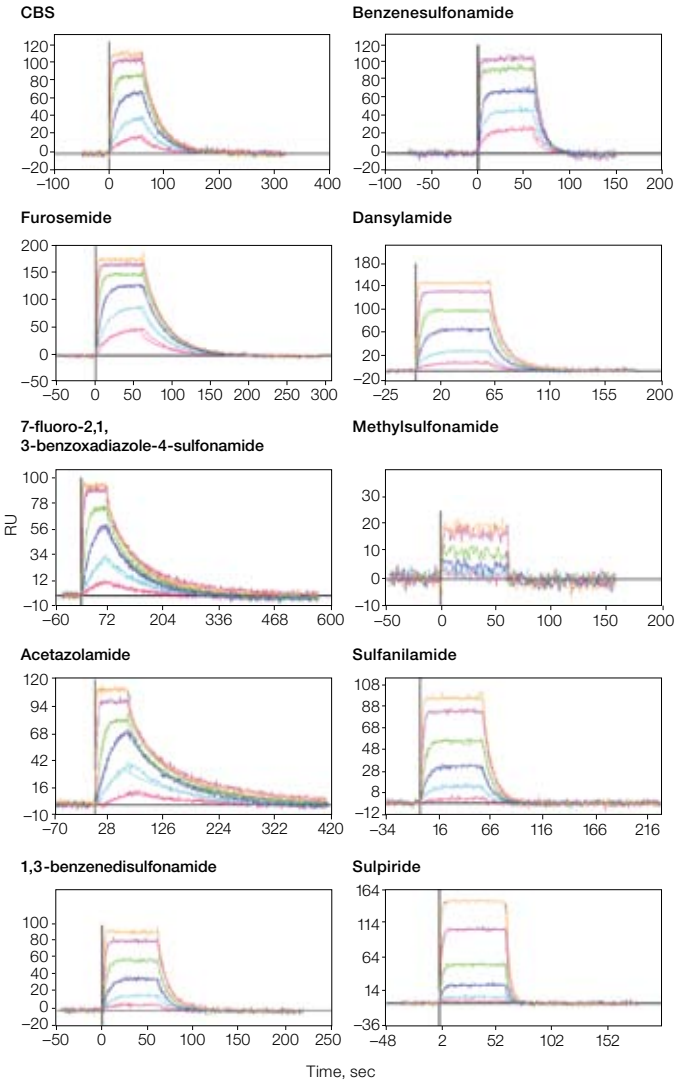

Fig. 3. Sensorgrams and analysis fit from each of the kinetic studies of CAII (20,000 RU) and the pertinent inhibitor. The kinetic parameters are shown in Table 2.

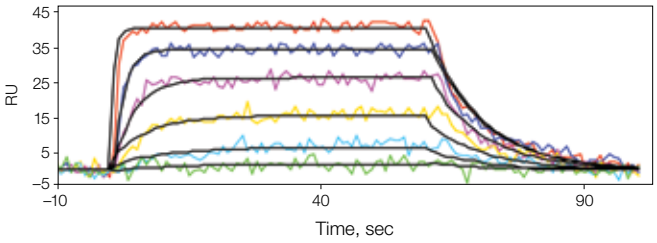

Fig. 4. Sensorgrams and analysis fit from the kinetic study of anti-DNP antibody (18,550 RU) and the DNP-valine analyte. The kinetic parameters are shown in Table 3.

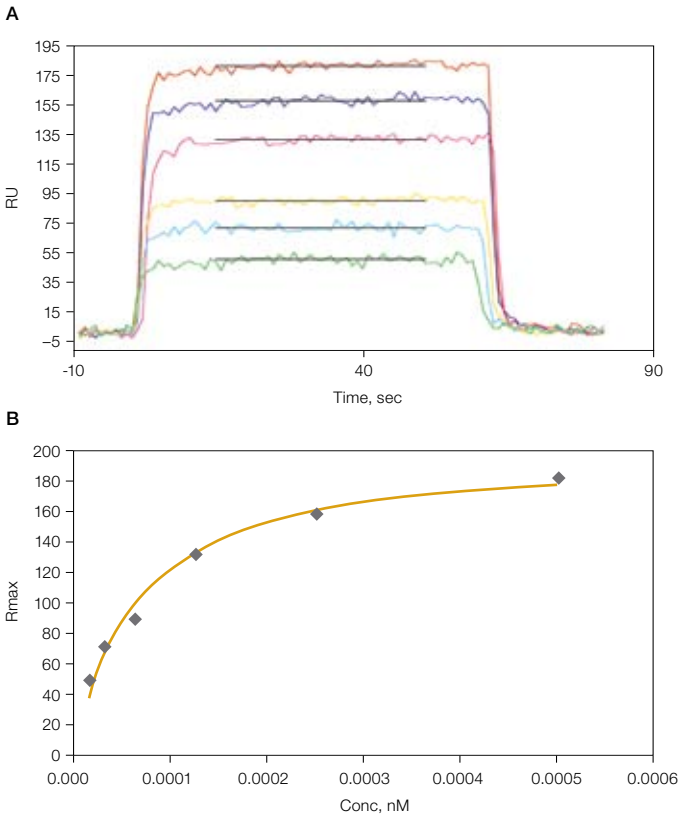

Fig. 5. Binding of Digitoxin to HSA (22,000 RU). A, Sensorgrams; **B**, Equilibrium analysis, indicating K_D = 63.6 µM.

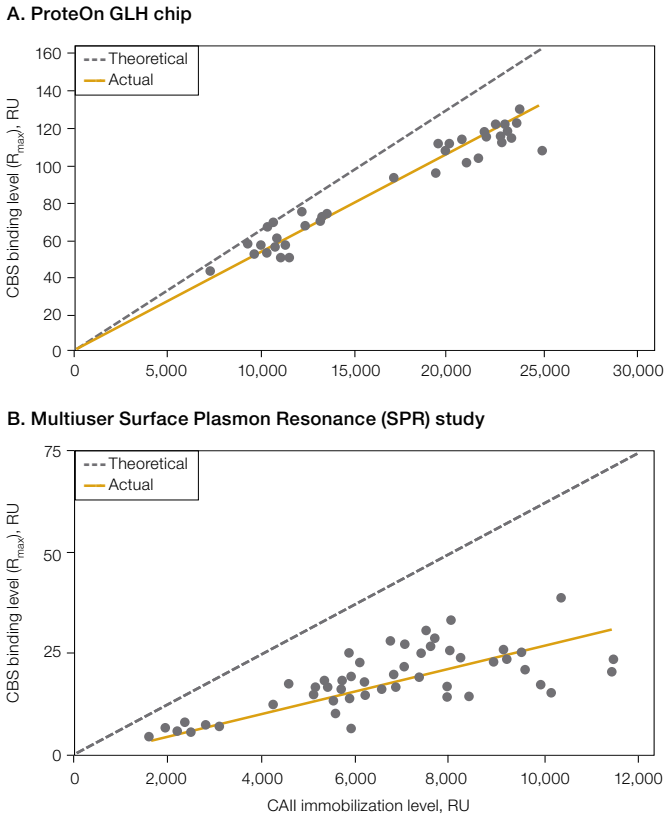

Fig. 6. Analytical response of CBS binding vs. the amount of CAII immobilized onto the sensor chip. A, ProteOn GLH chip; **B**, conventional chip (Myszka et al. 2003). The black dotted line shows the theoretical maximal response, assuming that 100% of the bound ligand molecules are active. The gold line is a linear fit of the actual response values. Actual ligand activity is 82% of theoretical for the GLH chip and 46% for the conventional chip surfaces.

HSA and the Drug Digitoxin

In this study we examined the binding of digitoxin (MW 768) to HSA (MW 66,000). The HSA ligand density was 22,000 RU. The sensorgrams of interaction with six concentrations of digitoxin and the thermodynamic analysis are shown in Figure 5. The K_D value was found to be 63.6 µM, with as much as 78% of the immobilized HSA being active.

Multi-Chip Study of the CAII/CBS Interaction

The bound amount of the CAII ligand ranged from 7,000 to more than 24,000 RU, depending on the level of surface activation. The kinetic analysis of the interaction with CBS was performed for each of the 35 sets of results; each set contained six analyte sensorgrams relating to one ligand density. The average results of the kinetic constants were: $k_a = 3.2 \pm 0.7 \times 10^4\ M^{-1}sec^{-1}$; $k_d = 0.037 \pm 0.003\ sec^{-1}$; $K_D = 1.2 \times 10^{-6} \pm 0.3 \times 10^{-6}$ M. These values are in agreement with published data (Myszka et al. 2003).

The mean ligand activity of the CAII was determined by plotting the maximal response of the analyte (R_{max}) vs. the ligand density (Figure 6A). Assuming a stoichiometric relationship between reactants in molar terms, the theoretical CBS binding response is 150-fold lower than the immobilized level of CAII due to the mass difference between the interacting pair. The dotted trend lines in Figures 6A and 6B represent the theoretical correlation between the surface density of CAII and maximal binding signal of CBS. Experimental data typically fall below this line because some of the immobilized proteins are inactive. However, the data for the ProteOn GLH chip (Figure 6A) show that actual CBS binding values lie very close to the theoretical trend line, indicating that more than 80% of the immobilized ligand is active. These results demonstrate exceptionally high ligand activity of the CAII/CBS interaction, and are a significant improvement over the published results of less than 50% ligand activity (Figure 6B, from Myszka et al. 2003). In absolute terms, analyte signals of more than 120 RU could be gained with the GLH chip, while less than 40 RU was the maximal value recorded with conventional surfaces.

Conclusions

The ProteOn GLH sensor chip offers exceptionally high binding capacities while preserving ligand activity, providing enhanced analyte signal in situations where the molecular weight ratio of ligand to analyte is very high (~100 or more). These advantages make the GLH chip an ideal choice for protein-small molecule and protein-protein interaction studies where highest sensitivity is desired. Used with the ProteOn XPR36 protein interaction array system, up to 36 biomolecular interactions can be assayed simultaneously in one experiment, yielding valuable kinetic, concentration, and equilibrium data, and reducing research time from days to hours. The GLH chip is a valuable tool for the lead identification and optimization processes of drug development, as well as areas of fundamental research in protein-small molecule interactions and developmental work in assay optimization.

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