Method for identification of heme-binding proteins and quantification of their interactions

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Abstract

The standard assay for characterization of interaction of heme with proteins is absorbance spectroscopy. However, this approach demands relatively large quantities of proteins and it is difficult to perform in high-throughput manner. Here, we describe an immunosorbent assay based on the covalent *in situ* conjugation of heme to a pre-coated carrier. Advantage of this assay is that it allows both identification of heme-binding proteins and quantification of their binding avidity, using only minimal amounts of protein (1-10 μg). Importantly, the same approach can be used for covalent linkage of other natural or synthetic compounds and analyzing their interactions with proteins.

Keywords: Heme, proteins, antibodies, protein-heme interactions, ELISA, conjugation.

1. Introduction

Heme is an indispensable constituent of hemoproteins (hemoglobin, myoglobin, cytochromes, peroxidases). Heme can also regulate functions of many cellular and plasma proteins by transient interactions [1, 2]. For example, it has been demonstrated that the normal immune repertoire contains antibodies that bind to heme and consequently acquire antigen binding polyreactivity [3, 4]. There is evidence suggesting that heme binds to the variable region of antibodies and serves as an interfacial cofactor for antigen recognition [4, 5]. In order to identify proteins capable of transient interaction with heme, UV-vis absorbance spectroscopy can be applied. This technique allows measurement of interaction between proteins and heme. However, absorbance spectroscopy is not appropriate for screening of large libraries of proteins; it is time-consuming and requires relatively large quantities of protein.

ELISA (enzyme-linked immunosorbent assay) is cost-efficient, high-throughput and technically simple to perform. It is widely used to determine binding of immunoglobulins to protein antigens. Proteins are easily coated on the polystyrene surface. However, being a small molecule (652 Da), heme does not possess sufficient molecular size for appropriate coating on the polystyrene surface. For their efficient immobilization, low molecular weight compounds (haptens) are usually conjugated to carrier proteins. Nonetheless, the preparation and purification of protein conjugates especially with hydrophobic haptens, such as heme, is a complex procedure and may not be routinely implemented in all laboratories.

Here, we describe an alternative experimental technique based on ELISA, where heme is covalently linked *in situ* to a carrier peptide pre-coated on the surface. This technique is fast, cost efficient and can be applied in high-throughput manner. Moreover, it allows the quantification and comparison of the binding avidity of heme-binding proteins. The assay can also be applied for the evaluation of the interaction of proteins with other synthetic or natural heterocyclic compounds.

2. Materials and methods

2.1. Materials

Hemin, Fe(III)-protoporphyrin IX chloride, was obtained from Frontier Scientific, Inc. (Logan, UT). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (Sulfo-NHS) were obtained from Thermo Fisher Scientific (Waltham, MA). DMSO was obtained from Sigma-Aldrich (St. Louis, MO). All chemicals were with the highest available purity. Human recombinant IgG1 antibodies cloned from B cells isolated from synovial tissue of rheumatoid arthritis patients (Ab15, Ab16, Ab17, Ab18, Ab19, Ab20, Ab21, Ab49, Ab56, Ab65, Ab72 and Ab137) were kindly provided by Prof. Claudia Berek (DRFZ, Berlin). Heme-binding antibody Ab21 was thoroughly dialyzed against PBS containing 10% sucrose and stored before use at –20 °C at concentration of 12 mg/mL. Human pooled immunoglobulin G (IVIg, Endobulin, Baxter USA) was dialyzed against PBS and stored before use at –20 °C at concentration of 80 mg/mL. Hemin was dissolved in DMSO to final concentration of 10 mM. Human C1q was obtained from Calbiochem (Burlington, MA). Human hemoglobin was obtained from Sigma-Aldrich (St. Louis, MO). Preparation of apo-hemoglobin (apo-Hb) was done by procedure described in [6].

2.2. Immunosorbent assay for assessment of antibody binding to heme

2.2.1. *In situ* conjugation of heme

Ninety-six-well polystyrene plates (Nunc MaxiSorp) were first coated with 100 µl /well of 0.5 % gelatin in PBS. For facilitating the solubilization of gelatin the solution was heated in water bath to 40-45 °C. After incubation for 1 h at room temperature, plates were washed (3 ×) with water. Solutions of 1 mM heme in solvent mixture DMSO/H_2O = 1/1 was added to the microtitration plate at 50 µl /well. Next either: a) deionized water; b) 60 mM EDC in water, or c) 60 mM EDC and 60 mM Sulfo-NHS in water, were added to the heme-containing wells in the volume ratio 2:1 (i.e. 25 µl /well). The solutions were well homogenized. Plates were incubated at room temperature in dark with a gentle shaking for 2 h. After, the wells were washed (3 ×) with water, then incubated for 5 min with 1M water solution of ethanolamine at pH 8, and again washed (3 ×) with water.

2.2.2. Assessment of binding of proteins to heme-conjugated surface

The residual binding sites on plates were blocked by incubation with PBS containing 0.25% Tween 20. For assessment of the antibody binding, Ab21 and Ab72 were diluted to 100 µg/mL (670 nM) with PBS-T (0.05% Tween 20) and were further serially diluted with PBS-T in the range of 100 − 0.195 µg/mL (670 – 1.3 nM, dilution factor of 2). Antibodies were incubated for 1 h at room temperature with plate coated with heme-modified gelatin. After incubation with the antibody, the plate was washed extensively (5×) with PBS-T and incubated with a HRP-conjugated mouse anti-human IgG (9040-05, clone JDC-10, Southern Biotech, Birmingham, AL) for 1 h at room temperature. Binding of Ab21 was revealed by measuring the absorbance at 492 nm after the addition of peroxidase substrate, *o*-phenylenediamine dihydrochloride (Sigma-Adrich) and stopping the reaction by the addition of 2 M HCl. Measurement of the absorbance was performed with a microplate reader (Infinite 200 Pro, Tecan, Männedorf, Switzerland).

Following sections describe variations of the procedure. Initial and final steps of the experiments are identical to those described above. For the covalent binding of heme to gelatin, activation of 1 mM heme was done with 60 mM EDC, unless stated otherwise.

2.2.3. Analyses of the binding of pooled human IgG

For binding assessment, pooled therapeutic human IgG (IVIg) preparation, was first diluted to 1000 µg/mL (6.7 µM) in PBS-T (0.05% Tween 20) and was further serially diluted in the range

of 1000 − 3.90 μg/mL (6.7 – 0.026 μM, dilution factor of 2). Antibodies were incubated for 1 h at room temperature with plate coated with heme-modified gelatin.

2.2.4. Comparison of antibodies binding to heme

For binding assessment, a set of monoclonal antibodies were diluted to 10 μg/mL (67 nM) with PBS-T (0.05% Tween 20) and incubated (50 μl /well) for 1 h at room temperature with microtiter plate coated with heme-modified gelatin.

2.2.5 Binding of C1q to heme

For binding assessment, C1q was diluted to 50 μg/mL (120 nM) with PBS-T (0.05% Tween 20) and was further serially diluted with PBS-T in the range, of 50 − 0.195 μg/mL (120 - 0.48 nM, dilution factor of 2). C1q was incubated for 1 h at room temperature with the plate coated with heme-modified gelatin. For detection of the binding of human C1q to conjugated heme, we used a sheep anti-human C1q conjugated with HRP (ab46191, Abcam, Cambridge, UK) diluted 1000 × in PBS-T. The plates were incubated with the antibody for 1 h at room temperature.

2.2.6. Binding affinity of apo-hemoglobin

For binding assessment, human apo-hemoglobin was diluted to 100 μg/mL (1.5 μM) with PBS-T (0.05% Tween 20) and was further serially diluted with PBS-T in the range of 100 − 0.39 μg/mL (1.5 – 0.006 μM, dilution factor of 2). Apo-hemoglobin was incubated for 1 h at room temperature with the plate coated with heme-modified gelatin. After washing (5×) with PBS-T, the plates were incubated for 1 h at room temperature with goat polyclonal IgG to human hemoglobin (LS-B13233, LSBio, Seattle WA), diluted 1000 × in PBS-T. The immunoreactivity was detected by incubation with Rabbit F(ab')2 Anti-Goat IgG(H+L)-HRP (6020-05, Southern Biotech) diluted 3000 × in PBS-T.

3. Results and discussion

In order to identify and quantify interactions of proteins with heme, we developed an assay where heme is covalently bound to a carrier peptide coated on the plate surface. Here, we describe a procedure that was refined following testing different experimental conditions. This procedure resulted in the most efficient and reproducible immobilization of heme and detection of heme-binding proteins and therefore we recommend these conditions for analyses of protein-heme interactions.

Gelatin was chosen as carrier because it consists of smaller peptides, obtained by hydrolysis of 300 kDa protein collagen, hence reducing the possibility for non-specific interactions. Moreover, as gelatin represents protein hydrolysate, it does not have any defined tertiary structure that might be compromised by exposure to harsh conditions necessary for the conjugation reaction. Carboxyl groups of heme require chemical activation in order to be coupled with primary amino groups of lysine residues of peptides. Typical carboxyl activating agent EDC activates carboxylic acids by creating a more reactive ester leaving group. Less labile, but still a good leaving group can be formed when EDC is used in the combination with Sulfo-NHS. For the initial optimization of the assay, a human recombinant IgG1 Ab21 was chosen as a well characterized heme-binding antibody [5].

1) *Preparation of binding surface*

Polystyrene 96-well microtitration plates were coated for 1 hour with 0.5 % solution of gelatin in PBS. In order to achieve in situ heme conjugation to the carrier, after washing with deionized water, 1 mM solution of hemin in 50 % DMSO was added, followed by the addition of excess of the activating reagents – final concentration of 60 mM EDC or a mixture of 60 mM EDC and 60 mM Sulfo-NHS and incubation for 2 hours at room temperature. After, the wells were briefly incubated with 1M solution of ethanolamine at pH 8, in order to saturate possibly activated carboxyl groups on gelatin and to dissolve and wash away any non-reacted hemin. After the incubation with ethanolamine, the binding surface was blocked by exposure to 0.25 % Tween 20 in PBS.

2) Assessment of protein-heme interactions

For binding assessment, increasing concentrations of Ab21 were incubated on the hemin-conjugated surface. Covalent coupling of hemin to gelatin was successful, which was indicated by the high intensity of Ab21 binding to gelatin exposed to hemin as compared to native gelatin (Figure 1A). Activation with EDC alone provided better results than the activation with the mixture of EDC and Sulfo-NHS (data not shown). It is possible that the less reactive intermediate formed with Sulfo-NHS requires longer reaction time or an increased temperature to react with primary amino groups from gelatin. The non-linear regression fit of the binding data allowed calculation of the apparent binding affinity (avidity) of the antibody to hemin. Thus, Ab21 recognized immobilized hemin with K_D of 75 ± 16 nM, suggesting that this antibody has relatively high binding avidity for heme.

Since a significant fraction of immunoglobulins in a human immune repertoire has been shown to interact with heme and acquire antigen binding polyreactivity [7, 8], it was expected that pooled IgG preparation obtained from plasma of large number of healthy donors would be able to bind to immobilized heme. To test this assumption, a commercially available pooled IgG preparation was essayed for hemin binding by the assay described here. The pooled human IgG exhibited a considerable heme-binding potential, confirming that normal immunoglobulin repertoire contain a fraction of heme-binding antibodies.

To further confirm that the assay can discriminate between heme-binding and heme-nonbinding monoclonal antibodies, binding of Ab21 to heme-modified gelatin was compared to the binding of another human monoclonal IgG1, Ab72. As can be observed in Figure 1B, the binding of Ab72 to heme was significantly lower than the binding of Ab21 in the same concentration range.

The screening was further expanded to a set of human recombinant IgG1 antibodies that were pre-selected to have or not have potential to interact with heme. All antibodies were screened at a single concentration, 10 μg/mL (0.5 μg/well). The obtained results clearly demonstrated that the assay can well discriminate between the antibodies that were able to bind heme and those that did not (Figure 1C).

3) Validation of assay with apo-hemoglobin and C1q

To further validate the utility of the method for assessing the typical heme-binding proteins, we used apo-hemoglobin i.e. hemoglobin that lacks its prosthetic group. The apo-hemoglobin showed concentration dependent binding to the immobilized hemin (Figure 2A). The estimated apparent K_D value was 182 ± 55 nM. Further, the test was also applied for study of the interaction of non-conventional heme-binding protein – complement C1q [9]. This protein also

demonstrated clear binding to surface immobilized heme with apparent K_D value of 21.4 ± 5.6 nM (Figure 2B). Of note C1q has six identical globular heads that are responsible for target recognition. This can explain the estimated high binding avidity of this protein for immobilized hemin.

4. Concluding remarks

Here, we present a procedure that can be used for identification of heme-binding proteins. The proposed technique is simple, requires low quantities of protein and can be performed in high-throughput manner. In addition, it allows estimation of the binding avidity of the heme-binding proteins. The technique can be coupled with mass spectrometry for identification of high affinity heme-binding proteins in complex mixtures, as for example lysates from eukaryotic cells or bacteria.

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Figure 1

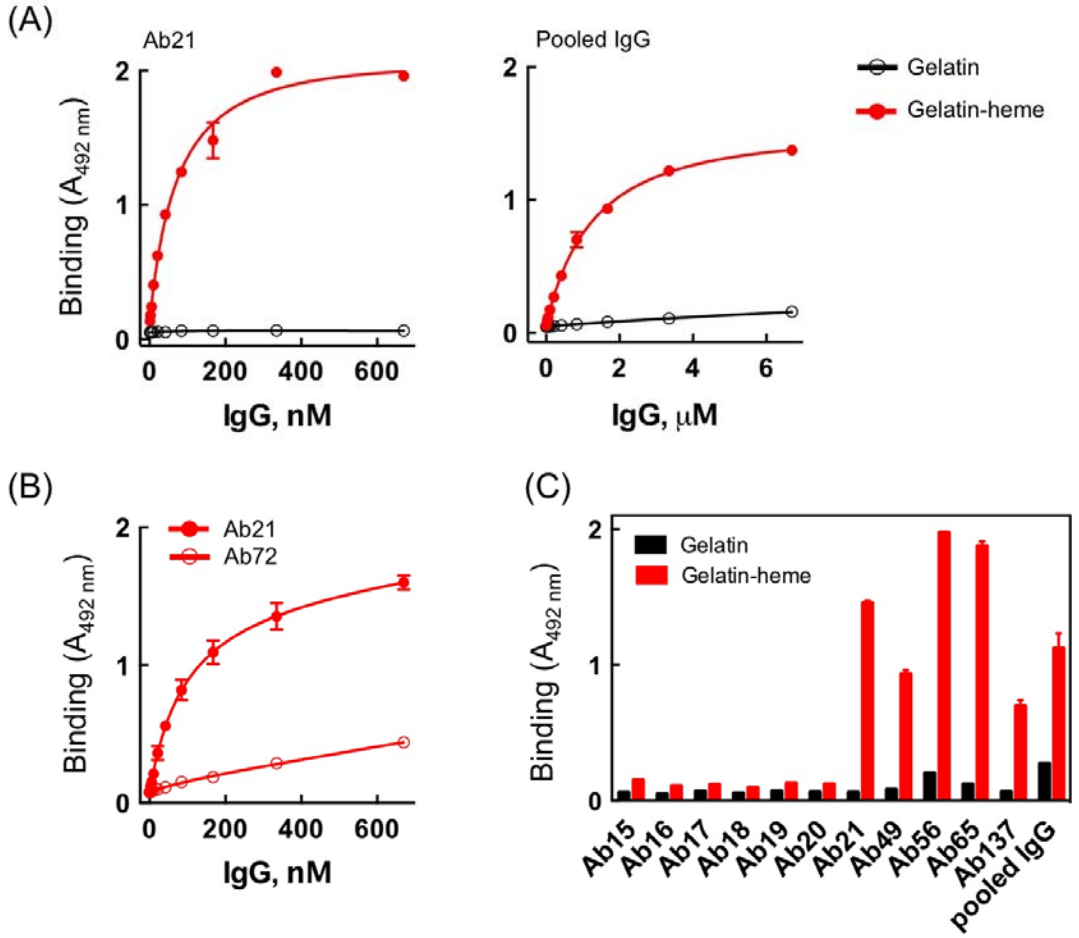

Figure 1. Analyses of interaction of antibodies with immobilized heme. (A) Evaluation of binding of human heme-binding IgG1 Ab21 (left panel) or pooled human IgG1 to *in situ* peptide-conjugated hemin. The binding of antibodies to carrier (gelatin) only was also determined. (B) Comparison of the hemin-binding capacity of increasing concentrations of human recombinant IgG1, Ab21 and Ab72. (C) Comparison of hemin-binding potential of a panel of human recombinant IgG1 antibodies and human pooled IgG. The monoclonal antibodies were tested at 10 μg/mL, pooled IgG was tested at 100 μg/mL. The binding of each antibody to non-conjugated carrier was also assessed. The binding isotherms in panels A and B were obtained by non-linear regression analyses using GraphPad Prisms v.6 software (GraphPad Software, San Diego, CA). The mean binding intensity ±SD (n=3) is presented.

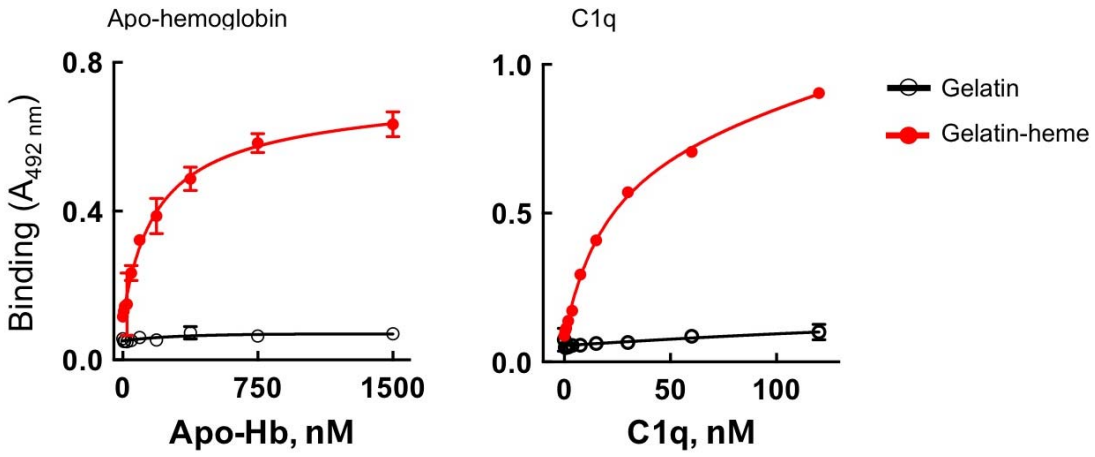

Figure 2. Analyses of interaction of apo-hemoglobin and C1q with immobilized heme. Binding of increasing concentrations of human apo-hemoglobin (A) and of human C1q (B) to hemin-conjugated to gelatin. The binding of the proteins to carrier (gelatin) only was also determined. The binding isotherms in panels A and B were obtained by non-linear regression analyses using GraphPad Prisms v.6 software. The mean binding intensity ±SD (n=3) is presented.