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Mechanism and Functional Implications of the Heme-Induced Binding Promiscuity of IgE

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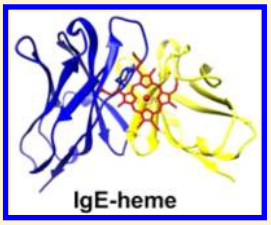
ABSTRACT: A fraction of antibodies from healthy immune repertoires binds to heme and acquires the ability to recognize multiple antigens. The mechanism and functional consequences of heme-mediated antigen binding promiscuity (polyreactivity) are not understood. Here, we used SPE7, a mouse monoclonal IgE specific for dinitrophenyl that has been thoroughly characterized at the molecular level, as a model antibody to elucidate the mechanism and functional consequences of heme-mediated polyreactivity. We first demonstrate that exposure of SPE7 to heme results in a substantial increase in its antigen binding polyreactivity. Comparison of the binding kinetics and thermodynamics of interaction of native and heme-bound SPE7 indicates that the binding of heme to SPE7 confers binding affinities in the low nanomolar range toward several antigens but has no influence on the mechanism of recognition of dinitrophenyl. *In vitro* cellular assays further demonstrate that heme-bound SPE7 does not promote the degranulation of basophils in the presence of new target antigens, while degranulation is observed in the presence of dinitrophenyl. Molecular docking and fluorescence spectroscopy revealed binding of heme to the variable region of SPE7 at a distance from the conventional binding site for dinitrophenyl, explaining the extension of binding polyreactivity without abrogation of the interaction with its cognate antigen. In addition, our data suggest that heme, when bound to IgE, is solvent-exposed and may serve as an interfacial cofactor mediating binding to diverse proteins. This study contributes to the understanding of the mechanisms of heme-induced antibody polyreactivity. It also paves the way toward the delineation of the functional impact of polyreactivity and cross-reactivity of IgE.



Binding and functional promiscuity are widespread phenomena in biology.[1,2] Many proteins are capable of interacting through a single active site with multiple structurally unrelated molecules, or catalyzing diverse reactions. Promiscuous interactions and activities might be essential for the acquisition of novel functions of proteins.[3,4] Besides, binding promiscuity plays an important role in host defense. The immune system must promptly cope with invading pathogens without eliciting at the same time damages to the host. Hence, the potential to evolve exquisite binding specificities for a limited period of time is fundamental for the survival of the organism. Evolution of any exquisite specificity depends, however, on the initial existence of a primordial one.[3,5,6] The molecular diversity of potential antigens that the immune system could encounter is practically unlimited. This diversity outcompetes the sequence diversity of adaptive immune receptors generated by stochastic recombination processes in B and T lymphocytes.[3,7] However, the existence of promiscuous immunoglobulins considerably augments the antigen recognition breadth of preimmune repertoires. Thus, low-affinity multispecific antigen receptors may contribute the necessarily primordial specificities for initiation of an immune response.

The antibodies (Abs) or B-cell receptors that are able to recognize multiple unrelated antigens are also defined as polyreactive or polyspecific.[8−12] They represent >20% of immunoglobulins in healthy immune repertoires, and their frequency could further increase during the course of some infectious or autoimmune diseases.[13−17] In addition to intrinsically polyreactive Abs, normal immune repertoires contain a fraction of Abs that acquire polyreactivity post-translationally. Thus, a brief exposure of these subset of Abs to different protein destabilizing conditions (pH ≤4, chaotropic agents, and high salt concentrations) or to substances released in vivo at sites of inflammation or tissue damage (ROS, heme) results in a transition to antigen binding polyreactivity.[18−25] The biological significance of induced antigen binding polyreactivity is not well-understood, but it may play an important role in the regulation of inflammation.[22,26]

Abs utilize different molecular mechanisms for achieving promiscuous recognition of antigens. Except in the case of polyreactivity mediated by differential epitope positioning, the molecular trait directly associated with antigen binding promiscuity of Abs is the structural dynamics of the antigen-binding sites.[5,7,12,27−31] The structural dynamics could contrib-

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ute in two ways to the polyreactivity of Abs. Some Abs use induced-fit interaction where conformational alterations in the binding site accompany the recognition of the antigen.[3,27,32] Another mechanism of binding heterogeneity is based on conformational selection of Abs, i.e., conformational isomerism. Thus, the antigen-binding sites of some Abs might exist as an ensemble of different structural variants with discrete energies in the absence of antigens.[3,33,34] Each conformer of a single Ab can recognize distinct antigens. The greater structural dynamics of the binding site would allow such an Ab to explore an extended conformational space and thus adapt to a larger number of possible structures. The molecular mechanism of post-translationally induced Ab polyreactivity is not well understood. Exposure to protein-destabilizing agents of susceptible Abs may result in an increase in the structural dynamics or level of disorder of antigen-binding sites.[22] In the case of binding polyreactivity induced by heme, Abs might use this promiscuous molecule as an interfacial cofactor for interacting with different antigens.[23,35]

SPE7 is an extensively studied mouse IgE Ab. It binds to its cognate antigen, dinitrophenyl (DNP), with a high affinity (K_D = 20 nM). SPE7 is polyreactive and also binds other unrelated aromatic compounds with high affinity.[36,37] Interestingly, structural and pre-steady state kinetic analyses revealed that SPE7 utilizes simultaneously two different molecular strategies for promiscuous antigen recognition: induced fit and conformational isomerism.[34] In its antigen-free state, at equilibrium, SPE7 was shown to exist in at least two conformational isomers: one less prevalent isomer with a deep and narrow binding site used for recognition of small aromatic haptens and another more prevalent isomer with a wide and shallow binding site for recognition of protein antigens.[34] Thus, the structural dynamics of its variable regions allows SPE7 to bind to completely different types of antigens.[34,36,37] Moreover, the less frequent isomer of SPE7 uses the induced-fit binding mode for promiscuous binding of distinct aromatic ligands. These unique molecular features and the available structural, kinetic, and functional data make SPE7 a valuable model for investigations of the mechanisms of binding promiscuity.

Here, we used SPE7 to study the mechanism and functional consequences of cofactor-induced promiscuity of Abs. We demonstrate that SPE7 further extends its intrinsic polyreactivity upon interaction with heme. The binding of heme to SPE7 did not influence the kinetics or thermodynamics of binding to its cognate antigen. The cofactor-induced binding promiscuity did not also impact the functional activity of SPE7. *In silico* molecular docking and fluorescence spectroscopy indicate that heme binds to a region of the antigen-binding site that is distant from the binding site for aromatic compounds and suggest that heme is used as an interfacial cofactor for contacting other proteins.

EXPERIMENTAL PROCEDURES

Treatment of SPE7 with Heme. IgE monoclonal anti-dinitrophenyl Ab produced in mouse, clone SPE7 (Sigma-Aldrich, St. Louis, MO), was dialyzed overnight at 4 °C against phosphate-buffered saline (PBS). The hematin stock solution was prepared by dissolving hemin dry substance (Fluka, St. Louis, MO) in 0.05 M NaOH. Unless noted otherwise, the treatment of IgE was performed at a protein concentration of 100 μg/mL (0.56 μM) with 10 μM heme for 5 min on ice, after which the antibody was diluted to the desired working concentration. A solution of NaOH (0.05N) without heme was added to SPE7 as a negative control.

Immunoblot Analysis. The binding of native and heme-treated SPE7 to a panel of antigens present in a lysate of *Bacillus anthracis* was studied by the immunoblot technique. The lysates were subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) using one-well NuPAGE Bis-Tris 4 to 12% gradient gels (Invitrogen, Life technologies, Novex). The gels were loaded with a total protein amount of 100 μg. After electrophoretic separation, the proteins were transferred onto nitrocellulose membranes using an *Invitrogen iBlot* system (Invitrogen). Following overnight blocking at 4 °C with TBS containing 0.1% Tween 20 (TBS-T), the membranes were mounted on a 28-channel miniblot system (Immunetics, Cambridge, MA) and incubated with increasing concentrations (0, 1.56−50 μg/mL) of native or heme-treated SPE7 antibody diluted in TBS-T buffer for 2 h at room temperature under gentle agitation. The membranes were washed and then incubated with secondary rabbit anti-mouse IgG H/L chain antibody (1:1000) coupled to alkaline phosphatase (Southern Biotech, Birmingham, AL). Immunoreactivities were revealed using the substrates 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (both from Sigma-Aldrich) diluted in a buffer containing 100 mM Tris base, 100 mM NaCl, and 5 mM MgCl (pH 9.5).

Enzyme-Linked Immunosorbent Assay (ELISA). Polystyrene 96-well ELISA plates (Nunc Maxisorp, Roskilde, Denmark) were coated with 10 μg/mL 2,4-dinitrophenyl-albumin (DNP-BSA) (Sigma-Aldrich) in PBS. The residual binding sites were blocked by incubation with PBS containing 0.25% Tween 20 for 1.30 h at 22 °C. After being washed, the plates were incubated with increasing concentrations (0, 0.013−6.7 μg/mL) of native and heme-treated SPE7 diluted in PBS and 0.05% Tween 20 (PBS-T) for 2 h at room temperature. The treatment of the Ab was performed at a concentration of 40 μg/mL (0.22 μM) in PBS with 5 μM heme. After being washed, the plates were incubated with the secondary goat anti-mouse IgE heavy chain antibody (1:2000) coupled to horseradish peroxidase (HRP) (AbD Serotec) for 1 h at room temperature. Immunoreactivity was revealed by measuring the absorbance at 492 nm using the substrate *o*-phenylenediamine dihydrochloride (Sigma-Aldrich).

Kinetic Analyses. Surface plasmon resonance-based kinetic analyses were performed with the Biacore 2000 system (GE Healthcare Biacore, Uppsala, Sweden). SPE7, DNP-BSA, and Asp f 1 (allergen from *Aspergillus fumigatus*) were immobilized by amino coupling procedure on research grade CM5 sensor chips (GE Healthcare, Biacore). Briefly, the carboxymethylated dextran surface of the chip was activated by injection of a mixture containing 50 mM *N*-hydroxysuccinimide and 200 mM *N*-ethyl-*N*′-dimethylaminopropyl carbodiimide (GE Healthcare Biacore). For immobilization on the activated surface, SPE7 was diluted to a final concentration of 56 nM in 5 mM maleate (pH 5), DNP-BSA was diluted to 496 nM in 10 mM glycine HCl buffer (pH 2.5), and Asp f 1 was diluted to 576 nM in maleate (pH 5). The protein were injected at a flow rate of 10 μL/min. The achieved immobilization levels for SPE7, DNP-BSA, and Asp f 1 were 1270, 200, and 2615 resonance units (RU), respectively. Running buffer used during the immobilization and binding analyses was HBS-EP [10 mM HEPES (pH 7.2), 150 mM NaCl, 3 mM EDTA, and 0.005% Tween 20] that had been filtered through a 0.22 μM filter and degassed under

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vacuum. The free activated carboxyl groups were blocked by injecting 1 M ethanolamine-HCl for 4 min.

To evaluate the binding kinetics of the interaction of SPE7 with its cognate antigen and the kinetics of homophilic binding, native and heme-exposed SPE7 were serially diluted (2-fold each step) in HBS-EP to concentrations ranging from 13.75 to 0.027 nM and injected over immobilized DNP-BSA or SPE7. The flow rate during injection of SPE7 was 10 μL/min. The association and dissociation phases of the interaction were monitored for 4 and 5 min, respectively. For regeneration of the sensor chip surface, a solution containing 0.5 M NaCl and 0.025 M NaOH was injected for 30 s. These measurements were performed at 20, 25, 30, and 35 °C. To estimate the kinetic parameters that characterize the interaction of native and heme-exposed SPE7 with Asp f 1, the IgE was serially diluted to concentrations ranging from 50 to 0.098 nM and injected over the immobilized protein. The flow rate was set to 20 μL/min. Association and dissociation phases were both monitored for 5 min. The surface of the sensor chip was regenerated by injection of a solution of 300 mM imidazole. The interaction analyses were performed at 25 °C. The kinetics of interaction of heme with immobilized SPE7 was evaluated by injection of freshly prepared heme solutions (dilutions ranging from 1 to 0.0078 μM) in HBS-EP. Association and dissociation phases of the interaction were monitored for 4 and 5 min, respectively. The flow rate during binding of heme to SPE7 was set at 30 μL/min. The regeneration of the sensor surface was performed by injection of 300 mM imidazole. The measurements were performed at 25 °C.

The evaluation of the kinetic data was performed with BIAevaluation version 4.1.1 (Biacore) by applying global analyses to the experimental data.

Evaluation of Binding Thermodynamics. We applied Eyring's approach for evaluation of the thermodynamic parameters of the interactions of native and heme-exposed SPE7 with DNP-BSA and its homophilic interaction. For evaluation of the thermodynamic parameters, the kinetic rate constants obtained at different temperatures were used to build Arrhenius plots. The values of slopes of the Arrhenius plots were calculated by using a linear regression analysis by using GraphPad Prism version 5 (GraphPad Prism Inc., La Jolla, CA) and substituted in the following equations:

$$E_a = -\text{slope} \times R$$

where slope = $\partial \ln(k_{a/d})/\partial(1/T)$ and E_a is the activation energy. The changes in enthalpy, entropy, and Gibbs free energy, characterizing the association and dissociation phases, were calculated using the following equations:

$$\Delta H^{\ddagger} = E_a - RT$$

$$\ln(k_{a/d}/T) = -\Delta H^{\ddagger}/RT + \Delta S^{\ddagger}/R + \ln(k'/h)$$

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$$

where T is the temperature in kelvin, k' is Boltzmann's constant, and h is Planck's constant.

The equilibrium values of the thermodynamic parameters were calculated using the equations

$$\Delta G_{eq} = \Delta G^{\ddagger}_a - \Delta G^{\ddagger}_d$$

$$\Delta H_{eq} = \Delta H^{\ddagger}_a - \Delta H^{\ddagger}_d$$

$$T\Delta S_{eq} = T\Delta S^{\ddagger}_a - T\Delta S^{\ddagger}_d$$

All thermodynamic parameters were determined at a reference temperature of 25 °C (298.15 K).

Molecular Docking of Heme to scFv of SPE7. The atomic coordinates of both antigen-free conformational isomers of SPE7 [Ab1, Protein Data Bank (PDB) entry 1OAQ; Ab2, PDB entry, 1OCW] were used for molecular docking. The Hex Protein Docking server[38] (www.hexserver.loria.fr) was used to accommodate the heme molecule with the scFv of SPE7. The server uses a spherical polar Fourier transform-based protein docking algorithm with rotational correlations, which reduces dramatically the execution times of the docking run to several minutes.[38] The advantages of this software compared to the other docking servers are the speed (returns results in a few minutes, compared to hours or days for the other servers) and, most importantly for this study, the possibility of docking nonprotein ligands (such as heme) to a protein molecule. The Hex server has been validated in the CAPRI blind docking experiment, and the prediction has often been found within the top 100 orientations in CAPRI scoring sections.[38,39] SPE7 and heme were considered as rigid-body structures. The atomic coordinates of six different heme molecules from PDB entries of different hemoproteins (three forms of hemoglobin, myoglobin, IsdI, and nitrophorin) were used for docking. They accounted for both planar and ruffled heme conformations. One hundred structures were generated in each docking run. We have analyzed in detail the top energy structures, proposed by the Hex server (between 11 and 20 complexes, depending on the SPE7 conformer and the heme molecule used). The criteria for the selection of final scFv–heme complexes were based on the total energy of binding and frequency of hits of the binding site. The top docking results of the six different heme molecules were taken into account. All complexes, in which heme docked at the plane of the antibody fragment, that are occupied by the constant immunoglobulin domain (when the antibody is intact) were excluded, irrespective of their energy scores. We used UCSF Chimera version 1.9 for visualization of the most probable molecular complexes.

Fluorescence Spectroscopy. Quenching of tryptophan fluorescence of SPE7 by heme was analyzed with a Hitachi F-2500 fluorescence spectrophotometer (Hitachi Instruments Inc., Wokingham, U.K.). SPE7 was diluted to 100 nM in PBS (pH 7.4). The emission spectra of tryptophans after excitation at 295 nm were recorded in 1 cm quartz cells. The excitation and emission slits were 10 nm. The spectra were recorded in the wavelength range of 300–450 nm with a scan speed of 300 nm/min. Aliquots of hematin stock solutions were added directly to the cuvette containing SPE7, resulting in final concentrations of 100, 200, and 400 nM. After intensive homogenization and incubation for 3 min in dark, the emission spectra were recorded. All measurements were performed at 22 °C.

IgE-Mediated Cell Degranulation Assay. The RBL-9 2H3 cell line was used for evaluation of the ability of the native and heme-treated SPE7 antibody to sensitize basophils for degranulation after allergen challenge. Cells were grown in Lonza RPMI 1640 with Ultraglutamine 1 medium supplemented with 5% heat-inactivated fetal bovine serum (Gibco, Life Technologies) and 1% penicillin/streptomycin (Gibco, Life Technologies). IgE-mediated degranulation of the cells was assayed by estimating the release of the enzyme β-

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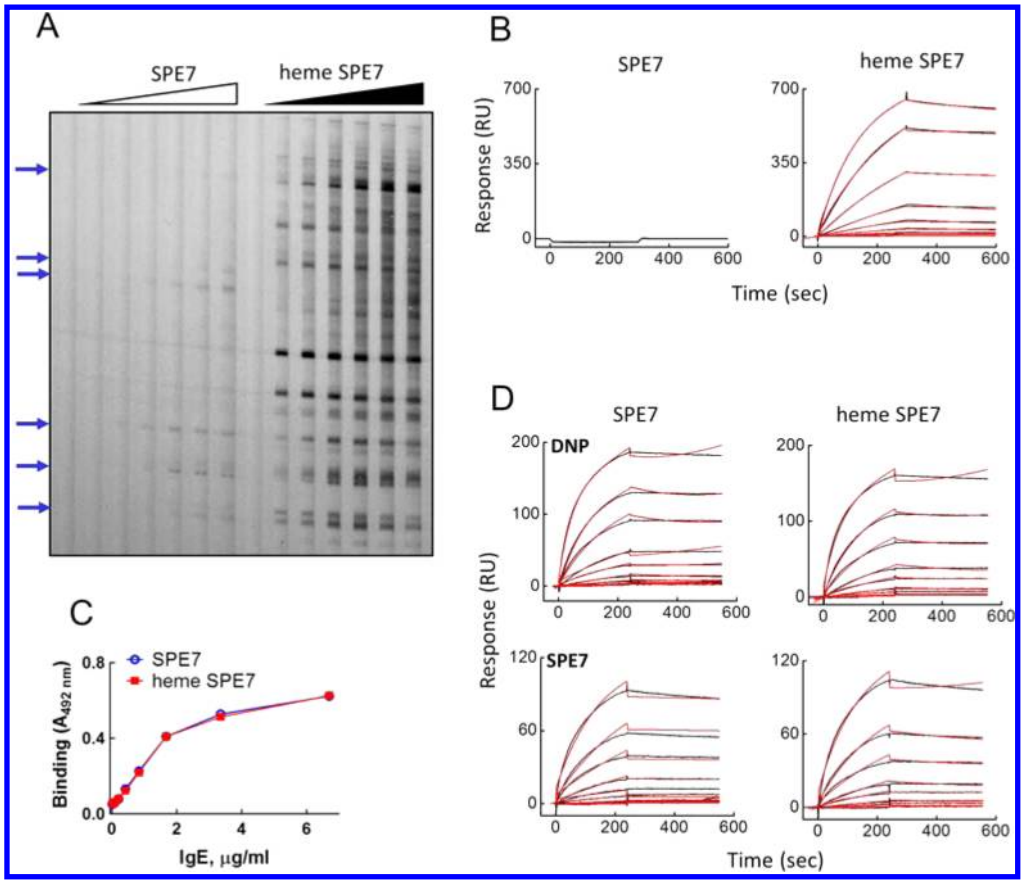

Figure 1. Antigen binding analyses of native and heme-exposed SPE7. (A) Immunoblot analyses of binding of native and heme-exposed SPE7 to proteins from *B. anthracis*. SPE7 at 100 μg/mL (550 nM) was exposed to 10 μM heme. The native Ab and heme-exposed Ab were diluted in TBS-T and incubated with a nitrocellulose membrane at concentrations of 0, 1.563, 3.125, 6.25, 12.5, 25, and 50 μg/mL. Blue arrows indicate the antigens that are recognized by native SPE7. (B). Real-time interaction profiles of binding of native or heme-exposed SPE7 to immobilized Asp f 1. The profile obtained after injection of native SPE7 diluted to 50 nM is presented in the left panel. The binding profiles of heme-exposed SPE7 at concentrations of 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.390, 0.195, and 0.098 nM are presented in the right panel. The kinetic analyses were performed at 25 °C. The graph represents experimentally obtained binding curves (black lines) and curves generated by globally fitting the data to the model of Langmuir binding with a drifting baseline by BIAevaluation (red line). Obtained kinetic parameters are listed in Table 1. (C). ELISA analyses of binding of increasing concentrations (0 and 0.013−6.7 μg/mL) of native (blue line and circles) and heme-exposed SPE7 (red line and squares) to immobilized DNP-BSA. (D) Real-time interaction profiles of binding of native or heme-exposed SPE7 to immobilized DNP-BSA (top) and immobilized SPE7 (bottom). The profiles obtained after injection of native SPE7 (from 13.75 to 0.027 nM, 2-fold dilutions) are presented in the left panel. The binding profiles of heme-exposed SPE7 at the same concentrations are presented in the right panel. The kinetic analyses were performed at 25 °C. The graph represents experimentally obtained binding curves (black lines) and curves generated by globally fitting the data to the model of Langmuir binding with a drifting baseline by BIAevaluation (red line). Obtained kinetic parameters are listed in Table 1.

hexosaminidase in the medium. Cells were seeded in 96-well plates at a density of 100000 cells/well in their complete grown medium. The next day, they were washed twice with Tyrode's buffer [130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, and 10 mM HEPES (pH 7.4)] and then sensitized with the SPE7 diluted in the same buffer for 30 min or 2 h for the antigen- or self-induced degranulation, respectively. Twofold dilutions of the SPE7 were used with a highest concentration of 312.5 ng/mL for the antigen-induced degranulation and 10 μg/mL for the self-induced degranulation. In the case of antigen-induced degranulation, the antigen DNP-BSA (10 ng/mL in Tyrode's buffer) was added to the plates for 15 min. Following stimulation with DNP-BSA or only IgE, 30 μL of the supernatants was transferred to 96-well ELISA plates. The supernatants were then incubated for 1 h at 37 °C with 50 μL of a substrate solution [1.3 mg/mL *p*-nitrophenyl *N*-acetyl-D-glucoseaminide (Sigma-Aldrich), dissolved in citric acid buffer (pH 4.5)]. The enzyme reaction was stopped by adding 80 μL of 0.5 M NaOH to each well, and the reaction product was measured at 405 nm. It was determined that maximal degranulation is achieved after cell sensitization with 160 ng/mL IgE and activation with 10 ng/mL DNP. Such conditions were used as a positive control in all the experiments. In the case of induction of degranulation by another noncognate antigen (Asp f 1),[40] cells were sensitized with 160 ng/mL IgE and then activated with 2-fold dilutions of Asp f 1, starting at 100 μg/mL.

RESULTS

Heme Extends Antigen Binding Polyreactivity of SPE7. SPE7 is a polyreactive mouse monoclonal IgE that interacts with aromatic and heterocyclic ligands as well as proteins.[34,36,37] Heme is a heterocyclic cofactor that has been demonstrated to bind to some Igs and to induce promiscuous antigen binding activity.[23,35] To study whether heme influences the binding characteristics of SPE7, we treated the Ab with the cofactor and assessed its antigen binding characteristics. In accordance with its intrinsic polyreactivity, native SPE7 at ≤50

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Table 1. Kinetic Constants of the Interaction of Native and Heme-Exposed SPE7 with Immobilized Asp f 1, DNP, and SPE7

	native SPE7					heme-exposed SPE7				
	k_a (M^{-1} s^{-1})	SE	k_d (s^{-1})	SE	K_D (nM)	k_a (M^{-1} s^{-1})	SE	k_d (s^{-1})	SE	K_D (nM)
Asp f 1						1.46×10^5	581	6.96×10^{-4}	4.34×10^{-5}	4.78
DNP	1.45×10^6	1.4×10^4	2.55×10^{-3}	2.55×10^{-3}	1.75	1.12×10^6	1.22×10^4	2.12×10^{-3}	2.12×10^{-3}	1.89
SPE7	8.42×10^5	9.76×10^3	2.09×10^{-3}	2.09×10^{-3}	2.48	6.18×10^5	9.32×10^3	1.3×10^{-3}	1.3×10^{-3}	2.11

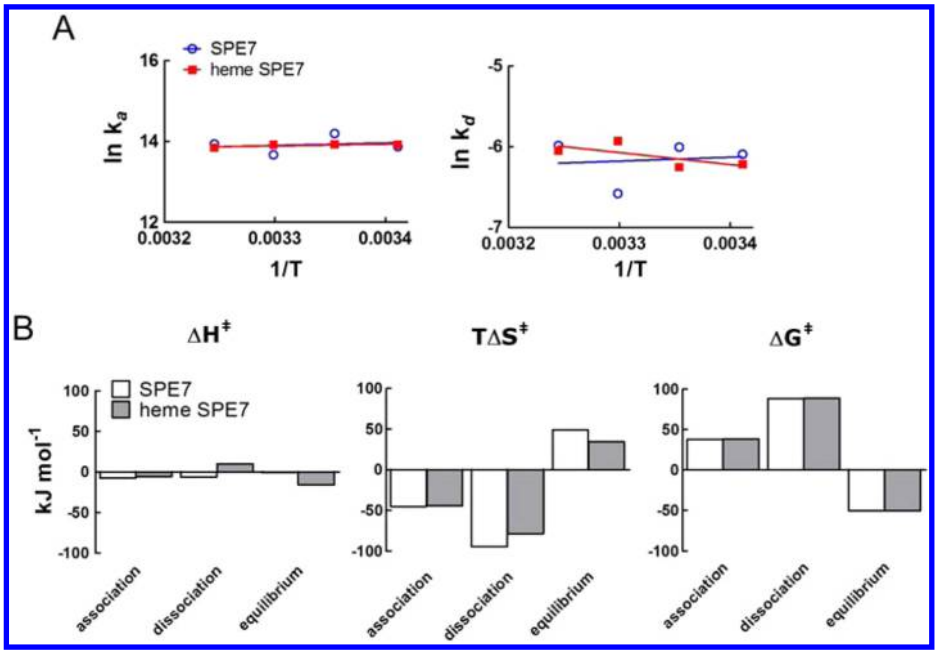

Figure 2. Binding thermodynamics of the interaction of SPE7 with DNP-BSA. (A) Arrhenius plots depicting the temperature dependency of association and dissociation rate constants of native SPE7 (blue circles and lines) and heme-exposed SPE7 (red squares and lines) obtained after interaction with DNP-BSA. The kinetic rate constants were determined by global analysis of sensorgrams generated after evaluation of binding kinetics of SPE7 at varying temperatures (20, 25, 30, and 35 °C). The slopes of the Arrhenius plots were determined by linear regression analyses with GraphPad version 5. (B) Activation and equilibrium thermodynamic parameters of binding of native and heme-exposed SPE7 to immobilized DNP-BSA. Changes in the enthalpy (left), entropy (middle), and Gibbs free energy (right) during the association and dissociation phases as well as at equilibrium of native SPE7 (white bars) and heme-exposed SPE7 (gray bars) with DNP-BSA. The changes in the thermodynamic parameters were evaluated by applying Eyring's analyses to the data from Arrhenius plots. All thermodynamic parameters were determined using a reference temperature of 298.15 K.

μg/mL showed reactivity with several bacterial antigens (Figure 1A). However, its contact with heme resulted in a marked additional increase in the number of recognized antigens. Interestingly, the contact with heme also resulted in an increase in the level of binding to some bacterial proteins that are recognized also by the Ab in its native form (Figure 1A). The heme-induced acquisition of novel antigen binding specificities of SPE7 was further confirmed by surface plasmon resonance (SPR)-based analyses. Incubation of SPE7 with heme resulted in binding to an immobilized allergen from *Aspergillus*, Asp f 1 (Figure 1B), with a high apparent binding affinity [K_D value of 4.78 nM (Table 1)]. Native SPE7 did not interact with Asp f 1 (Figure 1B). A high binding affinity (K_D in the low nanomolar range) was also measured for the binding of SPE7-heme to other proteins, Factor H and thyroglobulin (data not shown).

Next, we studied whether cofactor-mediated polyreactivity affects the binding of SPE7 to its cognate antigen, DNP. While inducing novel antigen binding specificities against foreign antigens (Figure 1A), heme treatment did not influence the binding of SPE7 to DNP, which is evident from ELISA and SPR analyses (Figure 1C,D). Kinetic analyses indicated that both native SPE7 and heme-induced polyreactive SPE7 recognize immobilized DNP-BSA with practically identical values of binding affinity (K_D values of 1.75 and 1.89 nM, respectively). This result suggests that the polyreactivity of IgE induced by heme is not associated with a change in the binding affinity for its cognate antigen DNP. The higher values of binding affinity obtained in this study, compared with values observed by solution-based kinetic assays for the interaction of DNP-Ser with SPE7 (K_D value of 20 nM),[34] might reflect the effect of the hapten carrier, surface immobilization of the ligand, or a contribution of Ab avidity.

Some of the biological effects of SPE7 have been hypothetically attributed to its tendency to interact homophilically.[41,42] Indeed, soluble SPE7 bound to immobilized SPE7 with a high apparent affinity (K_D value of 2.48 nM). The increase in the level of promiscuity of the IgE following heme exposure, however, did not influence the tendency for self-binding activity of the Ab (Figure 1D). The estimated K_D value of heme-exposed SPE7 in this interaction was 2.11 nM.

Heme-Induced Polyreactivity Does Not Influence the Kinetics or Thermodynamics of Interactions of SPE7. To improve our understanding of the effect of heme on antigen recognition by SPE7, we performed kinetic and thermodynamic analyses. The association of SPE7 with its cognate antigen at 25 °C was characterized by a high apparent bimolecular

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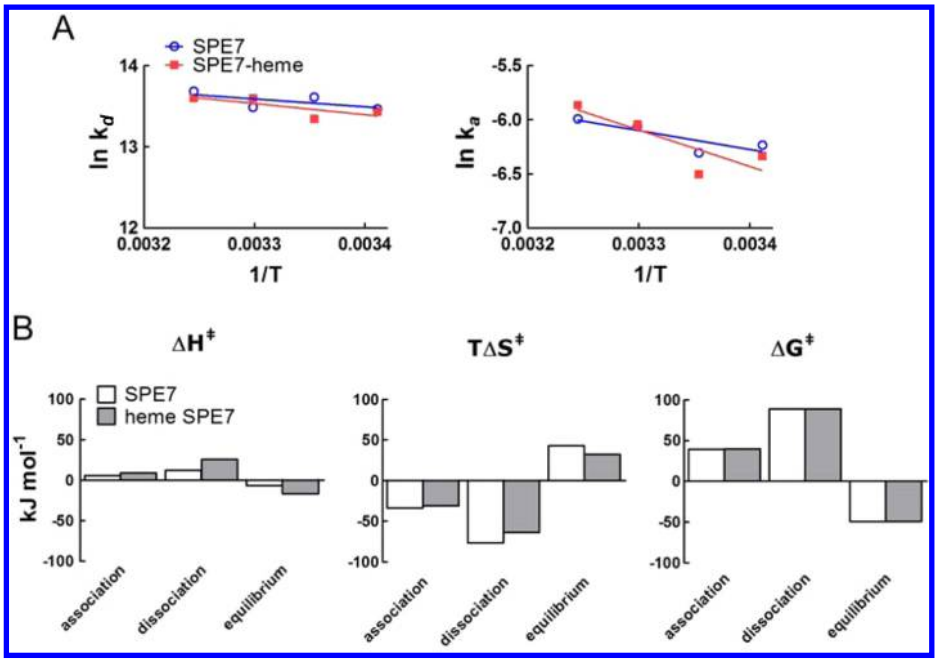

Figure 3. Binding thermodynamics of homophilic interaction of SPE7. (A) Arrhenius plots depicting the temperature dependence of association and dissociation rate constants of native SPE7 (blue circles and lines) and heme-exposed SPE7 (red squares and lines) obtained after interaction with immobilized SPE7. The kinetic rate constants were determined by global analysis of sensorgrams generated after evaluation of binding kinetics of SPE7 at varying temperatures (20, 25, 30, and 35 °C). The slopes of the Arrhenius plots were determined by linear regression analyses with GraphPad version 5. (B) Activation and equilibrium thermodynamic parameters of binding of native and heme-exposed soluble SPE7 to immobilized SPE7. Changes in the enthalpy (left), entropy (middle), and Gibbs free energy (right) during the association and dissociation phases as well as at equilibrium of native SPE7 (white bars) and heme-exposed SPE7 (gray bars) to DNP-BSA. The changes in the thermodynamic parameters were evaluated by applying Eyring's analyses to the data from Arrhenius plots. All thermodynamic parameters were determined by using a reference temperature of 298.15 K.

Table 2. Changes in Enthalpy (ΔH), Entropy ($T\Delta S$), and Free Energy (ΔG) during Interaction of SPE7 with DNP-BSA and Homophilic Binding

	native SPE7			heme-exposed SPE7		
	ΔH (kJ mol^{-1})	$T\Delta S$ (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔH (kJ mol^{-1})	$T\Delta S$ (kJ mol^{-1})	ΔG (kJ mol^{-1})
association						
DNP	−7.5	−45.3	37.8	−5.8	−44.3	38.5
SPE7	5.6	−33.7	39.2	8.9	−30.9	39.9
dissociation						
DNP	−6.5	−94.4	87.9	9.9	−78.6	88.5
SPE7	12.1	−76.5	88.6	25.6	−63.5	89.1
equilibrium						
DNP	−0.97	49.1	−50.1	−15.7	34.3	−50.0
SPE7	−6.5	42.9	−49.4	−16.6	32.6	−49.2

association rate constant [i.e., k_a = 1.45 × 10^6 M^{-1} s^{-1} (Table 1)]. The dissociation of SPE7 from immobilized DNP-BSA was relatively fast with a k_d value of 2.55 × 10^{-3} s^{-1}. The increase in antigen binding polyreactivity of SPE7 following exposure to heme did not significantly impact the rate constants of association and dissociation (Table 1). Thus, heme-treated SPE7 associated with DNP-BSA with an apparent k_a value of 1.12 × 10^6 M^{-1} s^{-1} and dissociated with a k_d value of 2.12 × 10^{-3} s^{-1}. The homophilic interaction of IgE was characterized by an ∼2-fold slower association rate and a similar dissociation rate (k_a of 8.42 × 10^5 M^{-1} s^{-1} and k_d 2.09 × 10^{-3} s^{-1}, respectively). Again, the extension of the antigen binding polyreactivity by exposure to heme did not significantly influence the kinetic parameters of self-binding (Table 1). We next studied the binding kinetics of native and heme-exposed IgE with DNP-BSA as a function of temperature. The kinetic rate constants of the binding of native and heme-exposed SPE7 to DNP-BSA were not affected by changes in the temperature of interaction (Figure 2A). The variation of the temperature had an only negligible effect on the rate constants characterizing the homophilic binding of SPE7 (Figure 3A). Thus, an increase in temperature from 20 to 35 °C was associated with an increase in both the rates of association and dissociation (Figure 3A).

Arrhenius plots (Figures 2A and 3A) and Eyring's analyses yielded the values of the thermodynamic parameters that characterize the interactions of native and heme-exposed SPE7 (Figures 2B and 3B and Table 2). The association of SPE7 with DNP-BSA was characterized by minor exothermic changes in the enthalpy that were not significantly affected with an increase in the antigen binding polyreactivity (Figure 2B and Table 2). The association entropy of binding to DNP-BSA was

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also not significantly modified after exposure of the Ab to heme (Figure 2B and Table 2). The additional extension of binding promiscuity of SPE7 after its exposure to heme did not influence the change in activation free energy either (Table 2). Although the values of dissociation $\Delta H^{\ddagger}$ differed qualitatively (−6.5 and 9.9 kJ mol^{-1} for native and heme-exposed IgE, respectively), this difference exists because the deviation from linearity of values of k_d determined at 25 °C (Figure 2A) and could not be attributed to heme-induced polyreactivity. The dissociation $T\Delta S^{\ddagger}$ and $\Delta G^{\ddagger}$ values were also not significantly impacted by induction of polyreactivity of SPE7 (Figure 2B and Table 2). Our analyses indicated that, at equilibrium, the binding of native SPE7 to DNP-BSA is an entropy-driven process ($T\Delta S$ = 49.1 kJ mol^{-1}) with only minimal contribution for the equilibrium ΔG of enthalpy changes (ΔH = −0.9 kJ mol^{-1}). Likewise, the interaction of heme-exposed IgE was also an entropy-driven process ($T\Delta S$ = 34.3 kJ mol^{-1}) with minimal contribution of favorable enthalpy changes (ΔH = −15.7 kJ mol^{-1}) (Table 2). The equal values of equilibrium ΔG detected for native and heme-exposed SPE7 upon binding to DNP-BSA (Table 2) are in full agreement with the identical values of the binding affinity obtained by kinetic analyses (Table 1).

We next evaluated the thermodynamic parameters that characterize the homophilic binding of soluble SPE7 with surface-immobilized SPE7 (Figure 3B and Table 2). The exposure of SPE7 to heme resulted in a minimal increase in the changes in activation enthalpy. During the homophilic association of SPE7, the entropy change was not significantly modified following exposure of SPE7 to heme (Figure 3B and Table 2). These data explain the absence of a difference in the energetic barrier for homophilic association of SPE7 (Table 2). During the dissociation phase of the interaction, a small change in enthalpy was detected, which was qualitatively different from the change during the dissociation from DNP-BSA (Figures 2B and 3B and Table 2). Heme-exposed SPE7 had a 2-fold increase in $\Delta H^{\ddagger}$. As seen for the dissociation from DNP-BSA, the homophilic dissociation of SPE7 had a negative value of entropy (Table 2). This value was reduced upon induction of polyreactivity (Table 2). The changes in the free energy of dissociation for self-binding of SPE7 were qualitatively identical to those observed for the interaction with DNP-BSA, and they did not differ between native and heme-exposed Ab (Figures 2B and 3B and Table 2). The equilibrium thermodynamics parameters indicate that the homophilic interaction of SPE7 is an entropy-driven process ($T\Delta S$ = 42.9 kJ mol^{-1}) with a small favorable contribution of enthalpy (ΔH = −6.5 kJ mol^{-1}). Thus, the mechanism of self-binding of SPE7 did not differ qualitatively from the mechanism of recognition of the cognate antigen (Figures 2B and 3B and Table 2). The augmentation of the antigen binding polyreactivity of SPE7 upon exposure to heme resulted in only minimal differences in the equilibrium thermodynamics parameters (Figure 3B). These differences were similar to those observed in the case of interaction of SPE7 with DNP-BSA (Figure 2B). Finally, almost identical values of equilibrium free energy changes were determined in the case of native and heme-exposed IgE (ΔG values of −49.4 and 49.2 kJ mol^{-1}, respectively).

Heme Binds to SPE7. Previous studies have demonstrated that induction of polyreactivity of Abs by heme is associated with direct binding of the cofactor molecule to immunoglobulins.[22,35] As SPE7 is shown to interact with various aromatic and heterocyclic compounds in addition to DNP, we studied whether SPE7 directly binds heme. To this end, first we applied a SPR-based assay. Injection of heme over immobilized SPE7 resulted in a dose-dependent increase in the magnitude of the resonance signal (Figure 4A), indicating an interaction of the

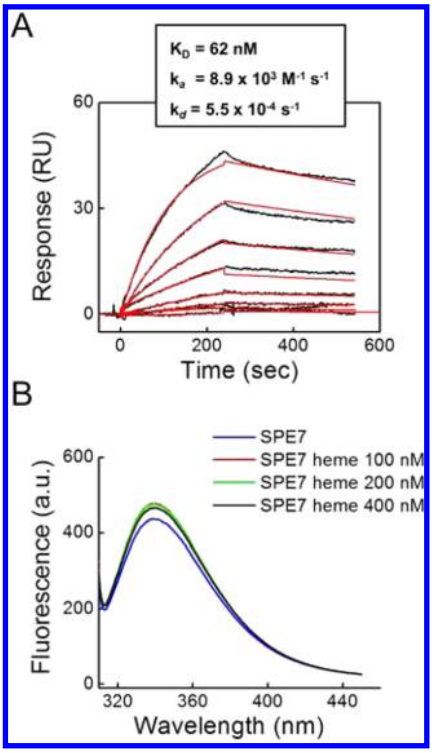

Figure 4. Binding of heme to SPE7. (A) Real-time interaction profiles of the binding of heme to immobilized SPE7. Heme (hematin) was injected at increasing concentrations (0.0078, 0.0156, 0.0312, 0.0625, 0.125, 0.25, 0.5, and 1 μM) over SPE7 immobilized on a sensor chip surface. Each heme dilution was prepared immediately before injection in HBS-EP buffer. The graph represents experimentally obtained binding curves (black lines) and curves generated by global analyses by a Langmuir binding model, included in BIAevaluation (red line). (B) Fluorescence spectroscopy analyses of binding of heme to SPE7. Fluorescence spectra of SPE7 (100 nM in PBS) were recorded after addition of increasing concentrations of hematin (0, 100, 200, and 400 nM). The graph shows the emission spectra after excitation of tryptophan residues in the antibody at a wavelength of 295 nm.

compound with the Ab. Results from global kinetic analyses of binding data indicate that heme associated with SPE7 with a moderate rate (k_a of 8.9 × 10^3 M^{-1} s^{-1}) and dissociated relatively slowly from the Ab (k_d of 5.5 × 10^{-4} s^{-1}). The apparent binding affinity was relatively high with a K_D value of 62 nM. This value is close to K_D values for high-affinity binding of SPE7 to aromatic ligands. The absence of an effect of heme binding on the kinetics and thermodynamics of binding of SPE7 to DNP suggests that heme binding occurs at a site different from the binding sites for aromatic ligands.

To predict the most probable binding site of heme on SPE7, we performed molecular docking analyses with the Hex Protein Docking server. The docking analyses were performed by using atomic coordinates of both conformers of the variable regions of SPE7: Ab1, protein-binding conformer (PDB entry 1OAQ); Ab2, DNP-binding conformer (PDB entry 1OCW). Four binding areas, each accommodating heme molecules with slightly different orientations, were found in the case of Ab1. In contrast, the binding of heme to Ab2 was clustered to only one area. In Figure 5 are depicted examples of complexes from the

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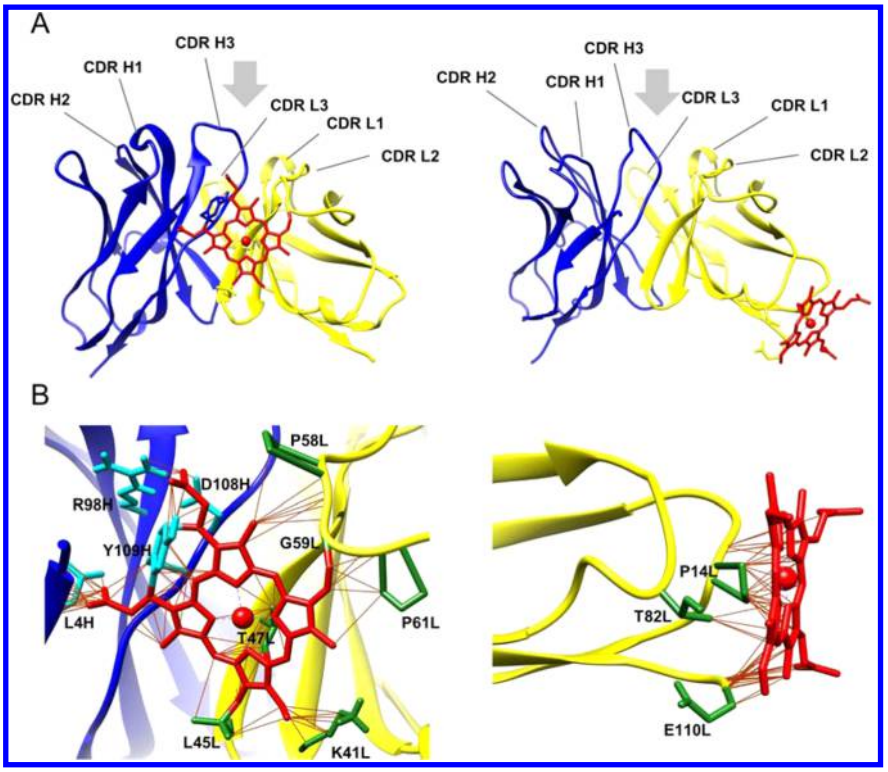

Figure 5. Most probable binding sites for heme on the variable region of SPE7. (A) Molecular model of the complex of heme with the scFv of the protein-binding isomer (PDB entry 1OAQ, left) and DNP-binding isomer of SPE7 (PDB entry 1OCW, right). (B) Close-up of the predicted heme-binding sites on protein-binding (left) and DNP-binding (right) isomers of SPE7. The amino acid residues that establish contacts with heme are presented as sticks (cyan and green for heavy and light immunoglobulin chains, respectively). The molecular contacts are depicted with brown lines. The Hex molecular docking server was used to generate the models, and the most probable complexes and the contact residues were visualized by using UCSF Chimera version 1.9. The examples presented in this figure have been generated with heme in a planar conformation. The heavy chain variable region is colored blue, the light chain of the variable region yellow, and the heme red. The gray arrows indicate the position of the antigen-binding sites of the antibody. Different complementarity-determining regions (CDRs) of both isomers of SPE7 are indicated.

most probable areas of heme binding with the variable regions of SPE7. The molecular docking analyses predicted that heme could bind to the Ab1 conformer between the light and heavy chains (Figure 5A). The binding site for heme was perpendicular to the antigen-binding site. As shown in Figure 5A, the most probable binding site for heme on the DNP-binding isomer was situated on the light chain of SPE7, a long distance from the antigen-binding site and on a different surface. The analyses of the putative heme binding sites on both conformers of SPE7 revealed that heme makes contact with the antibody through the polypeptide chain backbone as well as with side chains of various amino acids (Figure 5B). Different types of noncovalent interactions contributed to the binding: hydrophobic contacts, hydrogen bonds, and electrostatic interactions. However, in the cases of the most energetically favorable complexes with both conformers of SPE7, there was no evidence of coordination of the iron ion in heme. Docking of heme to SPE7 in complex with aromatic ligands (DNP or alizarin red) showed a loss of the preference for the heme binding site predicted for the free DNP-binding conformer of SPE7 and the appearance of complexes, where heme clusters within the most probable binding area for the protein-binding conformer (data not shown). We speculate that this effect might be explained by the conformational changes in the variable region of SPE7, observed as a result of interaction with aromatic ligands.[34,43]

The binding site for aromatic ligands of SPE7 contains a tryptophan residue (Trp93L) that directly participates in the interaction with the haptens.[34] Therefore, binding of DNP or other aromatic ligands of SPE7 results in marked quenching of Trp fluorescence.[34,37] To provide further experimental support to the molecular docking analyses, we performed fluorescence spectroscopy. Exposure of SPE7 to heme did not result in quenching of intrinsic Trp fluorescence (Figure 4B), thus confirming that heme does not bind to the conventional binding site of SPE7 for aromatic ligands.

Heme-Induced Polyreactivity Does Not Impact the Functional Activity of SPE7. To evaluate the functional impact of heme-induced extension of polyreactivity of SPE7, we performed functional degranulation assays on rat basophilic leukemia (RBL) 2H3 cells. The β-hexosaminidase releasing assay was used to measure the ability of the native and heme-exposed SPE7 to induce degranulation. The native SPE7 induced DNP-specific degranulation in a concentration-dependent manner (Figure 6A). No difference in the degranulation, however, was observed after pretreatment of SPE7 with heme. The peak of degranulation was observed when RBL-9 2H3 cells were sensitized with 100−150 ng/mL IgE and then challenged with 10 ng/mL DNP (Figure 6A). We next investigated whether degranulation can be induced by stimulating the cells with antigens to which SPE7 binds only after exposure to heme. Despite the high binding affinity (4.78 nM) characterized by SPR for the binding of heme-SPE7 to Asp f 1, Asp f 1 did not induce any degranulation in RBL-2H3 cells (Figure 6B); neither did factor IX or other antigens (data not shown). Interestingly, native SPE7 alone, when incubated

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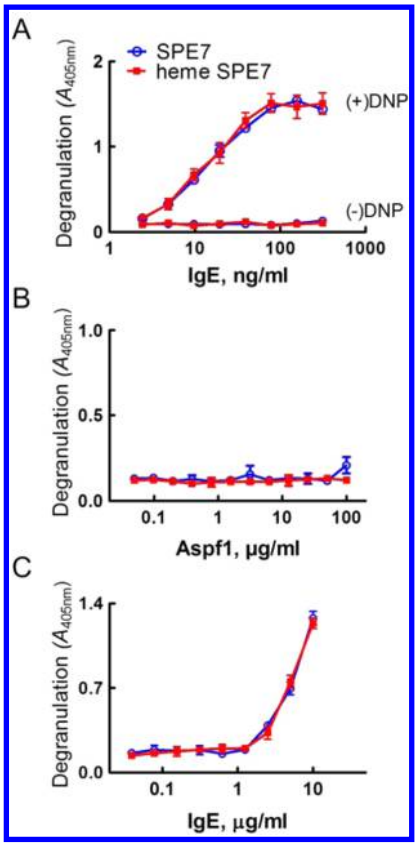

Figure 6. Degranulation of basophilic cell line RBL-9 2H3 by SPE7. (A) Assessment of the degranulation of rat basophils mediated by native and heme-exposed SPE7 in the absence or presence of a cognate antigen (DNP-BSA). The cell line was first sensitized by preincubation with an increasing concentration of native or heme-exposed SPE7 (2.44−312.5 ng/mL, 2-fold dilution) and then incubated in the presence or absence of 10 ng/mL DNP-BSA. The degranulation of the cells was assayed by estimation of the enzyme activity of extracellular β-hexosaminidase. The graph depicts a representative example obtained from three independent experiments. Each data point is the average of duplicate wells ± the standard deviation (SD). (B) Degranulation of RBL-9 2H3 cells by SPE7 and Asp f 1. The cell line was preincubated with 160 ng/mL native and heme-exposed SPE7. After being washed, the cells were challenged with increasing concentrations of Asp f 1 (0, 0.488−100 μg/mL, 2-fold dilutions). The degranulation was assessed as described above. Each data point is the average ± SD of duplicate wells. (C) Direct degranulation of RBL-9 2H3 cells by SPE7. The basophilic cell line was incubated with native and heme-exposed SPE7 at increasing concentrations (0, 0.039−10 μg/mL, 2-fold dilutions). The release of β-hexosaminidase was evaluated after incubation with the Ab for 2 h. Each data point is the average ± SD of three wells.

at high concentrations (micrograms per milliliter range) without a DNP, was able to induce basophil degranulation (Figure 6C). This could be explained by the observed high affinity for homophilic binding (Figure 1 and Table 1). Heme treatment of SPE7 did, however, not influence the degranulation in the absence of antigen (Figure 6C).

Taken together, results from the functional analyses are in agreement with those from binding assays wherein the induction of polyreactivity by heme failed to affect the binding of SPE7 to DNP-BSA or homophilic interactions.

DISCUSSION

Here, we demonstrate that the mouse monoclonal IgE SPE7 substantially augments its antigen binding promiscuity upon interaction with heme. The heme-mediated increase in the polyreactivity of SPE7 does, however, not influence the mechanism of recognition of the cognate antigen and the functional activity of the Ab. Molecular docking analyses predicted that binding of heme to the variable region of SPE7 occurs at a site distinct from the antigen-binding site. This observation might explain the absence of perturbation of the interaction of SPE7 with aromatic ligands.

SPE7 is an intrinsically polyreactive Ab that has undergone affinity maturation. In the absence of antigen, the antigen-binding site of SPE7 adopts at least two unrelated conformations:[37] one of the conformers recognizes DNP and numerous other unrelated aromatic compounds, another more energetically favorable conformer of SPE7 is suited for the recognition of protein antigens. James and Tawfik have examined the molecular mechanism of the promiscuity of binding of SPE7 to aromatic ligands.[34,43] They demonstrated that, although SPE7 is promiscuous, it is able to discriminate between very closely related molecules, such as dinitrophenol and nitrophenol. Moreover, it was shown that the ligand binding promiscuity of SPE7 is not mediated by nonspecific hydrophobic interactions, but rather by the ability of the Ab to provide versatile patterns of hydrogen bonds.[37] On the basis of analyses of kinetic and structural analyses, a model has been proposed that explains the simultaneous existence of promiscuity and specificity of SPE7 for aromatic ligands.[43] Initially, both specific and nonspecific aromatic compounds dock to the antigen-binding site of SPE7 with identical association rates. Specific ligands such as DNP, however, are capable of "locking in" via formation of hydrogen bonds into a second high-affinity conformation. Conversely, nonspecific ligands cannot establish hydrogen bonds and rapidly dissociate from the Ab. This induced-fit isomerization of SPE7, combined with the prebinding isomerization to an energetically favorable protein-binding isomer, results in a "kinetic proofreading" mechanism allowing SPE7 to distinguish by a factor of $>10^3$ in the K_D value between closely related ligands that initially bind with the same association rate.[43]

Heme is a heterocyclic aromatic compound used by many proteins as a cofactor for catalysis of various redox reactions and electron and gas transport. Previous studies have shown that binding of heme to a fraction of Abs present in normal immune Ig repertoires results in the acquisition of antigen binding promiscuity.[23,35] The interactions of these Abs with antigens are characterized by high values of the binding affinities (K_D in the range of 10^{-7}−10^{-8} M), suggesting that they may be physiologically relevant. The prevalence of Abs with cofactor-induced polyreactivity in human immunoglobulin repertoires might be high. Indeed, a recent study estimated that approximately 24% of Abs in a human IgG repertoire exhibit novel antigen binding specificity upon contact with heme.[44] This phenomenon has been demonstrated to occur in Abs belonging to different isotypes (IgG, IgA, and IgM), but never for IgE.[21,23,35]

As SPE7 has a potential to bind various aromatic compounds, we studied whether heme could interact with the Ab and further extend its intrinsic polyreactivity. Indeed, we observed that SPE7 binds heme with a relatively high affinity (62 nM). Moreover, exposure of SPE7 to heme resulted in a

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substantial increase in the repertoire of recognized protein antigens, as evaluated by immunoblot analyses. Further, we demonstrated that new heme-induced specificities of SPE7 are characterized by very high values of binding affinity. Thus, although native SPE7 did not show binding to Asp f 1, heme-treated SPE7 recognized this protein with an apparent K_D of 4.8 nM. The considerable increase in the repertoire of protein antigens recognized by SPE7 might be the result of a heme-mediated shift in the equilibrium in the conformational ensemble of the Ab. In contrast to the recognition of protein antigens, the cofactor-mediated increase in the polyreactivity of SPE7 had no effect on the recognition of the cognate antigen, the aromatic compound DNP, which was made evident by kinetic and thermodynamic analyses. Both native and heme-exposed SPE7 recognized immobilized DNP-BSA with similarly fast bimolecular association rates (in the range of 10^6 M^{-1} s^{-1}). A high association rate implies that the studied Ab recognizes DNP with a preoptimized binding site, a result in full agreement with data from other kinetic studies of interactions of SPE7. The absence of an effect of heme on the association rate constant for binding to DNP ruled out binding of heme to the binding site used for the recognition of aromatic ligands. Identical association kinetics excluded also the possibility that heme induces a shift in the ensemble of conformational isomers of the IgE. Results obtained by thermodynamic analyses are in agreement with the kinetic data. Induction of polyreactivity has no significant impact on the thermodynamic changes during association of SPE7 with DNP.

Many aspects of the mechanisms of cofactor-induced Ab polyreactivity are not well understood. It is shown in the case of IgG that induction of polyreactivity is associated with direct binding of heme to Abs and that coordinative interactions of the iron ion of the cofactor play an important role in this process.[23,35] Interaction of Abs with heme could induce antigen binding promiscuity by two mutually nonexclusive mechanisms. Thus, the binding of a cofactor molecule can provoke structural reorganizations of the antigen-binding sites. Alternatively, Ab-bound heme could contribute versatile noncovalent contacts, thus extending the potential for antigen recognition inherited in the polypeptide chain. It is noteworthy that heme is a highly promiscuous compound and could interact with numerous distinct proteins, which are unrelated to the conventional hemoproteins.[45,46] Accordingly, our previous analyses provided evidence of the involvement of heme as an interfacial cofactor for the recognition of gp120 from HIV-1 by a heme-bound monoclonal IgG.[35] In this study, we performed molecular docking analyses to improve our understanding of the mechanism of heme-induced polyreactivity of SPE7 and the absence of an effect on the recognition of the cognate antigen. Supporting our binding assay, the molecular docking revealed that heme could form a complex with the variable regions of SPE7. Heme was able to form a complex with both conformational isomers of SPE7. The complex of heme with the isomer that recognizes aromatic ligands revealed that the cofactor molecule binds to the light variable chain of SPE7 some distance from the binding site for aromatic compounds. This result might explain the absence of an effect of heme binding on the kinetics and thermodynamics of binding to DNP-BSA. The putative site of binding of heme to the protein-binding isomer of the Ab is between the heavy and light variable chains in the vicinity, but on a perpendicular surface as compared to the antigen-binding site. The observed considerable difference in the predicted binding areas between protein- and aromatic ligand-binding isomers of SPE7 may be explained by the structural difference between the two conformers, which concerns mostly the antigen-binding site but also includes area (on the protein-binding isomer) that was predicted to interact with heme.[34] These structural alterations, together with the observed differences in the electrostatic potential in the two SPE7 conformers, may explain the substantial difference in the predicted binding sites.

It is noteworthy that, when heme is bound to any of the conformational isomers of SPE7, its surface is exposed to the solvent but not intercalated in the protein interior. Thus, there is a possibility that the cofactor molecule serves as an interfacial bridge in the recognition of other proteins, thus explaining broadening of the antigen binding promiscuity of the Ab. Our data could not exclude, however, the induction by heme of some conformational alterations in the variable regions. Such structural changes would well explain the observation that some proteins recognized by native SPE7 are recognized with substantially higher intensity by heme-exposed SPE7 (Figure 1A).

Biological Implications. IgE is the least abundant immunoglobulin in plasma but can elicit strong inflammatory reactions by inducing the degranulation of sensitized mast cells and basophils with subsequent release of pro-inflammatory compounds and cytokines, which cause the symptoms of allergies. Some IgEs, in the absence of a specific antigen, were shown to promote mast cell survival and growth under conditions of growth factor limitation.[47−49] These effects, together with the augmentation of FcεRI expression on the surface of mast cells, might contribute to the amplification of allergic reactions.[42,48,49] Human and mouse IgE antibodies show heterogeneity with respect to modulation of mast cell physiology in the absence of an allergen.[41,49−51] Thus, highly cytokinergic (HC) IgEs are at the one extreme end and poorly cytokinergic (PC) IgEs at the other. HC IgEs are superior to PC IgEs in promoting the survival of mast cells, receptor aggregation, degranulation, and cytokine production and secretion.[48−50] Interestingly, it has been recently shown that the cytokinergic potential of IgEs positively correlates with the polyreactivity of Abs.[49,51] Accordingly, it has been demonstrated that one of the most potent HC IgE is SPE7.[51] In this study, we investigated whether extension of the antigen binding promiscuity of SPE7 by interaction with a cofactor renders this Ab more powerful in exerting its cytokinergic potential. Our data reveal that heme exposure does not affect the ability of SPE7 to induce degranulation of a sensitized rat basophil cell line in the presence or absence of the cognate antigen. These observations are in full agreement with kinetic and thermodynamic analyses of binding of SPE7 to DNP-BSA or to itself. Strikingly, although heme-exposed SPE7 exhibits a high binding affinity for Asp f 1 (K_D of 4.78 nM), this allergen was not able to degranulate cells sensitized with the heme-exposed Ab. Taken together, these data reveal important functional aspects about the heme-induced antigen binding promiscuity of IgE. The cofactor-mediated binding of antigen that occurs outside the conventional antigen-binding site might not fulfill the stereochemical requirements for aggregation of FcεRI.

Polyreactivity and cross-reactivity of IgE may have important undesirable consequence in allergy. Aggressive agents that may induce Ab polyreactivity (e.g., ROS) are released at sites of inflammation that accompanies allergic reactions. Therefore, further studies of mechanisms and physiological consequences of post-translationally acquired IgE polyreactivity are necessary

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to fully appreciate the physiopathological relevance of the phenomenon.

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Notes

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