Antibodies Use Heme as a Cofactor to Extend Their Pathogen Elimination Activity and to Acquire New Effector Functions*

Received for publication, March 30, 2007, and in revised form, July 10, 2007. Published, JBC Papers in Press, July 18, 2007, DOI 10.1074/jbc.M702751200

Jordan D. Dimitrov†1,2, Lubka T. Roumenina***, Virjinia R. Doltchinova†‡, Nikolina M. Mihaylova†, Sebastien Lacroix-Desmazes†‡§, Srinivas V. Kaveri‡¶, and Tchavdar L. Vassilev‡¶

From the †Department of Immunology, Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria, the **Department of Biochemistry, Sofia University, St. Kliment Ohridsky, 1164 Sofia, Bulgaria, the ‡†Department of Biophysics and Radiobiology, Sofia University, St. Kliment Ohridsky, 1164 Sofia, Bulgaria, §Centre de Recherche des Cordeliers, Université Pierre et Marie Curie-Paris 6, UMR S 872, F-75006 Paris, France, ††Université Paris Descartes, UMR S 872, F-75006 Paris, France, and ¶INSERM, U872, F-75006 Paris, France

Various pathological processes are accompanied by release of high amounts of free heme into the circulation. We demonstrated by kinetic, thermodynamic, and spectroscopic analyses that antibodies have an intrinsic ability to bind heme. This binding resulted in a decrease in the conformational freedom of the antibody paratopes and in a change in the nature of the nonvalent forces responsible for the antigen binding. The antibodies use the molecular imprint of the heme molecule to interact with an enlarged panel of structurally unrelated epitopes. Upon heme binding, monoclonal as well as pooled immunoglobulin G gained an ability to interact with previously unrecognized bacterial antigens and intact bacteria. IgG-heme complexes had an enhanced ability to trigger complement-mediated bacterial killing. It was also shown that heme, bound to immunoglobulins, acted as a cofactor in redox reactions. The potentiation of the antibacterial activity of IgG after contact with heme may represent a novel and inducible innate-type defense mechanism against invading pathogens.

Heme is a pivotal molecule for prokaryote and eukaryote organisms. As a prosthetic group of different proteins, it performs versatile biological functions, e.g. oxygen transport and storage, oxygen activation, and electron transport. The characteristics that make heme a key component of the aerobic metabolism (easy acceptance or donation of electrons by the iron ion) also make it inherently dangerous. Heme is redox-active and when liberated from hemoproteins may induce or aggravate the oxidative stress (1, 2). Heme could be released in large amounts as a result of many pathological conditions such as hemolysis, rhabdomyolysis, hemoglobinopathies, ischemia/reperfusion, malaria, severe inflammation, etc. (2–8). Usually under these conditions, the systems that protect the organism from free heme toxicity are saturated, and high plasma concentrations of this pro-oxidative molecule are reached (more than 20 μM). Heme, when liberated from its protein-bound state, may interact and oxidize lipids (9, 10), proteins (11, 12), and nucleic acids (13). Free heme is also implicated in the catalytic nitration of tyrosine residues of proteins in a number of pathological conditions (14, 15).

In addition to its pro-oxidative potential, it has been found that free heme also possesses potent pro-inflammatory activity. In vivo it induces increase in the vascular permeability, an increase in the expression of the adhesion molecules on the endothelial cells, and leukocyte recruitment to the organs (6, 16). Furthermore, it was shown that the transient exposure of neutrophils to heme triggers the oxidative burst and delays spontaneous apoptosis (17, 18). Heme also acts as a potent chemotactant for neutrophils in vitro and in vivo (17). In addition, heme possesses potent mitogenic activity for human peripheral blood mononuclear cells and increases the expression of the pro-inflammatory cytokines tumor necrosis factor-α and interferon-γ from these cells (19, 20). Because of its pro-oxidative and pro-inflammatory properties, free heme is implicated in the pathogenesis of many disease conditions such as acute renal failure after hemolysis, tissue injury induced by ischemia/reperfusion, as well as chronic inflammation that could be observed in some hemolytic diseases (6, 7, 21).

Immunoglobulins are the main serum glycoproteins responsible for detection and destruction of pathogens or their products. In disease conditions accompanied by the release of large amounts of heme from hemoproteins, Igs could be exposed to high concentrations of this molecule. This may influence their biological functions. Indeed, it has been observed recently that the in vitro exposure of polyclonal antibodies from healthy individuals to heme resulted in the appearance of new strong reactivities toward various autoantigens (22–25). The molecular mechanisms responsible for the effects on heme on the antigen binding activity of Igs are not understood. It is also not clear what is the biological significance of the interaction of the heme with the Igs. In an attempt to address these questions and because of the importance of such studies for understanding...
the pathophysiological mechanisms of diseases accompanied by release of heme, this study was undertaken.

Our results demonstrated that an exposure of antibodies to heme resulted in a substantial increase in their binding to bacterial antigens. Kinetic, thermodynamic, and spectroscopic analyses revealed that antibodies have an intrinsic property to bind heme and to use it for acquiring promiscuous antigen binding. It was also found that this caused more efficient pathogen elimination by complement. The heme-mediated broadening of the antibody repertoire may provide an inducible innate-like defense against invading pathogens. However, it may also contribute to the pathogenesis of inflammation and autoimmunity.

**EXPERIMENTAL PROCEDURES**

**Immunoglobulin Preparations**—As sources of polyclonal IgG (pIgG)\(^4\) human therapeutic polyclonal intravenous immunoglobulin preparations Intraglobin F (Biotest AG, Dreieich, Germany) and Sandoglobulin (Novartis, Pharmaceuticals, NJ) were used. Preparation containing Fc fragments from polyclonal human IgG was a kind gift from Dr. Marianne Debré (Hospital Necker, Paris, France) (26). F(ab')\(_2\) fragments from polyclonal IgG were obtained by pepsin hydrolysis and chromatography on protein G-Sepharose (Amersham Biosciences). Z2 hybridoma that produced a mouse IgG2b antibody with specificity for mouse IgG2a (27) and the IP2-11-1 hybridoma producing a monoclonal IgG2a (28) were kindly provided by Dr. Eva Rajnavolgyi (University of Lorand Eötvös, Budapest, Hungary). The growth of mouse IgG2a was monitored on protein G-Sepharose (Amersham Biosciences). Z2 hybridoma which produced a mouse IgG2b antibody with specificity for mouse IgG2a (27) and the IP2-11-1 hybridoma producing a mouse IgG2a were kindly provided by Dr. Eva Rajnavolgyi (University of Lorand Eötvös, Budapest, Hungary). The growth of hybridomas and purification of antibodies were performed as described in Ref. 28. All chemicals, except where indicated, were from Sigma. Exposure of immunoglobulins to hemin is described in the supplemental Experimental Procedures.

**Evaluation of Immunoreactivities of Native and Hematin-exposed IgG**—The ability of native and hematin-exposed IgG to bind to bacterial antigens or myosin was assessed by immunoblot analyses or by ELISA as described previously for ferrous ion-treated IgG (28). The binding to intact bacteria was evaluated as in Ref. 29. More details for the procedures are given in the supplemental Experimental Procedures.

**Surface Plasmon Resonance Analysis**—The kinetic constants of the interactions between native or hematin-exposed monoclonal antibodies were determined by using a surface plasmon resonance BIAcore 2000 (Biacore AB, Uppsala, Sweden). Puriﬁed mouse IgG2a (clone IP2-11-1) was immobilized on research grade CM5 sensor chip (Biacore) using amino-coupling kit as described by the manufacturer (Biacore). After that, the monoclonal IgG2a antibody solutions in 5 mM maleate, pH 4, with final concentrations of 25, 10, or 5 \(\mu\)g/ml, were injected with a flow rate of 10 \(\mu\)l/min and a contact time of 7 min on the surfaces of three independent flow cells. All the experiments were performed using HEPES-buffered saline (0.01 M HEPES, pH 7.4, containing 0.15 M NaCl, 3 mM EDTA, and 0.005% polysorbate) as running buffer and sample dilution buffer. All solutions were filtered through 0.22-\(\mu\)m filters and degassed under vacuum. Concentrations ranging from 37.5 to 600 \(\mu\)M of the native or 5 \(\mu\)M hematin-exposed Z2 antibody were injected on the immobilized monoclonal IgG2a with flow rate of 10 \(\mu\)l/min. Association phase was monitored for 10 min and then the chip surfaces were exposed to running buffer for 10 min to monitor the dissociation phase. For the regeneration of the chip surface, a 3 M solution of potassium thiocyanate (Sigma) was used. All kinetic measurements were performed at 5, 10, 15, 20, 25, and 30 °C. The binding to the surface of the control flow cell was always subtracted from the binding to the antigen-coated cells. The details of surface plasmon resonance measurements of the interactions of 771P52H7 antibody with FVIII are given in the supplemental Experimental Procedures.

BIAnormalization version 4.1 software (Biacore) was used for the calculation of the kinetic rate constants of association and of dissociation. Analysis was performed by global analysis of the experimental data using the kinetic models, included in the software, fitting the data with lowest value of \(\chi^2\). The values of the equilibrium dissociation constant were determined as the result of the ratio between the kinetic rate constants \((K_D = k_{off}/k_{on})\). The evaluation of the thermodynamic parameters of the interactions of Z2 antibody was described under the supplemental Experimental Procedures.

**Determination of the Salt Concentration Dependence of the Kinetic Rate Constants**—The salt concentration dependence of the kinetic rate constants of native and of hematin-exposed Z2 were determined by using BIACore. For this, the kinetic measurements were performed consecutively in variants of phosphate buffer (5 mM \(\text{Na}_2\text{HPO}_4\), 1.5 mM \(\text{KH}_2\text{PO}_4\), pH 7.4, and 0.005% Tween 20) differing by the NaCl concentrations (0, 50, 100, 200, 400, or 800 mM). The native and hematin-exposed Z2 antibodies were diluted in various buffers to concentrations ranging from 18.75 to 300 \(\text{NM}\) and injected on the immobilized monoclonal IgG2a with a flow rate of 20 \(\mu\)l/min. Association times of 5 min and dissociation times of 10 min were monitored. For the regeneration of the chip surface, a 3 M solution of KSCN was used. All measurements were performed at 25 °C.

**UV-visible Absorption Spectroscopy**—UV-visible spectroscopic assays for the binding of heme to polyclonal IgG were taken in PBS, pH 7.4, or in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl with Ultraspec 1000 spectrophotometer (Amersham Biosciences). A stock solution (2.5 mM) of hematin in 0.1 M NaOH was added to the buffer containing IgG (0.125, 0.25, 0.5, or 1 mM) to a final concentration of 2 \(\mu\)M. After hematin addition, the samples were homogenized and allowed to stand for 5 min at room temperature. The absorption spectra of the solutions were then recorded in wavelength range from 350 to 650 nm. In some cases potassium cyanide or imidazole were added (from 25 mM stock solutions) to buffers containing hematin or IgG-hemin. The absorption spectra of IgG alone in the range 350–650 nm were also recorded. It was found not to be significantly different from the background absorbance of the buffer alone. Titration of binding of heme to IgG, aliquots from a hematin stock solution were added to 1 \(\mu\)M solution of IgG and to reference cuvette containing buffer only. After each addition, samples in two cuvettes were homogenized and incubated for 5 min in dark at 25 °C. Thus, difference absorption spectra were

---

4 The abbreviations used are: pIgG, normal human polyclonal IgG; ROS, reactive oxygen species; IC, indigo carmine; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; CFU, colony-forming unit; 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid).
Antibodies Use Heme as a Cofactor

recorded. The IgG heme binding curve was built by plotting the difference in absorbance ($A_{\text{heme-IgG}} - A_{\text{heme}}$) at 390 nm versus molar concentrations of heme. For determination of heme binding to Fc-$\gamma$ or to F(ab$^\prime$)$_2$ fragments from human plgG, a stock solution (2.5 mM) of hematin was added to the PBS containing 50 $\mu$M F(ab$^\prime$)$_2$ fragments or 100 $\mu$M Fc-$\gamma$ fragments to a final concentration of 20 $\mu$M.

Assessment of Peroxidase Activity—For measurement of the peroxidase-like activity of the complexes of hematin with plgG, 2,2$'$-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was used as the chromogenic substrate. ABTS was added to 0.1 M phosphate citrate buffer, pH 5, to a final concentration of 1 mM. Samples under study were added to the reaction buffer at quantities resulting in final hematin concentrations of 0.2 or 2 $\mu$M. The hydrogen peroxide to a concentration of 6 $\mu$M was then added. The peroxidase activities were followed by an increase in absorbance at 414 nm. The measurements were performed in a 1-cm cuvette at 20-s time intervals for at least 600 s. For preparation of the samples, hematin from 2.5 mM stock solution in 0.1M NaOH was added to PBS alone or to PBS containing plgG (50 or 100 $\mu$M), Fc-$\gamma$ (50 $\mu$M), or F(ab$^\prime$)$_2$ (50 $\mu$M) to final concentrations 10 or 50 $\mu$M. In some cases, 10 $\mu$M NaN$_3$ was also added. After incubation for 30 min at 25 °C, samples were tested for their peroxidase activity as described above.

Oxidation of Indigo Carmine by a Metal-catalyzed Oxidation System—Fe(II) ions (from ferrous sulfate) and EDTA were both added in PBS (150 mM NaCl, 5 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, pH 7.4) to final concentrations of 100 $\mu$M. The reaction buffer also contained 300 $\mu$M indigo carmine. Also added to the reaction buffer were plgG to a final concentration of 1 $\mu$M, hematin to a final concentration of 1 $\mu$M, or F(ab$^\prime$)$_2$ (50 $\mu$M) to final concentrations of 10 and 50 $\mu$M. The Fenton reaction was started by addition of hydrogen peroxide to a concentration of 1 $\mu$M. After 10 min of incubation at 25 °C, the absorption at 610 nm was measured. In some cases, reaction buffer contained 200 units/ml CuZn-superoxide dismutase from bovine erythrocytes or 40 units/ml crystalline bovine liver catalase. The redox activity of the plgG-hematin complex preincubated with 1000-fold molar excess (in relation to hematin) of KCN or NaN$_3$ was evaluated by using the same assay.

Bactericidal Activity by Hydrogen Peroxide—in a typical experiment, a culture of ampicillin-resistant Escherichia coli (HB101 strain) in log phase growth, $A_{600}$ = 0.2–0.3) was repeatedly pelleted (three times at 3000 rpm) and resuspended in PBS, pH 7.4. The PBS suspended cells were then added to sterile Eppendorf tubes. Pooled human IgG (final concentration 100 $\mu$M) was pretreated with hematin at final concentrations of 0, 1, 5, 10, 25, 50, and 100 $\mu$M. Control preparations of hematin with the same final concentration but without IgG were also prepared. After 1 h, four series of E. coli were incubated as follows: with IgG (10 $\mu$M final concentration), preincubated with increasing hematin concentrations; IgG, preincubated with increasing hematin concentrations in the presence of 0.5 mM H$_2$O$_2$ (this concentration was estimated to cause 50% killing); PBS with increasing hematin concentrations (0–10 $\mu$M); PBS with increasing hematin concentrations (0–10 $\mu$M) in the presence of 0.5 mM H$_2$O$_2$ for 1 h at 37 °C. Viability of bacteria was assessed by recovery of colony-forming units (CFUs) on agar plates after overnight incubation at 37 °C. Each experiment was performed at least in duplicate. Data are given as percent of the viability of E. coli without any treatment.

Bacterial Killing by Complement—E. coli was incubated for 1 h at 37 °C with hematin (0, 10, 25, 50, and 100 $\mu$M)-pretreated plgG. Subsequently, guinea pig complement (1:100, 1:40, or 1:20 dilutions, or PBS) was added, and again bacteria were incubated for 1 h at 37 °C. Viability was determined by recovery of CFUs on agar plates after 12 h of incubation at 37 °C. Each experiment was performed in at least duplicate. As a control, the experiment was performed with two different preparations of plgG, and the results were comparable. Data are given as percent of the viability of E. coli without any treatment.

RESULTS

Exposure of Polyclonal IgG to Heme Resulted in Increased Antibody Binding to Foreign Antigens and to Myosin—We first addressed the question whether the heme exposure could influence the ability of antibodies to recognize foreign antigens. Normal human plgG was briefly incubated in the presence of increasing concentrations of hematin, and its ability to interact with bacterial proteins was then evaluated by an immunoblot technique. A significant hematin dose-dependent increase in the binding of plgG to these antigens was observed (Fig. 1A). Moreover, we tested the ability of hematin-exposed plgG to bind to intact E. coli cells, immobilized on a solid matrix. A considerable increase in the binding of heme-exposed plgG to bacteria was detected (Fig. 1B). Interestingly, the appearance of new binding specificities of plgG for E. coli antigens was detected after exposure to concentrations of heme that were lower than those reached in vivo under certain pathological conditions (3). The binding of secondary antibodies to native or heme-exposed plgG, immobilized on ELISA plates, did not differ significantly (data not shown). This observation ruled out the possibility that heme treatment of IgG results in enhanced recognition by secondary antibodies.

Furthermore, we studied the interaction of heme-exposed plgG with myosin. A significant increase in the binding of plgG to this self-protein was detected after exposure to heme (Fig. 1D). Antibodies from native plgG interacted only negligibly with myosin, whereas the plgG exposure to even 1 $\mu$M hematin resulted in an ∼2-fold increase in the binding. In addition, by using label-free real time interaction measurements, a significant increase in the binding of heme-exposed plgG or of heme-exposed F(ab$^\prime$)$_2$ fragments (obtained from plgG) to human complement factor H was observed (data not shown).

To rule out possibility that increased antigen binding activity of IgG is caused by heme-induced IgG multimer formation, size-exclusion chromatography was performed. The exposure of plgG to hematin, at concentrations that considerably influence antigen binding behavior (2, 10, 20, and 40 $\mu$M), did not result in a significant increase in the proportion of IgG dimers or multimers (supplemental Fig. S1A). Moreover, the reactivity of the monomer fraction isolated from hematin-exposed IgG with human factor VIII was considerably higher than that of the monomer fraction from native plgG (Fig. S1B), as seen in the case of unfractionated IgG (Fig. 1).
Antibodies Use Heme as a Cofactor

**Kinetic and Thermodynamic Analyses of the Interactions of Heme-exposed IgG**—To elucidate the molecular mechanisms responsible for the effects of heme on the antigen-binding properties of antibodies, kinetic and thermodynamic analyses were performed. The characteristics of the interactions of the mouse monoclonal IgG2b antibody Z2 to its cognate antigen—mouse IgG2a were deciphered by surface plasmon resonance. The exposure of Z2 to heme resulted in the appearance of new antigen binding specificities (Fig. 1C). This allows us to use Z2 as a suitable model system for studies on the biophysical mechanisms of heme-induced changes in antigen binding behavior of antibodies. Comparison of the interaction profiles of the native with those of hematin-exposed Z2 antibody revealed a qualitative difference in the interactions with the same antigen between the two forms of the antibody (supplementary Fig. S2A). In contrast, the exposure to hematin of monoclonal antibody 771P52H7 resulted only in small difference in the interaction profiles (supplementary Fig. S2B). These data clearly indicate that heme does not influence the antigen-binding properties of all antibodies and rule out nonspecific effects of heme on immunoglobulin molecules.

The studies of the interactions of Z2 allowed us to determine the binding affinity for the target antigen. The value of the equilibrium dissociation constant ($K_{D}$) measured at 25 °C was 140 nm (±12, n = 3) for the native antibody. The binding affinity of Z2 was increased more than twice after exposure to hematin (to $K_{D}$ value of 56 nm (±10, n = 3)). We then evaluated the effect of temperature on the $K_{D}$. For the native form of Z2, the correlation between the interaction temperatures and the change in the $K_{D}$ values was not significant (Fig. 2A). In contrast, the values of $K_{D}$ characterizing the binding of the heme-exposed antibody showed significant dependence on the change in the temperature. Interestingly, with increasing temperatures from 5 to 25 °C, 2-fold elevation of the antigen binding affinity of the antibody was observed (Fig. 2A). The value of the equilibrium dissociation constant depends on the values of the kinetic rate constants that describe the dissociation and the association phases of the interaction process. The comparison of the kinetic rate constants of the native and of heme-exposed Z2, measured at 25 °C, revealed that the increased antigen binding affinity after contact with heme was because of both an increase of the association rate constant ($k_{on}$) and a decrease of dissociation rate constant ($k_{off}$). Thus, the value of $k_{on}$ for native antibody was $3.60 \times 10^{4} \text{M}^{-1} \text{s}^{-1}$ (±0.24 × 10^4, n = 3), whereas it was $5.00 \times 10^{4} \text{M}^{-1} \text{s}^{-1}$ (±0.026 × 10^4, n = 3) for the heme-exposed antibody. The $k_{off}$ values of $5.10 \times 10^{-3} \text{s}^{-1}$ (±0.126 × 10^{-3}, n = 3) and of $3.11 \times 10^{-3} \text{s}^{-1}$ (±0.0156 × 10^{-3}, n = 3), respectively, were measured. Furthermore, the temperature dependences of the kinetic rate constants were evaluated. On Fig. 2B Arrhenius plots depict the temperature dependences of the $k_{on}$ and of $k_{off}$ constants, characterizing the interaction of the native and heme-exposed Z2. The rate of association for both forms of the antibody was weakly dependent on temperature change. However, although an increase of the temperature resulted in a decrease of the rate of association of native Z2, a reverse temperature dependence of $k_{on}$ was observed for heme-exposed Z2 (Fig. 2B). It is interesting to note that the rates of association at low temperatures (10 and 15 °C) were slightly slower for heme-exposed Z2 than those for the native one. At higher temperatures the opposite tendency was seen. In contrast to the results for $k_{on}$, the temperature sensitivity of the dissociation kinetic rate constant was similar for the native and heme-treated antibodies (Fig. 2B). Moreover, at all studied temperatures, the values of $k_{off}$ of the hematin-treated antibody were lower than those of native one. The kinetic measurements performed on surfaces with different quantities of the target antigen or by using different flow rates gave similar values of the
Antibodies Use Heme as a Cofactor

**FIGURE 2.** Kinetic and thermodynamic analyses of the binding of the native and of heme-Z2 monoclonal antibody. A, comparison of the temperature dependences of $K_D$ for the cognate antigen of native (crosses) and of heme-exposed Z2 (open triangles). The slopes were determined by linear regression ($R^2 > 0.96$ for the heme-Z2 and $R^2 > 0.87$ for native Z2, indicating a significant fit for the $K_D$ values of heme-exposed Z2, but not for the $K_D$ values of the native ones). B, Arrhenius plots showing the temperature dependences of the association and of the dissociation kinetic rate constants of native (open circles) and of heme-exposed Z2 antibody (closed circles). Reported data are representative of three independent measurements with at least five analyte concentrations. Each data point represents the mean ± S.D., $n = 3$. The slopes were determined by linear regression ($R^2 > 0.98$ for the heme-Z2 and $R^2 > 0.96$ for native Z2, indicating significant fits). Data shown are from one of two independent experiments. C, bar graphs (mean ± S.D., $n = 3$) depict changes in entropy (upper panel), enthalpy (middle panel), and Gibbs free energy (lower panel) for the association and dissociation interaction phases and for the equilibrium of native (empty bars) and of heme-exposed Z2 IgG (filled bars). Data shown are from one of two separate experiments.

kinetic parameters. This rules out a skewing of the kinetic data by effects of the mass transport or by other rebinding artifacts. The activation energies derived from the Arrhenius plots were used to evaluate the changes in the thermodynamic parameters that characterize association, dissociation, and equilibrium phases of the interactions of the native and of heme-exposed Z2 (Fig. 2C). The change in the entropy term ($T \Delta S$) for the association step was with a negative value for the interactions of both forms of the antibody. However, considerable differences were observed in the extent of the change of this parameter. Thus, the unfavorable $T \Delta S$ of $-75.90 \pm 1.10$ kJ mol$^{-1}$ for the association of native Z2 was significantly attenuated to $-36.34 \pm 2.23$ kJ mol$^{-1}$ in the case of the heme-exposed antibody (Fig. 2C). Interestingly, the change in the enthalpy ($\Delta H$) differed qualitatively between both forms of Z2. Although the association $\Delta H$ for the native antibody was favorable with a value of $-28.96 \pm 0.4$ kJ mol$^{-1}$, the change of the same parameter for the heme-treated antibody was unfavorable with a value of $9.58 \pm 0.6$ kJ mol$^{-1}$. No significant difference in the change of the Gibbs free energy ($\Delta G$) of association was found between two forms of the Z2. During the dissociation phase, neither of the thermodynamic parameters differed between native and heme-exposed antibody. The value of the changes in enthalpy at equilibrium was favorable for the native antibody and highly unfavorable for the heme-exposed one ($-9.66 \pm 1.45$ and $27.62 \pm 4.46$ kJ mol$^{-1}$, respectively). A favorable $T \Delta S$ at equilibrium was observed to be characteristic for the interactions of both forms of the antibody ($T \Delta S = 36.84 \pm 5.60$ kJ mol$^{-1}$, in the case of native antibody, and $T \Delta S = 68.96 \pm 11.14$ kJ mol$^{-1}$, in the case of heme-exposed one). This result indicates that the binding of IgG2a by Z2 is essentially an entropy-driven process. No significant differences were observed between the values of $\Delta G$ characterizing the equilibrium phase of the binding to antigen of native and of the hematin-traded Z2.

Salt Concentration Dependence of the Interaction Kinetics of Z2—To elucidate the origin of the dramatic effect of hematin exposure on the association $\Delta H$ of Z2, we studied the interactions of the antibody as a function of the salt concentration. The qualitative comparison of the interaction profiles obtained at different salt concentrations revealed that the binding to cognate antigen of native Z2 was extremely sensitive to the ionic strength of the buffer (Fig. 3A). Thus, increase of the concentration of NaCl from 50 to 100 mM resulted in a decrease of the resonance signal by more than 1000 resonance units. In contrast, the interactions of heme-pretreated Z2 were highly resistant to changes of the salt concentration. Then we evaluated the antigen binding kinetics of Z2 in buffers with different ionic strength. The association and dissociation kinetic rate constants of the native form of Z2 were highly sensitive to changes of the salt concentration. In contrast, the values of the rate constants characterizing the interactions of heme-exposed Z2 were not affected within the range of NaCl concentration used (Fig. 3B).

Absorption and Fluorescence Spectroscopy Analyses of Heme Binding to Immunoglobulins—The data obtained as a result of the thermodynamic analyses suggested that heme can directly bind to immunoglobulins and thus modulate their antigen binding function. To test this possibility, absorption spectroscopy...
hematin is able to bind to immunoglobulin G, preferentially to their F(ab′)2 fragments.

To further support the data, indicating that hematin binds to IgG, we performed fluorescence spectroscopy analyses. Addition of aliquots of hematin to solution containing IgG resulted in concentration-dependent quenching of protein fluorescence excited at 280 nm (supplemental Fig. S3). The high sensitivity of IgG fluorescence to hematin once again proves that Iggs can bind heme.

Suppression of the Increased Antigen Binding Activity of pIgG by CN⁻—The spectroscopic data indicated that the coordination sphere of iron remained unoccupied after its binding to immunoglobulins. Indeed, the incubation of the complex of hematin-pIgG with high molar excess of CN⁻ resulted in a significant inhibition of the elevated antigen binding activity of pIgG (Fig. 5). However, even a large excess of CN⁻ (in relation to heme) was not able to inhibit completely the appearance of new antigen binding specificities of the antibodies. Moreover, the increased binding of hematin-exposed pIgG to myosin was not inhibited by CN⁻ (data not shown). These results proved that the coordination of iron ion in hematin, bound to IgG, contributes to the binding of IgG to antigens. However, this is not the sole mechanism responsible for the appearance of new antigen binding specificities of the antibodies upon heme exposure.

Peroxidase-like Activity of Complexes of Heme with Immunoglobulins—The binding of heme to antibodies may give rise not only to appearance of new antigen binding specificities but also in the appearance of catalytic activities. Next, the peroxidase-like activity of the pIgG-hematin complex was examined. The kinetics of the oxidation of the chromogenic co-substrate for peroxidase ABTS was followed. The complex of pIgG with hematin exhibited significantly greater peroxidase-like activity than free hematin. A preincubation of the pIgG-hematin complex with an excess of NaN₃ (a inhibitor for heme enzymes) abrogated the elevated peroxidase activity. A similar intensity of the oxidation of ABTS as that seen for the free hematin was detected (Fig. 6A). The peroxidase-like activity of F(ab′)2 was also markedly elevated (data not shown) upon coupling with hematin.

Oxidation of Indigo Carmine by IgG-Hematin Complex—To get a better understanding of the involvement of IgG-hematin complexes in redox processes, the oxidation of indigo carmine
Antibodies Use Heme as a Cofactor

FIGURE 4. Normal pooled IgG forms complexes with heme. A, absorption spectra (350–650 nm) of hematin alone (2 μM), of hematin (2 μM) in the presence of 1 μM plgG, and of the same concentrations of hematin and plgG in the presence of 3 mM potassium cyanide. B, spectrophotometric titration of hematin (1 μM) with increasing concentrations of plgG. Absorption spectra were recorded after stepwise addition of plgG to the hematin-containing buffer. C, spectrophotometric titration of the hematin binding capacity of plgG. Immunoglobulins (1 μM) were mixed with increasing concentrations of hematin (0–20 μM). Similar amounts of hematin were added to a reference cuvette containing buffer only. The IgG hem binding curve was generated by plotting the difference in absorbance at 390 nm versus the molar concentrations of heme. D, absorption spectra of hematin (20 μM) alone, of 20 μM hematin in the presence 50 μM of F(ab′)2, or in the presence of 100 μM Fe-γ. All spectroscopic measurements were performed at 25 °C.

FIGURE 5. Cyanide partly reverses the heme-induced augment of IgG immunoreactivity. The effect of the complete heme iron coordination on the increased antigen binding of heme-IgG complexes was assessed by immunoblot analysis. The nitrocellulose membrane-immobilized bacterial proteins were left to interact with 50 μg/ml of native plgG (1); plgG treated with 3 μM hematin (2); plgG treated with 3 μM hematin and then with 5 mM KCN (3); plgG treated with 9 μM hematin (4); plgG treated with 9 μM hematin and then with 5 mM KCN (5); plgG treated with 27 μM hematin (6); plgG treated with 27 μM hematin and then with 5 mM KCN (7). Binding of secondary antibodies to their target antigens, innate effector mechanisms such as complement and/or phagocytic cells are activated. In most of the cases this results in rapid pathogen elimination. In this study we demonstrated that the exposure of plgG to hematin resulted in increased binding to intact E. coli or its proteins (Fig. 1B). It is tempting to speculate that heme-exposed IgG will be more efficient in pathogen destruction by effector systems. To examine this hypothesis we evaluated the ability of native and of heme-exposed IgG to initiate complement-mediated killing of E. coli. Hematin-exposed IgG caused a significantly
Mechanisms of Heme-induced Enlargement of the Available Antibody Repertoire—It is known that very high plasma levels of free heme may be achieved as a result of certain pathological conditions. We demonstrated here that the in vitro contact of antibodies with concentrations of heme that are much lower than those observed in vivo influenced dramatically their antigen-binding properties. Thus, exposure of pooled IgG to heme resulted in a considerable increase of the available antibody repertoire for bacterial antigens. These as well as previous findings (24) point out a previously unknown biochemical mechanism for modulation of the repertoire of circulating IgG. This mechanism may exist in vivo in physiological and/or pathological conditions and contributes to the immune surveillance.

To test this hypothesis, we first sought to decipher the biophysical mechanisms responsible for effects of heme on immunoglobulins. Kinetic and thermodynamic analyses of the interaction of free heme may be achieved as a result of certain pathological conditions. We demonstrated here that the IgG-heme complexes possess redox activities. Each data bar represents the mean ± S.D. of three independent experiments. **, *p < 0.01; ***, *p < 0.005 (paired Student’s t test).
Antibodies Use Heme as a Cofactor

tions of a heme-sensitive monoclonal IgG (Z2) were performed. The native form of Z2 is monoreactive, but after exposure to hematin it behaves as a typical polyreactive one. As expected, this change in the mode of interaction was accompanied by significant modifications in the kinetics and thermodynamics of antigen binding. The thermodynamic parameters, characterizing the association phase of the interaction with antigen, were most strongly affected. We observed that the exposure of Z2 to hematin markedly affected the changes in enthalpy during the association. Although the interaction of the native antibody was characterized by a favorable $\Delta H$ (with a negative value), highly unfavorable $\Delta H$ was found to be typical for the interaction of heme-exposed Z2. The changes in enthalpy (heat absorbed or released from the system) during intermolecular interactions depend on the nature and quantity of the noncovalent forces contributing to the complex formation (31, 32). This allowed us to propose that the heme-induced polyreactivity of IgG may be due to qualitative and quantitative changes in the noncovalent forces that contribute to the antigen binding. Indeed, the salt concentration dependence of the kinetic rate constants indicated that the native antibody used electrostatic interactions, whereas the heme-exposed antibody relied mainly on nonpolar forces for binding to the same antigen. It has been shown that antibodies that use the hydrophobic effect for antigen binding are usually polyreactive (33–36). Our observation on the enlargement of the antibody repertoire upon heme exposure is in full agreement with these findings. It was shown that the change in the entropy during the association of native Z2 to its cognate antigen was unfavorable (with negative value) for the overall affinity. Interestingly, heme exposure of the antibody resulted in more than 2-fold decrease in the unfavorable change in association entropy. $\Delta S$ during intermolecular associations depends on the degree of the structural/conformational changes in the interacting molecular surfaces (31, 32, 37). Thus, marked decrease of the unfavorable change in entropy induced by the exposure of Z2 to hematin could be due to restriction of the conformational freedom of the paratope. The heme in IgG seems to have the ability to rigidify the antigen-combining site of the antibody. Our data imply that hematin may bind directly to the antibody, thus influencing its antigen-binding properties. The decrease in the unfavorable $\Delta S$ penalty for heme-exposed Z2 correlated with an elevation of the binding affinity for the cognate antigen. Heme is a compound that may provide versatile types of noncovalent interactions-electrostatic interactions by the propionate residues, axial binding at the iron ion, and $\pi$-aromatic interactions by the tetrapyrrole ring. Accordingly, it has been shown previously that heme may easily bind to various proteins such as calmodulin (38), amyloid-$\beta$ (39, 40), prone protein (41), myosin (42), apolipoproteins (10), histidine-rich glycoprotein (43), and myelin basic protein (44). Interestingly, the binding of heme to unstructured or partly structured proteins induces and stabilizes the protein structure (45–47). The CD loops that build the antigen-binding sites of Igs are particularly rich in tyrosine and tryptophan residues (33, 48). The side chains of these amino acids could easily interact with the planar hydrophobic macrocyclic structure of heme. These facts, as well as the propensity of heme to adsorb to different proteins and to impose strong constraints on their structures, allowed us to hypothesize that the increase in the value of the association entropy after the Z2 exposure to hematin could be explained by the hematin binding to the Ig molecules. Thus, the propensity of heme to rigidify a flexible protein structure may explain the observed restriction of the conformational freedom of the paratope of Z2. The results obtained by spectroscopic analyses clearly indicated that hematin could bind to Ig molecules, preferentially to their Fab fragments. Moreover, the titration of the polyclonal IgG with hematin showed that $\sim$16 molecules of hematin bind per Ig molecule. The binding was not accompanied by the coordination of the heme iron by amino acid side chains. In addition, the spectral data indicated that the tetrapyrrrole ring of hematin was bound to Ig in such a way that one of the planes of the molecule remained fully exposed to the solvent. Our findings are in agreement with previous studies demonstrating that protoporphyrin IX (compound similar to hematin but without iron) is able to bind to Ig molecules (49, 50). It was shown in previous studies (49, 50) that protoporphyrin binds to Ig molecules in a similar way as was observed for hematin in this study. Therefore, it seems likely that Igs have an inherent capability to bind heme. Furthermore, we investigated the possibility that heme is able to bind to Igs as an interface cofactor that assists their promiscuous antigen binding. The complete coordination of heme iron in the IgG-heme complex significantly inhibited the heme-induced elevation of the antigen binding activities of IgG. However, the complete inhibition of this polyreactivity was never reached. The latter would well be explained by the data from the thermodynamic analyses and salt concentration dependence showing an increase in the contribution of nonpolar forces for antigen binding. These findings suggest that the antigen binding promiscuity of the IgG-heme complex is a result of the synergistic effects of the hydrophobicity and of the axial iron binding. Both are provided by hematin. It is important to note that not all antibodies change their antigen binding behavior after heme exposure (24). It is tempting to speculate that only a fraction of IgG is able to bind heme in a way that results in a further broadening of their antigen binding spectrum.

Our data suggest that the biophysical mechanisms of the antigen binding promiscuity of heme-exposed antibodies differed qualitatively from those of natural polyreactive antibodies (51–55). They also differed from the mechanisms of in vitro-induced polyreactivity of IgG (28, 36, 56). It was demonstrated that the association of natural polyreactive antibodies was accompanied by highly unfavorable changes in entropy, reflecting high structural flexibility of the paratope (57, 58). Moreover, it was shown that the extent of the unfavorable $\Delta S$ quantitatively determined the degree of antibody polyreactivity (58). In contrast to the mechanisms of natural antibody polyreactivity, the promiscuous antigen binding induced by hematin is accompanied by a restriction of the conformational freedom of the antibody paratope. At first sight this would seem to hamper binding to unrelated antigens (as is the case with affinity matured antibodies). However, when an antibody binds heme it inherits a specific molecular pattern that may impose by itself high interaction promiscuity. Thus, two general thermodynamic types of antibody polyreactivity may be defined, entropy-
driven (for natural antibodies) and enthalpy-driven (for antibodies that use cofactor for promiscuous antigen binding).

**Functional Implications**—The fact that polyclonal IgG could bind heme prompted us to investigate the ability of the IgG-heme complex to catalyze redox reactions. Binding of heme to normal human polyclonal IgG resulted in the appearance of a significant peroxidase activity. It has been reported before that antibodies, specifically generated against metalloporphyrins, acquire peroxidase activity upon binding to the respective cofactor (59). We have shown for the first time that antibodies with unrelated specificity also acquire peroxidase activity when complexed with heme. The binding of cofactors to Igs is not a novel observation. Igs have been shown to be the main serum factor responsible for riboflavin transport (60, 61). In addition, IgG could use riboflavin for catalyzing oxidation reactions (62).

Interestingly, all antibodies regardless of their isotype or antigen specificity catalyze sophisticated redox processes (63, 64). Is use highly conservative catalytic sites for this activity. On the basis of our results it is tempting to speculate that the heme binding to Ig molecule resulted in the formation of a new catalytic redox center. It is possible that the reactions catalyzed by the conservative and by the novel redox centers are interrelated. Indeed, the IgG-heme complex was able to convert ROS, produced by the Fenton reaction, to the products of antibody-catalyzed water oxidation with a significantly higher yield. It is important to note that the antibody-catalyzed water oxidation reaction takes place in an environment where free heme may be available (29). To study the potential biological significance of the above observations, we further analyzed the effects of the IgG-heme complex on the survival of H_2O_2-challenged bacteria. Interestingly, when bound to IgG, heme not only lost its pro-oxidative potential but in addition it also protected the bacteria from the toxic effects of H_2O_2. Thus, the IgG-heme complex behaved as a typical antioxidant. On the basis of these results one could speculate that in pathological conditions accompanied by the release of large amounts of heme, the binding of heme to Igs may represent an alternative defense system against free heme toxicity. The formation of this antioxidant system may be beneficial in conditions of severe inflammation and ischemia-reperfusion, where released heme and ROS could interact with circulating Igs.

The ability of natural polyreactive antibodies to interact with a large panel of unrelated antigens is responsible for their function as a first line of defense against pathogens (65, 66). We hypothesized that the enlargement of the available antibody repertoire after exposure to heme may result in enhanced pathogen elimination. Indeed, complement-mediated bactericidal activity was elevated when bacteria were opsonized with heme-exposed antibodies. The broadening of the antibody repertoires by free heme may represent an innate-type defense mechanism against invading pathogens. The host cell damage, induced by infection could result in the leakage of heme and saturation of heme-binding proteins. Then free heme may complex with circulating antibody molecules resulting in an increase in their antigen elimination potential. In accordance with the Matzinger’s “danger” model (67), the heme molecule is a *bona fide* danger signal that possesses the ability to alert the cells of the immune system (16, 17, 20). We demonstrated here that it also influenced the biological functions of the main recognition molecules of the immune system. In addition to its beneficial effects, the heme-induced broadening of the antibody repertoire may contribute to tissue damage. Ischemia-reperfusion injury represents a severe inflammatory response, mediated mainly by an inappropriate activation of the complement system (68). It has been demonstrated recently that the binding of natural antibodies to hypoxia-induced neoepitopes is responsible for the initial activation of complement in ischemia-reperfusion sites (69). As high amounts of free heme are released in the sites involved (2), we speculate that local heme-modified antibodies start binding to intact cell surface antigens resulting in triggering the complement system and in the tissue damage.

We have previously observed that exposure of C1q to hematin inhibits its binding to immunoglobulin (25), an opposite effect compared with those described for IgG. Indeed, in pathological conditions, released heme may affect both partners of the interaction, thus compensating for increased antigen binding activity of Igs. The physiological relevance of simultaneous exposure of both Igs and C1q remains to be evaluated.

**Conclusions**—Treatment of pooled IgG with heme results in increased binding of antibodies to bacterial antigens and to intact bacteria. Thus, heme exposure reveals not only autoantibody reactivities of antibodies as previously reported but also toward bacterial antigens. Immunoglobulins possess intrinsic ability to bind heme. Igs exploit the physicochemical properties of heme for promiscuous antigen binding and for more efficient activation of the innate effector systems.

As opposed to the polyreactivity of natural antibodies, polyreactivity induced by heme is accompanied by a significant decrease in the conformational freedom of the antigen-binding site. This finding has never been reported before. The IgG-heme complex possesses catalytic redox-activity and acts as a potent antioxidant system.

**Acknowledgments**—We thank Drs. Boris Atanasov and Bharath Woolta for stimulating discussions.

**REFERENCES**

1. Darley-Usmar, V., and Halliwell, B. (1996) *Pharm. Res.* (N. Y.) 13, 649–662
2. Kumar, S., and Bandyopadhyay, U. (2005) *Toxicol. Lett.* 157, 175–188
3. Muller-Eberhard, U., Javid, J., Liem, H. H., Hanstein, A., and Hanna, M. (1968) *Blood* 32, 811–815
4. Sears, D. A. (1970) *J. Clin. Investig.* 49, 5–14
5. Comporti, M., Signorini, C., Buonocore, G., and Ciccolì, L. (2002) *Free Radic. Biol. Med.* 32, 568–576
6. Wagener, F. A., Volk, H. D., Willis, D., Abraham, N. G., Soares, M. P., Adema, G. J., and Fidgert, C. G. (2003) *Pharmacol. Rev.* 55, 551–571
7. Balla, J., Vercellotti, G. M., Nath, K., Yachie, A., Nagy, E., Eaton, J. W., and Balla, G. (2003) *Nephrol. Dial. Transplant.* 5, 8–12
8. Arruda, M. A., Graça-Souza, A. V., and Barja-Fidalgo, C. (2005) *Mem. Inst. Oswaldo Cruz* 100, 799–803
9. Camejo, G., Halberg, C., Manschik-Lundin, A., Hurt-Camejo, E., Rosen gren, B., Olsson, H., Hansson, G. I., Forsberg, G. B., and Ylhen, B. (1998) *J. Lipid Res.* 39, 755–766
10. Grinshtein, N., Banum, V. V., Tsemakhovich, V. A., and Shaklai, N. (2003) *Biochemistry* 42, 6977–6985
11. Aft, R. L., and Mueller, G. C. (1984) *J. Biol. Chem.* 259, 301–305
Antibodies Use Heme as a Cofactor