ADSORPTION OF THE PROTEIN ANTIGEN MYOGLOBIN AFFECTS THE BINDING OF CONFORMATION-SPECIFIC MONOCLONAL ANTIBODIES

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ABSTRACT Five monoclonal antibodies against sperm whale myoglobin have been used to investigate the physical state of the antigen adsorbed onto a polydimethylsiloxane surface. The binding of each antibody is sensitive to the antigen's conformation in solution while the locations of the antigenic sites on the myoglobin molecule for three of the antibodies have been determined (Berzofsky, J. A., G. K. Buckenmeyer, G. Hicks, F. R. N. Gurd, R. J. Feldmann, and J. Minna. 1982. *J. Biol. Chem.* 257:3189–3198). The binding of the fluorescein isothiocyanate–labeled IgG and Fab antibodies to previously adsorbed myoglobin has been observed using total internal reflection fluorescence. Three of the antibodies bind specifically to surface-adsorbed myoglobin with affinities at least 50% relative to myoglobin in solution whereas two of the antibodies show affinities for the surface-adsorbed myoglobin diminished by at least two orders of magnitude relative to myoglobin in solution. The specific loss of certain antigenic determinants on the adsorbed myoglobin, coupled with the retention of others, indicates a nonrandom adsorption of the myoglobin molecules.

INTRODUCTION

Proteins and other macromolecules generally adsorb onto solid/liquid interfaces. The conformation and orientation of adsorbed proteins are thought to affect a number of processes. For example, the physical state of adsorbed proteins is believed to influence cell/substrate interactions (1–3). It is recognized that the thrombogenic response of blood towards artificial surfaces is influenced by proteins in the adsorbed layer (4, 5). Protein adsorption accompanied by alterations in molecular conformation or by spatial ordering on the surface may also have important implications for the use of antibody–antigen assays involving the adsorption of protein antigen onto a solid surface (6). In this regard, it is interesting to note documented examples of hybridoma supernatants that exhibit false positives in solid-phase immunoadsorbent screening assays. That is, a monoclonal antibody is found that reacts strongly with the antigen adsorbed to microtiter plates but is subsequently found not to react well with the antigen in solution (7–10). Such antibodies sometimes bind more strongly to the antigen in solution after the antigen has been denatured by heat or by the reduction of disulfide bonds (9).

Very little direct information is available concerning the relationship between protein adsorption and conformation. Probably the most compelling evidence that conformational changes can occur subsequent to the adsorption of proteins to solid surfaces is the observation, in some cases, of alterations in the circular dichroism spectra of desorbed protein as compared with native protein (11, 12). Conclusions about the effects of surface-adsorption on protein conformation have also been inferred from results obtained using ellipsometry and infrared spectroscopy (13). microcalorimetry and potentiometric titrations (14), and total internal reflection fluorescence (15). Virtually no information is available at the molecular level concerning the conformation and orientation of an adsorbed protein.

With the goal of defining the physical state of a surface-adsorbed protein in terms of its conformation and orientation, we have undertaken a study of the adsorption of sperm whale myoglobin (Mb) to a polydimethylsiloxane (PDMS; filler-free silicone rubber) surface. A preliminary characterization of the adsorption behavior of Mb on PDMS has been presented (16). The Mb/PDMS system used in this study serves as a model system of protein adsorption onto solid surfaces. PDMS forms a relatively hydrophobic surface and thus the noncovalent interactions involved in the adsorption process and the qualitative aspects of the results obtained herein are expected to apply to other proteins adsorbing onto hydrophobic surfaces in a general sense.

The use of Mb offers several advantages. Mb is a thoroughly studied and characterized protein with respect to its solution properties and structure (17, 18). In addition, monoclonal antibodies that have been characterized with respect to their antigenic sites on the Mb molecule are available (19, 20). The observation that none of these antibodies recognize denatured fragments of Mb suggest that the binding of each antibody is sensitive to the antigen's conformation. Furthermore, the positions on the

protein antigen of amino acid residues essential for the binding of three of the antibodies have been determined. Strong arguments have been presented that these essential amino acids are contained within the antigenic sites for the three antibodies (20). Two of these antibodies recognize groups of residues which are far apart in the primary sequence but close together in the folded, tertiary structure of the antigen, demonstrating the topographic, as opposed to sequential, nature of the antigenic sites. Here we present an investigation of the binding characteristics of five of these monoclonal antibodies with Mb adsorbed onto a PDMS surface.

MATERIALS AND METHODS

Myoglobin

Sperm whale Mb (Biozyme, Batch 2, Accurate Chemical and Scientific, Hicksville, NY) was repurified by the method of Hapner et al. (21) as described previously (22). The major component IV, using the notation of Garner et al. (23), was used throughout. Mb concentration was determined from the visible absorption at 540 nm using an extinction coefficient of 10.4 mM^{-1} cm^{-1} for the ferric cyanide derivative (24). A molecular weight of 17,800 was used in any calculations.

Antibodies

The preparation, subclass, affinity, and binding site characterizations of the monoclonal anti-Mb IgG antibodies have been described previously (19, 20). Clones 1, 3.4, 4, and 5 are $\gamma_1\kappa$, whereas clone 2 is $\gamma_{2a}\kappa$. All were made by fusing spleens of hyperimmunized A.SW mice with NS1, a nonsecreting derivative of the P3 × 63 plasmacytoma cell line. The antibodies were affinity purified from culture supernatant (clone 1) or ascites (all others) by affinity chromatography on Mb-Sepharose, elution with 0.1 M glycine · HCl (pH 3), and immediate neutralization with Tris, pH 8.

For studies incorporating non-Mb-specific antibodies, purified mouse MOPC 21 myeloma protein (lot 03604; Litton Bionetics, Kensington, MD) was used.

Fab Fragments

Fab fragments of each of the IgG antibodies were prepared by digestion with papain immobilized on an agarose gel (Pierce Chemical Co., Rockford, IL). Typically, 0.5 ml of the papain–agarose (~130 μg papain) was added to 5 mg antibody dissolved in 1 ml of 20 mM sodium phosphate, 50 mM cysteine · HCl, 10 mM sodium EDTA, pH 6.2. The mixture was incubated for 5 h at 37°C with gentle rocking. The immobilized papain was separated by centrifugation (8,800 *g* for 10 min). The supernatant was desalted by size exclusion chromatography (1 × 10 cm column) in 10 mM sodium phosphate, pH 8.50. The protein fraction was then applied to a 5-ml column of protein A–Sepharose CL-4B (Pharmacia, Uppsala, Sweden) equilibrated in 10 mM sodium phosphate, pH 8.50, to remove Fc fragments and undigested IgG. Although protein A binds mouse IgG_1 (clones 1, 3.4, 4, and 5) only very weakly at neutral pH, IgG_1 and IgG_{2a} (clone 2) antibodies are bound quantitatively and with high affinity above pH 8.0 (25). The eluted Fab fragments were collected and stored for further use. The protein A column was regenerated with 0.1 M glycine · HCl, pH 2.80.

The IgG digestion and Fab purification steps were followed by size-exclusion high-performance liquid chromatography (HPLC) (Bio-Sil TSK-250 column, 7.5 × 300 mm; Bio-Rad Laboratories, Richmond, CA) in 20 mM sodium phosphate, 0.1 m Na_2SO_4, pH 6.80. Undigested IgG was found to have an elution volume of 8.60 ml at the conditions used. As the papain digestion proceeded, the IgG peak, monitored by the absorbance at 280 nm, decreased in intensity while a new peak with an elution volume of 10.2 ml appeared. This was presumably Fab and Fc fragments coeluting due to their similar molecular weights. After 5 h of digestion, the area under the IgG peak was <5% of the Fab–Fc peak. After the protein A–affinity chromatography, any IgG left in the sample was too dilute to be detected by its absorbance at 280 nm.

[^{3}H]Fluorescein Isothiocyanate Antibodies

Clone 4 IgG and each of the Fab samples, prepared as described above, were labeled with fluorescein isothiocyanate (FITC) by covalent attachment to primary amine groups (mainly lysine residues) of the proteins (26). Antibody was incubated with a 100 times molar excess of FITC in 0.1 M sodium borate, pH 9.5, for 1 h. The unreacted FITC was removed by size-exclusion chromatography (1 × 20 cm column) in 0.1 M sodium borate, pH 9.0. The labeled protein and unreacted FITC were observed as well-defined, yellow bands separated by more than half the column length when the protein fraction eluted from the column.

The FITC-antibody was immediately tritiated by a reductive methylation technique (27). At 0°C, the FITC-antibody was mixed with a 20 times molar excess of formaldehyde and a five times molar excess of sodium [^{3}H]borohydride (lot 2273-097, 62.0 Ci/mmol; New England Nuclear, Boston, MA). After 1 min, a 500 times molar excess of lysine was added to quench the reaction. The labeled protein was separated from low molecular weight components of the mixture by size-exclusion chromatography (1 × 20 cm column) in 10 mM sodium phosphate, 150 mM NaCl, 3 mM NaN_3, pH 7.40. Each of the labeled samples behaved identically to unlabeled samples of the same protein upon size-exclusion HPLC as described above. The labeled samples were stored at 4°C and used within 1 wk.

Protein concentrations and fluorescein labeling densities were determined spectrophotometrically. Extinction coefficients for IgG ($\epsilon_{276\ nm}$ = 200 $mM^{-1}cm^{-1}$) and for antibody-bound fluorescein ($\epsilon_{276\ nm}$ = 22 $mM^{-1}cm^{-1}$ and $\epsilon_{493\ nm}$ = 60 $mM^{-1}cm^{-1}$ at pH 7.4) have been determined previously (26). An extinction coefficient of 78 $mM^{-1}cm^{-1}$ at 276 nm was used for Fab concentration determination (28). Fluorescein labeling densities were typically 1 to 2 (moles fluorescein per mole antibody). The specific activities of the labeled antibodies, determined by liquid scintillation counting, ranged from 0.5 to 1 Ci/mmol. Molecular weights for IgG and Fab of 150,000 and 50,000, respectively, were used in any calculations.

Radioimmunoassay

The immunological activities of the labeled samples of clone 4 IgG and clones 1, 2, 3.4, 4, and 5 Fab were determined by solution radioimmunoassays. Mb was labeled with *N*-succinimidyl-[2,3-^{3}H]propionate (batch 39, 105 Ci/mmol; Amersham Corp., Arlington Heights, IL) as described previously (19).

The radiobinding assay for the labeled clone 4 IgG using polyethylene glycol (6,000–7,000 mol w; final concentration, 10% by weight) to precipitate the IgG plus bound antigen, was performed as described previously (22). A Scatchard analysis (data not shown) indicated that the labeled clone 4 IgG affinity for Mb was 5 × 10^8 M^{-1} and essentially 100% of the protein in the sample was active. The measured affinity is well within experimental error of the affinity for clone 4 IgG (7.1 × 10^8 M^{-1}) determined previously (19).

Because polyethylene glycol does not precipitate Fab fragments effectively, a second-antibody precipitation scheme was used. Fab, carrier antibody (mouse IgG, lot 0071; Miles Scientific Div., Miles Laboratories Inc., Naperville, IL) and [^{3}H]Mb were mixed in 10 mM sodium phosphate, 150 mM NaCl, 3 mM NaN_3, pH 7.40. After incubation for 2 h at room temperature, second antibody was added (affinity-purified goat anti-mouse IgG, $F(ab')_2$, lot 4922; Pel Freez Biologicals, Rogers, AK). The final Fab, carrier, and second antibody concentrations were 0.20, 0.67 and 7.2 μM, respectively, while the Mb concentration was varied. The final volume was 0.10 ml. The precipitation reaction was allowed to proceed for 48 h at 4°C. After centrifugation at 8,800 *g* for 30 min, the

supernatant was removed and counted. Controls with Mb-specific Fab absent precipitated less than 5% of the radioactive Mb, whereas >95% of the radioactive Mb could be precipitated by the specific samples. From Scatchard analyses of the data (not shown), each labeled Fab sample exhibited an affinity for Mb within 50% of the affinity determined for the undigested and unlabeled antibody (19, 20). At least 80% of the protein in each sample was active. These results were taken to indicate that, for the purposes of this study, the immunological activity of the Fab fragments was not significantly perturbed by the digestion and labeling procedures.

Total Internal Reflection Fluorescence Apparatus and PDMS Films

A total internal reflection fluorescence (TIRF) instrument described previously (29) was used. Also discussed in this prior publication are the techniques used to deposit and characterize PDMS films on glass slides. Adsorbed protein surface concentrations were determined and related to the TIRF signals as described elsewhere (29) with the modifications of Darst et al. (16). For both [^{3}H]FITC-IgG and [^{3}H]FITC-Fab, the fluorescence signals were found to be proportional to the surface concentration over the range of surface concentrations encountered.

TIRF Experimental Protocol

The same general experimental methods described previously (29) were used for the TIRF experiments. Prior to an experiment, the protein solution reservoirs were incubated for several hours with a Mb or antibody solution of equal concentration to that used in the experiment to prevent loss of protein by adsorption onto the glass walls of the reservoir. Unless otherwise specified, the following protocol was used for the TIRF experiments. Mb was adsorbed from a flowing solution (wall shear rate 94 s^{-1}) onto a PDMS film for 10 h. The Mb solution concentration was 8.5 μg/ml in 10 mM sodium phosphate, 150 mM NaCl, 3 mM NaN_3, pH 7.40. The temperature was controlled at 37 ± 0.5°C. Under these conditions, the Mb surface concentration was found to be 94 ± 6 ng/cm^2 (5.3 ± 0.3 pmol/cm^2). After 10 h, the flowing Mb solution was replaced with a solution of [^{3}H]FITC-antibody. The adsorption of the [^{3}H]FITC-antibody to the previously adsorbed Mb was then monitored continuously using TIRF.

Nonspecific Adsorption of Antibody

To determine the specific interactions of anti-Mb antibodies with adsorbed Mb, the extent of nonspecific antibody adsorption must be known. The adsorption of FITC-bovine-γ-globulins to adsorbed Mb have been investigated previously (16). For the purposes of this study, nonspecific adsorption of antibody was determined by exposing layers of adsorbed Mb to various concentrations of [^{3}H]FITC-IgG and [^{3}H]FITC-Fab fragments of the MOPC 21 myeloma protein of P3 × 63. This molecule originates from the protein-secreting parent of NS1 and, being also $\gamma_1\kappa$ but without specificity for Mb, serves as a good control.

When the PDMS surface was exposed to high solution concentrations of Mb (30 μg/ml or higher), a saturating amount of Mb was adsorbed (~130 ng/cm^2). As found previously (16), the nonspecific adsorption of antibody to the Mb-saturated surface was negligible for up to 20 h. The amount of nonspecifically adsorbed antibody increased as the surface concentration of Mb decreased. Nonspecifically adsorbed antibody thus appears to interact with surface sites distinct from the adsorbed Mb molecules, probably vacant regions remaining on the surface when the Mb surface concentration fails to reach saturation.

For the anti-Mb antibody studies reported herein, the PDMS surfaces were ~70% saturated with Mb (94 ng/cm^2 vs. 130 ng/cm^2 for a saturated surface). Exposure of such a surface to anti-Mb antibody in solution will result in nonspecific antibody/PDMS interactions as well as the potential antibody/adsorbed Mb interactions of interest. The amount of specifically adsorbed antibody for a given antibody solution concentration was determined by simply subtracting from the total amount of adsorbed antibody the amount of nonspecifically adsorbed antibody determined at the same solution concentration. MOPC 21 IgG adsorption was used to correct for the specific IgG adsorption results whereas MOPC 21 Fab adsorption was used to correct for the specific Fab results. At the highest IgG solution concentration observed (125 nM), after 2 min of antibody adsorption to previously adsorbed Mb, the total amount of adsorbed clone 4 IgG was 40 ng/cm^2, whereas in a separate experiment the amount of adsorbed MOPC 21 IgG was 4.6 ng/cm^2. At lower antibody solution concentrations, when specific antibody/adsorbed Mb interactions were observed, the nonspecific antibody adsorption was always less than 10% of the total antibody adsorption for both IgG and Fab binding. In performing these minor corrections, the assumption is made that small numbers of amino acid changes within the binding site regions of each antibody do not significantly affect the nonspecific adsorption properties and thus the nonspecific binding does not vary from antibody to antibody. This is supported by the finding described above that the nonspecific binding results using MOPC 21 myeloma protein are essentially identical to those obtained using a heterogeneous bovine-γ-globulins sample.

Model Used to Interpret the Antibody Binding Results

The simple model used to interpret the antibody binding results is shown schematically in Fig. 1. Nonspecific adsorption of antibody to the PDMS surface is neglected in this model because only relatively small amounts of nonspecific antibody binding were observed and this was taken into account as described above. The model is essentially the same as those used to describe the equilibrium binding of antibodies or ligands to cell surface receptors (30) with the conceptual difference that the adsorbed Mb is probably not able to diffuse freely in the surface plane. Although the mobility of adsorbed protein in the surface plane could affect the kinetics of the binding process, assumptions regarding surface mobility are not required to analyze the equilibrium binding results. The binding of an antibody is described by the linked equilibria (* indicates surface bound species):

$$Ab + Mb^* \rightleftharpoons Ab_1^* \qquad K_1 = \frac{[Ab_1^*]}{[Ab][Mb^*]}. \tag{1}$$

$$Ab_1^* + Mb^* \rightleftharpoons Ab_2^* \qquad K_2 = \frac{[Ab_2^*]}{[Ab_1^*][Mb^*]}, \tag{2}$$

where Ab denotes free antibody in solution, Mb* denotes adsorbed Mb, and Ab_1^* and Ab_2^* denote the mono- and bivalently bound antibody, respectively. K_1 and K_2 are the univalent and bivalent association constants, respectively. The quantity measured experimentally is the total

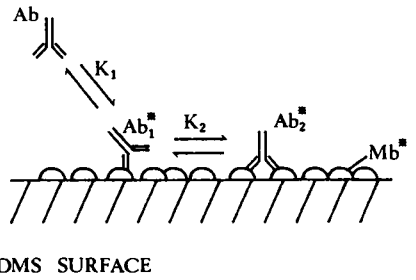

FIGURE 1 Schematic representation of the model used to interpret the antibody binding results. *Denotes a surface-bound species. According to this scheme Mb is bound in some unknown manner to the PDMS surface. Antibody in the bulk solution (Ab) is in equilibrium with a univalently-bound antibody/surface-adsorbed Mb complex (Ab_1^*) with an association constant K_1. For bivalent IgG antibodies, Ab_1^* is also in equilibrium with the bivalently-bound species, Ab_2^*, with an association constant K_2.

bound antibody ($[Ab_1^*] + [Ab_2^*]$). A surface coverage, θ, can be defined in the following manner:

$$\theta = \frac{[Ab_1^*] + [Ab_2^*]}{[Mb^*]_0}, \quad (3)$$

where $[Mb^*]_0$ denotes the total surface concentration of antibody-bound and free Mb, a known quantity. θ can be expressed as

$$\theta = \frac{K_1[Ab] + K_1K_2[Ab][Mb^*]}{1 + K_1[Ab] + 2K_1K_2[Ab][Mb^*]}, \quad (4)$$

or in the form of a Scatchard analysis:

$$\frac{\theta}{[Ab]} = K_1 + K_1K_2[Mb^*] - (K + 2K_1K_2[Mb^*])\theta. \quad (5)$$

For univalent Fab fragments, $K_2 = 0$ and the expression reduces to

$$\frac{\theta}{[Ab]} = K_1 - K_1\theta \quad (6)$$

RESULTS

Clone 4 IgG and Fab Antibodies Interact Strongly with Adsorbed Mb

Typical experimental results for the binding of the clone 4 antibody to surface-adsorbed Mb are shown in Fig. 2 (see figure caption for details). Fab and IgG antibodies behave qualitatively the same.

To derive conclusions from a Scatchard analysis, it must be demonstrated that a reversible equilibrium exists between the antibody in solution and the Mb-bound species. Evidence for this arises from two observations. First, when the antibody solution concentration is stepped, either up or down, the new antibody surface concentration is the same as if the experiment was performed in one step. Second, the Mb-bound antibody can be desorbed by flushing the flow cell with buffer or other solutions, such as bovine serum albumin. It should be noted that for high antibody solution concentrations, these equilibria conditions are observed as long as the Mb surface is exposed to the antibody solution for short times. If antibody adsorption is allowed to continue, a very slow increase in the antibody surface concentration occurs (in excess of nonspecific antibody adsorption) and the surface-antibody desorbs at an increasingly lower rate. For instance, at an IgG solution concentration of 63 nM, the initial rapid adsorption plateaus after ~5 min at a surface coverage of ~0.03. The antibody surface concentration continues to slowly increase, however. After 24 h, the surface coverage of specifically adsorbed antibody is ~0.13 and appears to have attained steady state. If the antibody adsorption process is interrupted after 5 h, virtually no antibody desorption occurs for up to 20 h. This long-term process may be due to aggregation of the surface-bound antibody but is not entirely understood and is not addressed further herein. All of the isotherms presented in this work were determined using data from the initial plateau of the antibody binding, the time period of which depends on the antibody solution concentration.

Isotherms for the binding of the antibodies to previously adsorbed Mb were determined by measuring the antibody binding over a range of antibody solution concentrations (0.5–200 nM). A Scatchard analysis of the isotherms for the clone 4 IgG and Fab antibodies is shown in Fig. 3.

Under conditions of random adsorption, a maximum surface packing of ~55% (surface area covered/total surface area) is expected (31). Using the Stokes radii of the IgG and Fab molecules calculated from the diffusion coefficients (28) as approximate molecular dimensions,

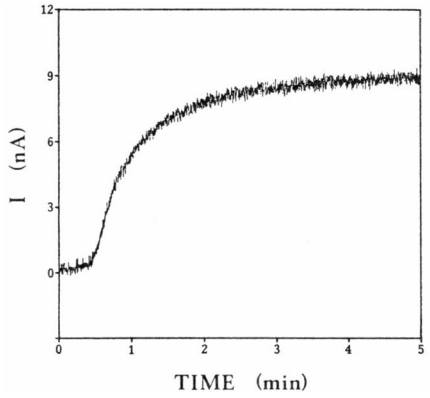

FIGURE 2 Surface-bound antibody fluorescence signal, in units of photomultiplier tube current, versus time for clone 4 FITC-IgG. Mb was allowed to adsorb onto a PDMS surface from a flowing solution for 10 h under the conditions described in Materials and Methods. At time zero, the Mb solution was replaced by a 63-nM solution of clone 4 FITC-IgG at a fluorescent labeling density of 1 mol FITC/mol IgG. The adsorption time lag is due to the time required for the antibody solution to displace the Mb solution up to the center of the flow cell and diffuse to the surface point of observation. Under the conditions of this experiment, the fluoresence signals from nonspecifically adsorbed and from bulk solution antibody are negligible. A fluoresence signal of 9 nA corresponds to an antibody surface concentration of 0.18 pmol/cm^2. The Mb surface concentration is 5.3 ± 0.3 pmol/cm^2, resulting in a surface coverage of 0.034.

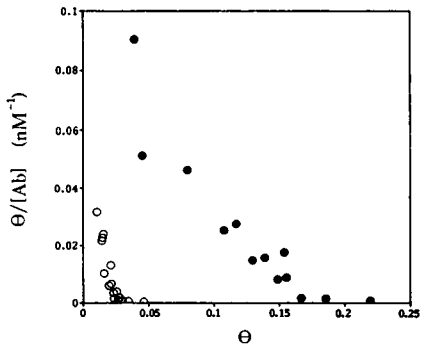

FIGURE 3 Scatchard analyses for clone 4 IgG (○) and Fab (●) binding to adsorbed Mb. θ is the fractional surface coverage as defined in the text (see Materials and Methods). [*Ab*] denotes the bulk solution antibody concentration in nM.

this surface packing corresponds to maximum surface coverages (θ) due to steric limitations for IgG and Fab of 0.09 and 0.25, respectively. This suggests that the clone 4 IgG and Fab adsorption may be limited by steric exclusion and explains the difference in maximum surface coverage observed between IgG and Fab, although additional experiments are required to confirm this point.

With Eq. 6, K_1 can be calculated from the slope of the Fab isotherm. The slope of the clone 4 Fab isotherm in Fig. 3 is estimated to be $-0.53\ nM^{-1}$, yielding a value for K_1 of $5.3 \times 10^8\ M^{-1}$. This compares very favorably with the value of the clone 4 affinity for Mb in solution, $7.1 \times 10^8\ M^{-1}$ (19). The limiting slope, as $\theta \rightarrow 0$, of the clone 4 IgG isotherm is estimated to be $-2.1\ nM^{-1}$. By comparison of Eqs. 5 and 6, the steeper slope of the IgG isotherm indicates that bivalent binding of the IgG molecules occurs. Using the value of K_1 determined above for the clone 4 Fab, K_2 can be calculated to be $2.8 \times 10^{11}\ cm^2/mol$.

Based on a comparison of the binding rates of the clone 4 antibodies to the adsorbed Mb with the results of a convection/diffusion model appropriate for the geometry of the TIRF apparatus (32), at the wall shear rate used ($94\ s^{-1}$) the binding rates of both the IgG and Fab antibodies are influenced by diffusion. Thus, the intrinsic kinetics of the forward reaction cannot be discerned from these data. The desorption rates, which are found to be kinetically limited, give further indication that the clone 4 IgG antibodies exhibit bivalent binding at low surface coverages. An apparent desorption rate constant, k_d, for the clone 4 IgG antibody was calculated assuming the following rate expression:

$$\frac{d\theta}{dt} = -k_d\theta. \tag{7}$$

The dependence of k_d on θ is tabulated in Table I. The significant increase in k_d with θ indicates that for small θ, the IgG binds bivalently to the adsorbed Mb. As θ increases, competition among the antibodies for a limited number of Mb molecules causes an increase in $[Ab_1^*]/[Ab_2^*]$. This results in an increased apparent k_d. This phenomenon should also result in an upward concave Scatchard plot, which is compatible with the clone 4 IgG isotherm shown in Fig. 3. However, it would be inappropriate to compare the clone 4 IgG isotherm with Eq. 5, owing to the possibility of significant steric limitations discussed previously.

TABLE I
DEPENDENCE OF THE DESORPTION RATE CONSTANT, k_d, ON θ FOR CLONE 4 IgG BOUND TO SURFACE-ADSORBED Mb

θ	$k_d(s^{-1} \times 10^4)$
0.020	2.1
0.034	2.6
0.045	4.3

Due to the complex binding behavior and the increased severity of the steric limitations for the IgG antibody, investigations of the binding of clones 1, 2, 3.4, and 5 to the adsorbed Mb were conducted using Fab fragments only.

Fab Fragments of Clones 1 and 2 Also Interact Strongly with Adsorbed Mb

The binding behavior of the clone 1 and 2 Fab antibodies to the adsorbed Mb was qualitatively similar to the behavior of the clone 4 Fab. The binding kinetics for both antibodies are diffusion-limited. Isotherms for these antibodies, in the form of a Scatchard plot, are shown in Fig. 4. From the estimated slopes of the isotherms, values for K_1 of $9.5 \times 10^8\ M^{-1}$ for clone 1 and $1.3 \times 10^9\ M^{-1}$ for clone 2 are determined. These affinity constants are within 50% of those for Mb in solution, $1.9 \times 10^9\ M^{-1}$ for clone 1 and $2.2 \times 10^9\ M^{-1}$ for clone 2.

Fab Fragments of Clones 3.4 and 5 Show Sharply Diminished Affinities for Adsorbed Mb

The affinities of the clone 3.4 and clone 5 Fab fragments for adsorbed Mb are several orders of magnitude less than their affinities for Mb in solution.

The clone 3.4 Fab fragments do not bind significantly to the adsorbed Mb. Under the conditions of these experiments, antibodies binding to the adsorbed Mb with an affinity of $\sim 10^6\ M^{-1}$ or less cannot be detected above background, nonspecific binding. Thus, the K_1 for the clone 3.4 Fab fragments is $10^6\ M^{-1}$ or less. This is compared to an affinity for Mb in solution of $2 \times 10^8\ M^{-1}$ (20).

The clone 5 Fab fragments interact weakly with the adsorbed Mb. The affinity is estimated to be $\sim 10^{-7}\ M^{-1}$, compared to an affinity of $1.6 \times 10^9\ M^{-1}$ for Mb in solution. In addition to the solution radioimmunoassay performed to determine the immunological activity of the clone 3.4 and 5 Fab fragments, another control was

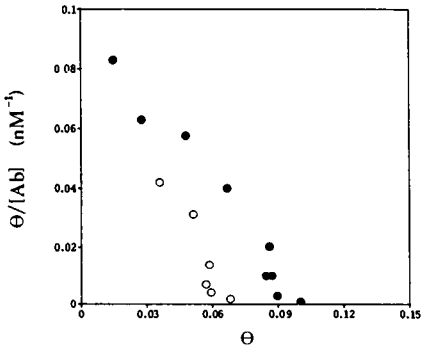

FIGURE 4 Scatchard analyses for clone 1 Fab (●) and clone 2 Fab (○) binding to adsorbed Mb. θ is defined in the text. [*Ab*] denotes the bulk solution antibody concentration in nM.

performed for the clone 5 Fab. It is known that the clone 4 and clone 5 antibodies can bind to the Mb molecule simultaneously in solution (33). Experiments were conducted in which clone 4 IgG was initially adsorbed to the PDMS surface and then exposed to Mb. Clone 5 Fab binds rapidly to the Mb-presenting surface. The maximum Fab surface concentration was 1.6 pmol/cm^2, close to the steric limit calculated previously. When the adsorbed clone 4 IgG is exposed to bovine serum albumin rather than Mb, the subsequent adsorption of clone 5 Fab is negligible. These results indicate that the clone 5 Fab sample used in this study is active under the conditions of these experiments and that the diminished binding to adsorbed Mb is due to an effect of the adsorption process on the Mb itself.

DISCUSSION

Table II summarizes the relevant properties of the antibodies investigated in this study, including their affinities for Mb in solution and for Mb adsorbed onto the PDMS surface. The amino acid residues essential for the binding of the clone 1, 3.4, and 5 antibodies are listed. Although the positions of amino acid residues within the antigenic sites of the clone 2 and 4 antibodies have not been determined, it is known that these antibodies can bind simultaneously with the clone 5 antibody to Mb in solution (33). Thus, the binding sites for the clone 2 and 4 antibodies must be distant from the clone 5 antibody site.

The main result of this study is that adsorption of the protein antigen Mb onto a PDMS surface results in the specific loss of some antigenic determinants (clones 3.4 and 5) whereas others are unaffected (clones 1, 2, and 4). The loss of antigenic determinants can be due to two effects. First, conformational changes of the adsorbed Mb could disrupt the antigenic sites. Second, the orientation of the adsorbed Mb on the surface could render the antigenic sites sterically inaccessible to antibody.

Significant conformational changes to the antigenic site for any of the antibodies investigated in this study would result in a decreased affinity (20). Conformational changes of the Mb molecule due to the adsorption process, if occurring at all, are confined to local regions of the adsorbed Mb. Although conformational changes to the antigenic sites of the clone 3.4 and 5 antibodies may cause the diminished binding of these antibodies, the binding affinities of the clone 1, 2, and 4 antibodies are essentially unaffected by the adsorption process, indicating the topography of the antigenic sites for these antibodies is not significantly altered by adsorption-related phenomena. In this regard it is of interest that the binding of the clone 1 and 4 antibodies, but not clone 5, perturb the spin-state equilibrium of the Mb heme slightly toward low spin relative to high spin at pH 8.7, near the dissociation constant (pK) (34). This effect is presumed to be due to the stabilization by antibody binding of a particular conformation of the protein. Although all of these antibodies bind to the "native" Mb conformation, in solution it is likely that the "native" state is an equilibrium mixture of several substates, all approximating the crystal structure but differing slightly from one another. Thus, one interpretation compatible with these results and those herein is that the clone 1 and 4 antibodies bind preferentially to a different conformational state from that preferred by the clone 5 antibody. Adsorption of Mb on the PDMS surface may stabilize the former state and disfavor the latter. A testable prediction of this hypothesis is that the ratio of low- to high-spin heme would be slightly greater, and the pK for dissociation of a proton from water bound at the heme would be slightly lower, on the PDMS surface than in solution.

The absence of more detailed knowledge concerning the locations on the Mb molecule of the clone 2 and 4 antibody binding sites makes it difficult to draw conclusions about possible orientation of the adsorbed Mb molecules.

Regardless of whether the diminished affinities of the clone 3.4 and 5 antibodies are due to conformational or steric effects, the specific loss of these antigenic sites in the adsorbed Mb, coupled with the retention of others, indicates the nonrandom nature of the adsorbed Mb molecules. The Mb molecules exhibit a preferred mode of adsorption, either in terms of their orientation or their conformation, because each antibody would be expected to bind to randomly adsorbed Mb with the same affinity relative to that for Mb in solution.

As random adsorption and native conformation of adsorbed protein antigen are important assumptions in many solid-phase immunoassay methods, these results have important implications for the interpretation of such studies. Conformational changes of adsorbed proteins and nonrandom adsorption may also influence cell/substrate interactions (1–3), and play a role in chromatographic separations of proteins and in thrombosis and other intravascular protein/surface interactions (4, 5). The use of conformation-specific monoclonal antibodies provides a sensitive tool to reveal the effects of surface adsorption on protein antigen.

TABLE II
SUMMARY OF THE Mb BINDING PROPERTIES OF THE MONOCLONAL ANTI-Mb ANTIBODIES

Clone	Essential Mb amino acid residues*	Affinity for Mb ($M^{-1} \times 10^9$)	
		Solution‡	Surface
1	Glu 83, Ala 144, Lys 145	1.9	0.95
2	Unknown	2.2	1.3
3.4	Glu 4, His 12, Lys 79	0.2	$\leq 10^{-3}$
4	Unknown	0.71	0.53
5	Lys 140	1.6	10^{-2}

*From reference 20. ‡From reference 19, except for clone 3.4, which is from reference 20.

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