Myoglobin Binding in Human Serum

Lawrence J. Kagen and A. Butt

Seventy-five of 82 human blood sera demonstrated binding for human myoglobin, the criterion for binding being the failure of myoglobin to pass through CF 50A (Amicon Corp.) ultrafilters. In most cases, when myoglobin was present in a concentration of 2 mg/liter, 50–87.5% of it was bound. Binding properties of sera were not related to age or sex of the donors. Dilution of serum by more than 50-fold removed its binding property. The percent binding could be increased by increasing the concentration of myoglobin to a maximum of 5–15 mg/liter. The binding factor of serum has a molecular weight exceeding 50,000 but less than 300,000, as determined by ultrafiltration experiments. Serum fractions that were devoid of albumin could bind myoglobin, as did two sera that lacked haptoglobin. Added hemoglobin did not interfere with myoglobin binding. Ultracentrifugation studies suggested that bound myoglobin exists in a complex that is slightly smaller in size than immunoglobulin G (i.e., approximate mol. wt. 100,000 to 150,000). Serum that bound myoglobin interfered with the complement fixation assay for the detection of myoglobin; serum that did not contain the binding factor had less such interference. We suggest that myoglobin binding factor may be responsible for this interference. Interference was not noticed in double-diffusion precipitin tests.

Additional Keyphrases: heart disease · infarction · proteins · cord-blood serum · complement fixation assay

Myoglobin, the oxygen-binding heme protein of striated muscle, may be released into the circulation and, after renal clearance, appear in the urine in several kinds of muscle disease or dysfunction (1). Recent interest has also focused upon serum myoglobin assay in the detection and assessment of myocardial infarction (2, 3). Among the factors to be considered in interpretation of serum assays and analyses of urine is the possibility of protein binding of myoglobin in the circulation. Two earlier studies yielded conflicting data regarding binding of myoglobin by serum proteins (4, 5). In this report we present evidence for the presence of binding of myoglobin by most human blood sera, along with partial characterization of this weak association system.

Materials and Methods

Ultracentrifugation of myoglobin serum mixtures. Purified human myoglobin, prepared as described (7), was added (final concn, 2 mg/liter) to human serum samples and to 0.15 mol/liter saline, and the mixtures were allowed to incubate at 37 °C for 1 h. They were subsequently ultrafiltered through cone-shaped “Diaflo” membranes (CF 50A; Amicon Corp., Lexington, Mass. 02173) at 1500 r.p.m. for 30 min. Myoglobin was assayed in the ultrafiltrates so obtained by hemagglutination-inhibition or complement fixation techniques, or both.

Ultracentrifugation of myoglobin serum mixtures. Myoglobin serum mixtures were subjected to ultracentrifugation in linear sucrose gradients (50 to 250 g/liter) in a Model L-2 Ultracentrifuge (Beckman Instruments Inc., Fullerton, Calif. 92634) at 35,000 r.p.m. for 17 h at 5 °C, in the SW 41 rotor. The final concentration of myoglobin in these experiments was 200 mg/liter. Thirty 0.3-ml fractions were collected after centrifugation. The volume of applied sample was 1.0 ml. Because some samples of frozen purified human myoglobin contained aggregated material, these were pre-centrifuged in the gradients described, and the unaggregated myoglobin was recovered and used for the preparation of mixtures for assay. Protein concentration was determined in the fractions by the modified Folin method (6).

Assays of myoglobin. Myoglobin and specific antiserum were prepared as described (7).

These immunological reagents were used in three assays: (a) Double diffusion in gels as described in 7 and 8. (b) Complement fixation as described in 2. (c) Hemagglutination-inhibition was performed with erythrocytes coated with myoglobin and reagents supplied by Ortho Diagnostics, Inc., Raritan, N. J. 08869. Reagents so prepared were mixed in microtiter plates
(Titerik; Linbro Scientific Inc., Hamden, Conn. 06517) as follows: 25 μl of sample for assay (e.g., ultrafiltrate preparations), 75 μl of diluted antibody, and 50 μl of a suspension (2.5 ml/dl) of myoglobin-coated erythrocytes. Setting patterns of the cells were assessed after 2–3 h. Because undiluted serum interfered with the settling patterns, only ultrafiltrates, which lacked interfering factors, were assayed by hemagglutination inhibition. The difference in titer between the amount of myoglobin present in ultrafiltrates prepared from myoglobin/saline mixtures and that in myoglobin/serum mixtures was used to calculate the percent binding in serum.

With the hemagglutination inhibition test we could detect 50 μg of myoglobin per liter. There was no reaction in the presence of normal serum or commercial preparations of human albumin, hemoglobin, or γ-globulin in concentrations ranging from 1 mg/liter to 30 g/liter.

Sources of proteins. Preparations of albumin (fraction V), γ-globulins (fraction II), and α-globulins (fraction IV) were obtained from Pentex Research Products Div., Kankakee, Ill.

Results

Binding of myoglobin by human blood serum. Myoglobin (2 mg/liter final concn) was added to myoglobin-free sera from 82 individuals (normal volunteers and patients awaiting elective orthopedic surgery). These sera were then centrifuged through CF 50 A Amicon filter cones, and the resulting ultrafiltrate was assayed for myoglobin. The difference in myoglobin content between that of the filtrates from serum and of those similarly prepared from 0.15 mol/liter saline was determined, to calculate the percent binding. As shown (Figure 1), myoglobin was retained in the high-molecular-weight fraction after filtration in all but five sera (6.1%). In most cases 50–87.5% of the added myoglobin was so retained. There was no relation between the subjects’ sex or age and myoglobin binding. Figure 2 includes data from 78 of the adults as well as from umbilical cord blood samples from 14 newborns. Although myoglobin was bound by 93.9% of the sera from adults and 100% of those from newborns, it was never bound completely. Free or low-molecular-weight myoglobin was detected in the ultrafiltrates in all cases. Myoglobin binding in eight serum samples studied did not change after freezing at −20 °C and thawing after storage for four days at 4 °C. Variation in the binding of myoglobin was studied in eight other individuals by repeated assay of serum samples obtained at different times (Table 1). In three cases there was no change after intervals of one to 60 days, in three apparent binding increased, and in two it diminished. The average change in binding was 14.1 ± 14.3%.

The effect of dilution of serum on binding is shown in Figure 3. Binding was no longer detectable at dilutions of 50-fold or more. Reduction in binding appeared with dilutions of fivefold or greater, in both sera tested.

Table 1. Repeated Assays of Myoglobin Binding In Sera of Eight Individuals (Two Samples from Each)

<table>
<thead>
<tr>
<th>Myoglobin binding, %</th>
<th>Interval, days</th>
<th>Change, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>First sample</td>
<td>Second sample</td>
<td></td>
</tr>
<tr>
<td>50.0</td>
<td>50.0</td>
<td>1</td>
</tr>
<tr>
<td>50.0</td>
<td>75.0</td>
<td>1</td>
</tr>
<tr>
<td>50.0</td>
<td>87.5</td>
<td>3</td>
</tr>
<tr>
<td>50.0</td>
<td>50.0</td>
<td>4</td>
</tr>
<tr>
<td>75.0</td>
<td>50.0</td>
<td>5</td>
</tr>
<tr>
<td>93.8</td>
<td>75.0</td>
<td>30</td>
</tr>
<tr>
<td>87.5</td>
<td>87.5</td>
<td>60</td>
</tr>
<tr>
<td>87.5</td>
<td>93.8</td>
<td>120</td>
</tr>
<tr>
<td>Av. 68.0</td>
<td>71.0</td>
<td>14.1</td>
</tr>
</tbody>
</table>

* Percent retention by CF 50A ultrafilter membranes of myoglobin, 2 mg/liter.

* Interval between obtaining the first and second serum sample from the same individual.
Binding of myoglobin by serum was related to the amount of myoglobin present (Figure 4). At concentrations of 1 mg/liter or less, less than 50% of the added myoglobin was present in the bound fraction. Myoglobin was not demonstrably bound at 0.5 mg/liter. Binding was maximum between 5 and 15 mg/liter. At higher concentrations, binding diminished slightly. Free myoglobin was also present at all concentrations, i.e., maximum binding never reached 100%.

Properties of the binding fraction of serum. Serum alone was perfused through CF 50A cones and myoglobin was added to the resulting ultrafiltrates. These ultrafiltrates were then refiltered through the same cones and, as shown (Table 2), myoglobin was now completely filterable, indicating the binding factor in serum was not present in the original CF 50 ultrafiltrate. In similar fashion, using XM 300 filtration of serum before addition of myoglobin and CF 50A assay, 87.5% of myoglobin was present in the bound fraction. With XM 100 prefiltration of serum, 50% of the added myoglobin could be bound. This indicated that the binding factor in human serum was itself not filterable through the CF 50A ultrafilters, but was completely filterable through XM 300 ultrafilters and partly filterable through XM 100 ultrafilters.

Two sera were obtained from patients undergoing severe hemolytic reactions at a time when neither had any haptoglobin detectable by precipitin analysis with specific antiserum. Each of these two sera bound myoglobin well (Table 3).

Partly purified commercial preparations of human serum proteins were tested for binding ability. At 30 g/liter, the most binding was found in the γ-globulin fraction II preparation, while little or no binding was evident in the albumin, or α-globulin fractions. The γ-globulin preparation, upon analysis by immunoelectrophoresis, was found to contain both immunoglobulins and β-globulin components, but no albumin.

The effect of addition of hemoglobin to serum is shown in Table 4. Hemoglobin in concentrations up to 500 mg/liter interfered very little, if at all, with myoglobin binding.

Ultracentrifugal pattern of myoglobin in the presence of human blood sera. Figure 5 demonstrates the results obtained after subjecting myoglobin to ultracentrifugation in linear gradients of sucrose-in-saline solutions (50–150 g/liter). Myoglobin alone under these conditions was found near the top of the gradient (max. near 75 g/liter). When myoglobin was mixed with “high binding serum,” it appeared deeper in the gradient, in a wider zone of sucrose concentration, from 80 to 130
g/liter. Serum with no demonstrable binding by the ultrafiltration technique did not bring about this increase in myoglobin density, although a slight increase in depth of migration to 103 g/liter was seen. Six sera obtained from patients with myoglobinemia, containing myoglobin from 10 to 184 mg/liter, had a distribution of myoglobin in the gradient from 61 to 105 g/liter. The positions occupied by albumin (100 g/liter) and immunoglobulin G (130 g/liter) are indicated, and suggest that the density of bound myoglobin was somewhat less that that of immunoglobulin G.

**Interference of serum with immunoassays for myoglobin.** Double-diffusion precipitin analysis in agar gels was not significantly altered by the presence of serum, although there was a slight tendency for the precipitin band to be more prominent or easily seen in serum compared to saline diluent (Table 5).

<table>
<thead>
<tr>
<th>Serum</th>
<th>% Mgb bound*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15 mol/liter saline + Mgb</td>
<td>0</td>
</tr>
<tr>
<td>Serum + Mgb</td>
<td>87.5</td>
</tr>
<tr>
<td>+ Hemoglobin, mg/liter</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>50.0</td>
</tr>
<tr>
<td>200</td>
<td>87.5</td>
</tr>
<tr>
<td>50</td>
<td>50.0</td>
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<tr>
<td>20</td>
<td>87.5</td>
</tr>
<tr>
<td>10</td>
<td>50.0</td>
</tr>
<tr>
<td>5</td>
<td>50.0</td>
</tr>
</tbody>
</table>

*Percent retention of myoglobin, 2 mg/liter, by CF 50A ultrafilter membranes.

Table 4. Effect of Addition of Hemoglobin to Myoglobin (Mgb) on Myoglobin Binding by Human Serum

**Certain sera, however, did interfere with complement fixation assay.** As shown in Figure 6, assay of 1 mg of myoglobin per liter, in saline diluent, demonstrated 94% complement fixation. Serum from an individual with no binding demonstrable after ultrafiltration, as described before, allowed less complement fixation (58%) when tested undiluted, but when diluted two- and fivefold it allowed complement fixation to increase to near expected values. However, serum which demonstrated high binding (87.5%) interfered to a greater degree with complement fixation, even when diluted. Maximum complement fixation was 43% at fivelfold dilution. Both sera were subjected to ultrafiltration through XM-100 ultrafilters. In both cases perfusates

**Fig. 5. Ultracentrifugation of myoglobin added to human serum samples in linear gradients (in 0.15 mol/liter NaCl) of sucrose (50–150 g/liter)**

Each fraction (0.3 ml) obtained after ultracentrifugation was tested for myoglobin by double-diffusion agar gel precipitin analysis. Bars indicate positions of positive fractions as functions of sucrose concentrations. Myoglobin 200 mg/liter in all cases except those of clinical sera. "Low binding" serum caused no demonstrable retention by CF 50A filters of myoglobin when tested at 2 mg/liter. "High binding" serum brought about 87.5% retention under these conditions. Dark bars indicate position of fractions positive for myoglobin. Numbers indicate number of determinations, with gray areas showing standard deviation of averages.

**Fig. 6. Complement fixation assay of myoglobin (1 mg/liter) in saline and two human serum samples and their filtered components**

In saline, myoglobin produced 94.5% complement fixation (broken line). "Low binding" serum caused no demonstrable retention of myoglobin by CF 50A ultrafilter assay. "High binding" serum produced 87.5% myoglobin retention. These sera were ultrafiltered through XM-100 membranes and myoglobin was then added to serum, the retentate, and the perfusate for testing by complement fixation assay.
so prepared did not interfere with complement fixation at dilutions of two- and fivefold, although the perfusate from the low binding serum did display some interference when tested undiluted. Interference was found primarily in the retained fraction and, as shown, accounted for the pattern of interference of the high binding serum.

Interference by whole serum in the hemagglutination-inhibition assay could not be determined because whole serum, as well as that retained by XM-100 ultrafilters, produced artifacts of erythrocyte settling in the hemagglutination-inhibition assay. Therefore this technique was only applied to CF 50A ultrafiltrate fractions.

Discussion

The apparent molecular size of myoglobin increased after it was added to most (77 of 82) human sera, so that it no longer passed through CF 50A membrane ultrafilters. This phenomenon could conceivably have resulted from myoglobin binding to serum proteins or other components, from myoglobin aggregation, or from interference with the function of the filters by other serum-related factors. We suggest that nonspecific aggregation or membrane artifacts were not responsible because the percent of binding described by this technique was related to the concentration of myoglobin, being lowest at low concentrations of myoglobin, 0.5–1.0 mg/liter and greatest at 5–15 mg/liter. Above this concentration, binding was again decreased. These observations are compatible with a binding system of low affinity and capacity rather than with nonspecific occlusion of membrane filtration surfaces. In addition, ultracentrifugation experiments also indicated that myoglobin is present in serum, in part, in a heavy form, slightly less dense than immunoglobulin G (mol. wt. approx. 150 000). For these reasons it seems likely that myoglobin was bound by a component of most human blood sera. This component was completely filterable through Amicon XM 300 filters, not filterable through CF 50A filters, and filterable through XM 100 filters. This suggests, from the manufacturer’s assessment of the molecular permeability ranges of the ultrafilters, that the binding factor had a molecular weight near 100 000. If this were the case and the binding factor, perhaps protein in nature, associated with one or two myoglobin molecules, the observed behavior in the ultracentrifuge would be expected (e.g., the size of the complex would be in the 120 000 to 145 000 range).

The binding factor in human serum was not haptoglobin, because two sera devoid of detectable haptoglobin bound myoglobin as well as other sera, and moreover, addition of hagemoglobin to serum did not inhibit its binding of myoglobin. Nor was albumin involved in this myoglobin binding, because partly purified serum fraction II, which contained no detectable albumin, bound myoglobin to the greatest extent of those commercial fractions tested. The myoglobin binding factor was present in most human sera tested (93.9%) and could be demonstrated in all of 14 samples of umbilical cord blood at birth. Its binding capacity was low, because binding could be prevented by diluting serum 50-fold, and weak, because free myoglobin was found to coexist with the bound form at all concentrations. Myoglobin was most efficiently bound in a concentration range of 5–15 mg/liter, where over 90% was present in the high-molecular-weight form. Above this concentration, although more myoglobin could be bound, the proportions of free myoglobin increased to a greater extent.

Previously, two reports on myoglobin binding in human serum came to differing conclusions. Javid et al. (4) failed to find any evidence of myoglobin binding to serum proteins after electrophoresis on starch gel and staining with benzidine or napththalene black, using myoglobin at a concentration of 1.25 g/liter. Wheby et al. (5), however, using similar staining techniques on both paper and starch gel electrophoresis, were able to find evidence of myoglobin binding by a non-haptoglobin globulin. The binding capacity of this protein was thought to be low and ranged from 0–230 mg/liter in the sera tested. Horse myoglobin was also thought to be bound to the $\beta_1$-globulin fraction of human serum after immunoelectrophoresis (11).

Studies in the dog (9) indicate that myoglobin is bound by an $\alpha_2$- or $\beta$-globulin to a maximum of 210 mg/liter and that binding is inhibited by hemoglobin. Protein-bound myoglobin was not subject to renal excretion.

The importance of myoglobin binding may be of two sorts: (a) its effect on renal clearance and (b) its effect on serum assay methods. Little is known about the renal clearance of myoglobin in relation to protein binding, but the weak binding observed would probably not impair renal excretion. One study determined the renal clearance of myoglobin to be 25-fold more rapid than that of hemoglobin (10).

The present studies propose a weak-affinity myoglobin binding factor in human serum capable of association with approximately two molecules of myoglobin and suggest that myoglobin binding by serum may be the mechanism of its interference with the complement fixation immunoassay. This may lead to underestimation of the myoglobin content of sera, particularly at low concentration where extensive serum dilution is not possible (2). Interference with less sensitive precipitation methods was not demonstrated.

The assistance of Dr. David Bishop, Ortho Diagnostics Inc., Raritan, N. J., in making available reagents for use in the hemagglutination-inhibition assay is gratefully acknowledged.

This study was supported in part by Grant 1 R01 AM 14650 from the USPHS and a grant from the Muscular Dystrophy Association, Inc.

References


