Isolation and Biochemical Characterization of α-2-Opsonic Glycoprotein from Rat Serum*

F. Blumenstock, P. Weber, and Thomas M. Saba

From the Departments of Physiology and Biochemistry, Neil H. Hellman Medical Research Building, Albany Medical College, Albany, New York 12208

The importance of a circulating α-2-globulin as a determinant of hepatic Kupffer cell phagocytosis of nonbacterial particulate matter has been well documented. This protein which manifests opsonic activity exerts a humoral control on hepatic reticuloendothelial phagocytic activity and deficiency of its serum level exists with advanced malignant disease or acutely following traumatic injury. The present study was designed to isolate this α-2-opsonic protein from rat serum by using a combination of gel filtration in 6 M urea on cross-linked Sepharose 6B with prior sequential steps of ammonium sulfate fractionation, high voltage preparative free flow electrophoresis, and conventional Sepharose 4B gel filtration. Immunochemical analysis utilizing nonabsorbed antiserum to the rat opsonic protein as well as polyacrylamide gel electrophoresis was used to evaluate the level of purification, which was in excess of 99%. The purified protein was selectively immunoreactive with monospecific antiserum and its serum concentration was quantified by electroimmunoassay. The protein consists of two subunits held together by disulfide bonds and the minimum molecular weight as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of each subunit was 2.29 × 10^6. Amino acid analysis revealed no detectable methionine and a high concentration of hydrophilic amino acid residues. Amino sugar analysis confirmed its classification as a glycoprotein with approximately 1.8% glucosamine and no galactosamine. The purification and biochemical analysis of this α-2-glycoprotein may provide insight as to its mechanism of action with respect to reticuloendothelial function.

The reticuloendothelial system (RES) has been shown to be involved in both specific and nonspecific aspects of host resistance to infection (1), neoplasia (2), and traumatic injury (3). The basis of this involvement in host defense centers around the recognition and phagocytic ability of RE cells. While the RES is diffuse in distribution, the major components are sessile RE cells in the liver and spleen which monitor the vascular compartment and remove circulating foreign and abnormal particulate matter. Among the substances removed from the blood by the RES, especially the hepatic Kupffer cells, are injured platelets and effete red blood cells, denatured proteins and fibrin aggregates, and a variety of inert and metabolizable test colloidal materials (4-7). Recently, several studies have indicated that RES phagocytic clearance of nonbacterial colloidal substances is conditioned, in part, by the opsonic ability of serum or plasma to support hepatic Kupffer cell phagocytic function (8-11). Depression of this clearance mechanism exists during advanced tumor growth (12, 13) and following traumatic as well as burn shock (3, 14) and is associated with an opsonic deficiency as detected by bioassay.

An understanding of the serum factor(s) that conditions RE cell phagocytic function may be an important step in the elucidation of the mechanism whereby the phagocytic system recognizes "self" from "nonself" or "self" from "altered self." While this recognition or discrimination mechanism with respect to bacterial phagocytosis has been shown to be related to complement as well as the immunoglobulin fraction of serum (15, 16), the recognition of circulating nonbacterial particulate and colloidal materials and their associated clearance appears modulated by a serum α-2-globulin (11, 17, 18). This α-2-globulin stimulates Kupffer cell colloid phagocytosis in vivo and modulates in vivo RES capacity especially during RES colloid blockade (6, 8, 17, 18).

Recently, we reported a high degree of purification of this rat serum α-2-globulin with retention of biological activity (18). Subsequent studies by Molnar et al. (19) and Molnar et al. (20) have confirmed its existence with additional documentation that it is unrelated to the complement system. The present study was designed to achieve purification of this α-2-opsonic protein to antigenic purity from rat serum so that chemical and structural analysis with respect to molecular weight and amino acid composition could be accomplished.

The abbreviations used are: RES, reticuloendothelial; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

(Received for publication, March 28, 1977)
Ammonium Sulfate Precipitation—The pooled serum was diluted with ice-chilled 0.016 M phosphate in buffered 0.154 M NaCl, pH 7.4 (PBS), and 2-mercaptoethanol was added to a final concentration of 0.01% (v/v). Addition of 2-mercaptoethanol increased the yield of active protein isolated (18). At 4°C, 1.4 volumes of 100% saturated (NH₄)₂SO₄ (Schwarz/Mann, ultrapure) in PBS was added dropwise with stirring to the diluted chilled serum yielding a final (NH₄)₂SO₄ saturation of 55%. After equilibration for 1.0 h at 4°C, the suspension was centrifuged at 8000 x g for 20 min at 4°C. Following decantation, the sediment was dissolved in cold distilled water containing 2-mercaptoethanol at a concentration of 0.01% (v/v) to a volume equal to the original volume of serum, and dialyzed overnight against 30 to 40 volumes of 0.053 M Tris/citrate buffer (0.01% 2-mercaptoethanol), pH 8.4, at 4°C.

High Voltage Flow Electrophoresis—The dialyzed opsonically active fraction was further fractionated using high voltage free flow electrophoresis (Brinkmann Instruments, FF-1) as previously described (11, 17, 18). Electrophoresis was conducted at a range of 120 to 140 mA and 2600 V. The buffer flow rate was 115 ml/h and sample application rate was 8.0 ml/h under a pressure head of 30 cm h of H₂O. The buffer was 0.01 M Tris/citrate electrophoresis buffer (23). The samples were allowed to stand at room temperature overnight. Thereafter, each sample was centrifuged at 8000 x g for 20 min and the sedimented protein stored at -78°C under N₂.

Sepharose 4B Gel Filtration—The combined (NH₄)₂SO₄-treated FF-1 fraction was dissolved in 6.0 ml of phosphate buffered saline containing 0.01% 2-mercaptoethanol. An aliquot was taken and diluted with phosphate-buffered saline to test for activity, and the rest was subjected to gel filtration and applied to a Sepharose 4B (Pharmacia) column (72 x 2 cm) previously equilibrated with 0.01% 2-mercaptoethanol, pH 8.4, at 4°C (24). The experimental samples and molecular weight standards for gel filtration in 6 M urea were subjected to gel filtration and applied to a Sepharose 4B (Pharmacia) column (72 x 2 cm) previously equilibrated with 0.01% 2-mercaptoethanol, pH 8.4, at 4°C. The buffer was 0.01 M Tris/citrate electrophoresis buffer (23). Gel electrophoresis of the opsonic protein in SDS—polyacrylamide gel electrophoresis on 5.6% gels was used to determine minimum molecular weight in accordance with the technique developed by Fairbanks et al. (23). Gel electrophoresis of the whole fraction to correct for destruction and delayed release of amino acids during ion exchange chromatography (30) of the fluorescent technique of Roth (27, 28). Using o-phthalaldehyde. The purified protein from gel filtration in 6 M urea was extensively dialedyzed against a volatile buffer system (0.072 M triethylammonium acetate, pH 8.0) to eliminate salt from the protein solution since a high salt content might have an adverse effect on the separation of the amino acids during ion exchange chromatography for amino acid analysis. Three equal aliquots of the purified protein solution were lyophilized and hydrolyzed in 5.7 N HCl under nitrogen for 24, 48, and 72 h to correct for destruction and delayed release of amino acids during hydrolysis. In the case of amino acids which showed an increasing trend, the 72-h hydrolysis value was used as the final value.

Cystine-Cysteine Analysis—Since the analysis of cystine-cysteine by the o-phthalaldehyde method is at least 10 times less sensitive than the analysis of most of the other amino acids (28), cystine plus cysteine was analyzed after oxidation to cystic acid using dimethyl sulfoxide in 6 M HCl following the method of Spencer and Wold (31). Estimation of Proline—As proline cannot be detected upon elution from the amino acid analyzer by the fluorogenic o-phthalaldehyde method, it was necessary to estimate the proline content of the opsonic protein by gas-liquid chromatography. For this, the amino acids in the 24-h 6 M HCl hydrolysate of the opsonic protein were converted into the N-acetyl n-propyl ester derivatives and quantitated after separation by gas-liquid chromatography following the modification of Adams (32) using a Hewlett Packard 5890 A gas chromatograph.
Fig. 1. Flow chart for sequential steps relative to the isolation and purification of α-2-opsonic protein from rat serum. The protein as isolated is called either opsonic protein, aspecific opsonin, or recognition factor.
Tryptophan Analysis—Due to its lability during acid hydrolysis, tryptophan was analyzed by a fluorometric method developed by Sasaki et al. (33).

A 0.400-ml aliquot of the opsonic protein in 0.072 M triethylammonium acetate buffer, pH 8.0, was dissolved in a large centrifuge tube and immersed in boiling water for 5 min to denature the protein. An identical volume of the buffer was treated in the same manner to act as a blank. When the solutions had cooled, 10 µl of a freshly prepared solution, containing 0.05% (w/v) each of chymotrypsin and pronase, were added to each tube. The mixtures were allowed to digest overnight at room temperature. The solutions were then diluted with 3.0 ml of 6 M urea in 0.072 M triethylammonium acetate buffer, pH 9.2, and 3.0 ml of these diluted solutions were transferred to quartz cells for fluorescence measurements.

Fluorescence measurements were made with an Aminco-Bowman spectrofluorometer (American Instrument Co., Silver Spring, Md.). The excitation wavelength was 228 nm and the emission wavelengths read sequentially to the solution in the cell, the solution mixed, and the fluorescence intensity read again after each 3-nmol addition. The amount of tryptophan in the opsonic protein was calculated by subtracting the X-axis extrapolation from that calculated for the opsonin.

Amino Sugar Analysis—Amino sugars were quantitated on the amino acid analyzer using an Aminex A-5 column (9.8 x 17 cm) in a manner identical with the technique used for quantitation of the basic amino acids. The amino sugars were released from the opsonic protein by hydrolysis in 3 N HCl for 4 h at 100°. Calibration standards of known amounts of amino sugars were used to calculate the fluorescent response.

**Immunoeassey Determinations of α-2-Opsonic Protein (Electroimmunossay)**—Electroimmunossay or "rocket" immunoelectrophoresis was utilized to quantify the serum concentration of the α-2-opsonic glycoprotein. A modification of the method developed by Laurel (34). Amino sugars present in the opsonic serum (21) were dissolved in 0.07 N barbitur buffer (pH 8.6 and 2 mM calcium lactate) to a concentration of 1% by careful heating to 65° while stirring. The 1% agarose was transferred to quartz cells for fluorescence measurements. The active protein as visualized by this immunoechemical technique, while the antigenically heterogeneous fractions (Rockets 4 to 7) were retarded fractions eluting from the column. The possibility that the major rocket to the opsonic protein masks precipitin rockets of impurities was remote since the concentration of any contaminant and antibody to such contaminants would have to be exactly the same ratios as that of the opsonin and antipsonin, respectively. Additionally, any significant molecular species contaminating the opsonic protein should show a discrete band on SDS gel electrophoresis (Fig. 4) in the unreduced state unless the contaminant also demonstrated heterogeneity in the absence of mercaptoethanol. "Rocket" immunossay was used rather than immunoelectrophoresis since the classic technique of immunoelectrophoresis was not as sensitive an index of immunoechemical purity.

The homogenous 2nd and 3rd fractions were pooled, protein concentration determined, and confirmed to contain the opsonic protein by electroimmunossay utilizing monospecific antiserum to the protein (21). With the demonstration that the opsonic protein was homogeneous by immunoechemical methods the minimum molecular weight was determined (Fig. 4). The active protein as isolated by gel filtration at 4° has a molecular weight of approximately 800,000, which was confirmed by gradient polyacrylamide gel electrophoresis at the same temperature (18). As presented in Fig. 4, SDS gel electrophoresis of the opsonic protein identified in position 5 revealed the presence of a lower molecular weight contaminant, which was less than 1% (0.8%) of the total protein (Fig. 5) as detected by scanning the stained gels. Parallel electrophoresis of molecular weight standards is also presented in Fig. 4, positions 1 to 4. Position 1 shows the electrophoresis of IgM and it is evident that very little of this nonreduced protein entered the gel. Position 2 depicts the IgG standard. In positions 3 and 4,
the electrophoresis of bovine serum albumin is shown. Position 3 was overloaded in order to visualize the albumin trimer and tetramer to provide additional molecular weight standards.

Presented in Fig. 6 is the plot of the logarithm of molecular weight against relative electrophoretic migration of both the molecular weight standards and the opsonic protein samples in the 3.3% gel. The high molecular weight α-2-globulin protein is indicated by (x), while the lower molecular weight contaminant (1%) is indicated by (x) on the plot. The minimum molecular weight of the subunit of the opsonic protein molecule as calculated by linear regression is 227,000 to 228,000. The molecular weight of the contaminant, which could be a breakdown product of the major protein is approximately 120,000.

When the reduced S-cyanoethylated opsonic protein was electrophoresed in the presence of SDS on 5.6% acrylamide gels at pH 8.5 using a modification of the Fairbanks (23) procedure, results similar to the results on 3.3% gels were achieved, as indicated in Fig. 7. These data are not as valid since the molecular weight of the opsonic protein does not fall on the line between the standard proteins; however, when used for the calculation of the molecular weight, an estimate of 232,000 was calculated, which is within 5% of the results obtained on 3.3% acrylamide gels at pH 7.2. Therefore, the minimum molecular weight of the subunits of the \( M_r = 440,000 \) species at 37°C approximated by these two systems was calculated to be approximately 229,000.

Since by gradient polyacrylamide gel electrophoresis, SDS gel electrophoresis, as well as electroimmunoassay, the opsonic protein was judged to be at least 99% homogeneous, an amino acid analysis of the opsonic protein was performed for further characterization. The complete amino acid analysis of
this protein is presented in Table I in terms of nanomoles per fraction as a function of hydrolysis time and residues per $M_r$ = 229,000 subunit. The most striking features are its low methionine content and high concentration of hydrophilic amino acid residues.

The presence of 1.8% glucosamine identifies the opsonic protein as a glycoprotein. It appears that the linkage of carbohydrate to protein is of the N-glycosidic type involving asparagine to glucosamine since no galactosamine was detectable on the chromatogram.

![Fig. 7](image)

**Fig. 7.** The relationship between molecular weight and relative migration of proteins with known molecular weights (○). The migration of the opsonic protein (×) is shown. Gel concentration was 5.6% at pH 8.5.

**TABLE I**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Amino acid composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/ fraction $^a$</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>3.48</td>
</tr>
<tr>
<td>Threonine</td>
<td>3.27</td>
</tr>
<tr>
<td>Serine</td>
<td>3.87</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>4.29</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.56</td>
</tr>
<tr>
<td>Alanine</td>
<td>2.77</td>
</tr>
<tr>
<td>Valine</td>
<td>2.91</td>
</tr>
<tr>
<td>Methionine</td>
<td>N.D. $^a$</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.47</td>
</tr>
<tr>
<td>Leucine</td>
<td>2.15</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.40</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.15</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.91</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.04 $^a$</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.28</td>
</tr>
<tr>
<td>Proline</td>
<td>0.86 $^b$</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>0.98 $^b$</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.25 $^b$</td>
</tr>
<tr>
<td>Glucosamine $^a$</td>
<td>0.39</td>
</tr>
</tbody>
</table>

$^a$ Each fraction = 4.91 μg of protein as assayed by immunoassay; extrapolated to 0 time.

$^b$ Determined by dividing all values by 0.25 (tryptophan).

$^c$ Nearest integer/$M_r$ = 229,000.

$^d$ N.D., none detected.

$^e$ Value is 72 h hydrolysis amount.

$^f$ Estimation by gas chromatographic analysis.

$^g$ Measured as cysteic acid after oxidation with dimethyl sulfoxide.

$^h$ Measured by spectrophotometric method.

$^i$ Measured after 4-h 3 N HCl hydrolysis of sample.

$^j$ Weight per cent.

**DISCUSSION**

Recently extensive documentation has appeared with respect to the role of a plasma α-2-globulin in both animals and humans as a determinant of hepatic Kupffer cell phagocytosis of nonbacterial particulate matter (12-14, 17, 18, 21). Previous studies resulting in the partial purification of this protein revealed that the biological activity of this α-2-globulin in terms of *in vitro* Kupffer cell phagocytosis is dependent on heparin and that the protein is of larger molecular weight (approximately 800,000 at 4°) as determined by gel filtration. However, at 37°, the activity elutes at a molecular weight of approximately 400,000, indicating that the 4° species is an aggregated complex of the 37° species (18, 20). It has been reported that the conversion from the 4° complex to the biologically active 37° species is not associated with a measurable change in conformation as detected by optical rotary dispersion (20).

The fact that most α-2-macroglobulins of serum are classically associated with serum proteinase inhibition or manifest some relationship to the complement system (or both) emphasizes the uniqueness of this α-2-opsonic protein, since it clearly lacks both of these characteristics. For example, recent studies by McLain *et al.* (19) indicate that this rat serum α-2-protein is independent of C3, C4, and C5 components of the complement system and is not associated with serum proteinase inhibition (19). Additionally, it also appears by immunological techniques that the protein from rat serum is not related to the rat plasma α-2-macroglobulin acute phase protein (19, 20). While one might suggest that this protein resembles the human α-2-macroglobulin with respect to method of isolation, molecular weight, and stability, purification of the analogous human opsonic protein has recently demonstrated that there is no cross-reactivity with monospecific antiserum to the human α-2-macroglobulin (36). Additionally, the serum levels of human α-2-macroglobulin are nearly 10-fold greater than the concentration of α-2-opsonic protein in both rat serum and human serum (21, 36). Although the α-2-macroglobulin and the presently isolated α-2-opsonic protein have similar molecular weights at 4°, the molecular weight of the α-2-opsonic protein at physiological temperatures is 400,000 (19, 21), which is approximately 50% of the molecular weight of the classical α-2-macroglobulin at comparable temperatures. Thus, these two proteins are distinct entities in both rat and man. Functionally, the dependence on heparin has been demonstrated to be involved in the ability of the opsonic protein to bind to particulates, although some evidence suggests that heparin may be involved in augmenting the direct phagocytic event (6, 17, 18, 20, 37, 38).

The present study demonstrates a high degree of purification of the rat α-2-opsonic protein as tested by immunochemical techniques. Excessive treatment of the protein with SDS and the reducing agent 2-mercaptoethanol failed to produce a significant quantity of a molecular species smaller than approximately 229,000 daltons, indicating either that the native subunit of this protein is a single polypeptide chain or that there are intermolecular disulfide bonds highly resistant to reduction by the method used. The fact that relatively gentle treatment of the protein with SDS and 2-mercaptoethanol produced a sharp band on SDS gels, whereas the unreduced protein treated with SDS consistently yielded a diffuse migration band suggests the production of a heterogeneous population. While the inclusion of a low concentration of 2-mercaptoethanol in all steps during the purification procedure helps to maintain the yield of biologically active protein, the
mechanism of this protection can only be speculated. This suggests the involvement of free sulfhydryl groups in the biological activity of this molecule.

The complete amino acid analysis of the purified α2-opsonic protein reveals a protein with no detectable methionine, a fairly high hydroxy amino acid content, and a strikingly low lysine but fairly high arginine content. The presence of glucosamine in the analysis of the opsonic protein confirms its classification as a glycoprotein, but whether its biological activity is dependent on the carbohydrate moiety is still unknown. The fact that we were unable to produce a molecular species less than approximately 229,000 indicates that the active 400,000- to 450,000-dalton species is probably made up of two of the monomeric polypeptide subunits held together by disulfide bonds. The tryptophan content expressed in mole per cent of bovine serum albumin as reported by Peters and Hawn (39) is nearly 50% of the amount found in the opsonic protein, while the tyrosine content is approximately the same in bovine serum albumin. This explains the difference in protein yields as measured by A280 measurements (tryptophan and tyrosine) as compared to the Lowry (25) method (tyrosine) based on albumin as a standard in both methods. Although there appears some similarity between the amino acid composition of the protein and human α2-macroglobulin, these proteins are different as judged by immunological and molecular weight studies. Moreover, the methionine content of the classical α2-macroglobulin is much greater than of the opsonic protein. Using the homogeneous protein as a standard for determining α2-opsonic protein levels by electrophoresis, we observed that in normal rat serum its concentration ranges between 650 and 750 μg/ml, which is distinctly less than the serum concentration of α2-macroglobulin and also much greater than the acute phase reactant α2-protein in normal serum.

The large molecular weight α2-opsonic protein as isolated in this study appears to be a previously uncharacterized rat serum protein from a biochemical and structural standpoint. Purification of the analogous human serum protein by the techniques described in the present study has led to an analogous protein, which is nearly homogeneous as revealed by gradient polyacrylamide gel electrophoresis and manifests high biological activity as measured in vitro (36). Further biochemical characterization of the human opsonic protein will be needed to determine whether this protein has been described but has not yet been assigned a function.

With respect to functional activities, the findings to date emphasize an important role in nonspecific host defense, especially with respect to macrophage function during tumor growth (12, 13) and following traumatic shock (14, 40). Its blood level as detected by bioassay appears to be related to Kupffer cell phagocytic activity and depletion or exhaustion (or both) of this protein appears most pronounced during advanced malignant disease as well as acutely after trauma in humans (3, 13, 14, 40). The isolation of the analogous human protein (36) and the development of the electrophoresis for the human protein will greatly facilitate the investigation of its biological significance and participation in disease processes.

Acknowledgment—The authors wish to thank Maureen Kaiser for secretarial assistance in the preparation of the manuscript.

REFERENCES
Isolation and biochemical characterization of alpha-2-opsonic glycoprotein from rat serum.
F Blumenstock, P Weber and T M Saba


Access the most updated version of this article at http://www.jbc.org/content/252/20/7156.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/252/20/7156.citation.full.html#ref-list-1