Purification and Characterization of Two Major DNA-binding Proteins in Human Serum*

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SALLIE O. HOCHÉ AND ELLA McVEY$§

From the Department of Microbiology, Scripps Clinic and Research Foundation, La Jolla, California 92037

The two major DNA-binding proteins (designated DNA-binding protein 1 and DNA-binding protein 2) in human serum have been purified and physically characterized. The two proteins co-purity through an ion exchange chromatographic step and DNA-cellulose affinity chromatography. Subsequently, DNA-binding protein 1 can be precipitated by 40% saturated ammonium sulfate; DNA-binding protein 2 precipitates in the 55% to saturation fraction. From these fractions, the two proteins are isolated by different protocols. Both purified proteins are homogeneous by the criteria of sodium dodecyl sulfate slab gel electrophoresis after reduction and denaturation and by sedimentation equilibrium centrifugation. DNA-binding protein 1 has a minimum molecular weight of 126,000; DNA-binding protein 2, 86,000. Amino acid analyses of the two proteins indicate that both are relatively rich in proline and cysteine and contain little methionine. Both proteins contain carbohydrate. Gel electrophoresis confirms the acidic nature of these proteins. DNA-binding protein 1 exhibits a single band upon isoelectric focusing, but DNA-binding protein 2 appears to be polymorphic, exhibiting three bands. NH₂-terminal end group analysis of DNA-binding protein 2 yields two major amino acids. DNA-binding protein 1 is an α-globulin as determined by immunoelectrophoresis; DNA-binding protein 2 is only weakly immunogenic. Neither of the proteins appears to be identical to any previously characterized serum protein.

This laboratory has previously reported the isolation of DNA-binding proteins in human serum using affinity chromatography on DNA-cellulose (1). Our procedure was designed to isolate the anionic DNA-binding proteins in serum. Our earlier work indicated that these proteins could be reproducibly isolated in quantity and that none of the proteins appeared to be identical to any previously characterized serum protein. The more than 20 species observed had a wide range of native molecular weights and comprised a significant fraction of the serum proteins. By radioimmunoelectrophoresis the proteins appeared to be α₁β-globulins (1), confirming that they were acidic. DNA binding was quantitatively assayed by nitrocellulose membrane filtration using human lymphocyte DNA. Using a similar membrane filter assay, Kubinski and Javid also reported the existence of DNA-binding proteins in human serum (2).

A modification of our original isolation procedure permitted a qualitative analysis of the distribution of the DNA-binding proteins in the sera of both normal and diseased individuals (3). The DNA-binding proteins isolated from 1-ml serum samples were analyzed by SDS-polyacrylamide slab gel electrophoresis. The most obvious change in the majority of serum samples from cancer patients was the appearance of a protein band of molecular weight about 36,000. This putative "malignancy-associated" protein has now been purified and characterized as a derivative of the third complement component (4). Several other changes were detected in the minor protein species. Throughout these studies of normal and abnormal sera, we consistently observed two predominant DNA-binding species. These two proteins have been designated DNA-binding protein 1 and DNA-binding protein 2, and their isolation and physicochemical characterization is the subject of this report.

EXPERIMENTAL PROCEDURES

Purification of DNA-binding Protein 1

All operations were carried out at 4°C and all buffers contained 1 mM 2-mercaptoethanol, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride.

Step 1: Quaternary Aminoethyl (QAE)-Sephadex Chromatography—A 250-ml sample of pooled adult human serum (Irvine Scientific Co.) was applied to a QAE-Sephadex column (11.5 x 20 cm) equilibrated in 10 mM potassium phosphate buffer, pH 6.5. The column was washed with the same buffer until the absorbance of the fractions, measured at 280 nm, approached zero. The column was then eluted with 10 mM potassium phosphate buffer, pH 6.5, containing 500 mM NaCl. The eluted protein was dialyzed for 64 h against 15 volumes of 10 mM potassium phosphate buffer, pH 6.8, containing 50 mM NaCl.

Step 2: DNA-cellulose Chromatography (Step Elution at pH 6.8)—The dialysate was applied to a DNA-cellulose column containing 1 g of DNA (approximately 100 g of DNA-cellulose). The column had been equilibrated in 10 mM potassium phosphate buffer, pH 6.8, and, after addition of the dialysate, was washed with this buffer until no further protein was eluted. The column was washed again with 10 mM potassium phosphate buffer, pH 6.8, containing 50 mM NaCl. The DNA-binding protein fraction was then eluted in 10 mM potassium phosphate buffer, pH 6.8, containing 500 mM NaCl.

Step 3: Ammonium Sulfate Fractionation—Solid enzyme-grade

1 The abbreviations used are SDS, sodium dodecyl sulfate; IgG, immunoglobulin G; dansyl, 5-dimethylaminonaphthalene-1-sulfonfyl fluoride.

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ammonium sulfate was added slowly to the DNA-binding protein fraction to 40% saturation. The solution was stirred overnight and then centrifuged for 15 min at 13,200 × g. The precipitate was removed by centrifugation at 100,000 rpm in 10 mM potassium phosphate buffer, pH 6.8, containing 50 mM NaCl, and dialyzed overnight against 50 volumes of this buffer.

**Step 4: DNA-cellulose Chromatography (Gradient Elution at pH 6.8)** — The dialysate was applied to a DNA-cellulose column containing 1.5 mg of DNA/mg of protein. The column was equilibrated in 10 mM potassium phosphate buffer, pH 6.8, containing 50 mM NaCl, and initially washed with 0.9 ml of buffer/g of DNA-cellulose after the adsorption of the protein sample. The adsorbed protein was eluted with a linear 0.05 to 0.4 M NaCl gradient (total volume = 9 ml of buffer/g of DNA-cellulose). DNA-binding protein 1 eluted at approximately 0.12 to 0.16 M NaCl. Samples from individual fractions across the gradient were applied to a 10% SDS slab gel to determine the distribution of DNA-binding protein 1. The peak tubes containing DNA-binding protein 1 were pooled and concentrated by the addition of ammonium sulfate to 55% saturation. The precipitate was collected by centrifugation for 15 min at 13,200 × g. It was resuspended at a concentration of 5 mg/ml in 10 mM potassium phosphate buffer, pH 7.8, containing 50 mM NaCl, and dialyzed overnight against 50 volumes of this buffer.

**Step 5: DNA-cellulose Chromatography (Gradient Elution at pH 7.8)** — The dialysate was applied to a DNA-cellulose column containing 2 mg of DNA/mg of protein. The column was equilibrated in 10 mM potassium phosphate buffer, pH 7.8, containing 50 mM NaCl; after adsorption of the protein sample, the column was washed with this buffer until no further protein was eluted. The adsorbed protein was eluted with a linear 0.05 to 0.4 M NaCl gradient (total volume = 9 ml of buffer/g of DNA-cellulose). The fractions containing DNA-binding protein 1 were again determined by SDS-slab gel electrophoresis. The peak tubes were pooled and concentrated by the addition of ammonium sulfate to 55% saturation. The precipitate was resuspended in 4 ml of 0.1 M Tris/HCl, pH 7.5, containing 0.1 M NaCl, and dialyzed overnight against 50 volumes of this buffer.

**Step 6: Sephadex G-200 Chromatography** — The dialysate was pumped through an upward flow G-200 column (1.6 × 80 cm) equilibrated in 0.1 M Tris/HCl, pH 7.5, containing 0.1 M NaCl.

**Purification of DNA-binding Protein 2**

All operations were carried out at 4°C and all buffers contained 1 mM 2-mercaptoethanol, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride. The first step (QAE Sephadex chromatography, DNA-cellulose chromatography, and ammonium sulfate fractionation) were as described in the purification of DNA-binding protein 1.

**Step 3: Ammonium Sulfate Fractionation** Ammonium sulfate was added slowly to the 40% ammonium sulfate supernatant to a concentration of 55% saturation. After 2 h, the solution was centrifuged at 13,200 × g for 15 min and the precipitate was discarded. The supernatant was saturated with ammonium sulfate and again stirred for 2 h before centrifugation. The precipitate was resuspended in a minimum volume (15 to 20 ml) of 10 mM potassium phosphate buffer, pH 6.8, and dialyzed overnight against 200 volumes of this buffer.

**Step 4: DNA-cellulose Chromatography** — The dialysate was applied to a DNA-cellulose column containing 5 mg of DNA/mg of protein. The column had been equilibrated in 10 mM potassium phosphate buffer, pH 7.8, containing 50 mM NaCl, and initially washed with 0.9 ml of buffer/g of DNA-cellulose after the adsorption of the protein sample. The adsorbed protein was eluted with a linear 0.05 to 0.4 M NaCl gradient (total volume = 9 ml of buffer/g of DNA-cellulose). DNA-binding protein 1 eluted at approximately 0.12 to 0.16 M NaCl. Samples from individual fractions across the gradient were applied to a 10% SDS slab gel to determine the distribution of DNA-binding protein 1. The peak tubes containing DNA-binding protein 1 were pooled and concentrated by the addition of ammonium sulfate to 55% saturation. The precipitate was collected by centrifugation for 15 min at 13,200 × g. It was resuspended at a concentration of 5 mg/ml in 10 mM potassium phosphate buffer, pH 7.8, containing 50 mM NaCl; after adsorption of the protein sample, the column was washed with this buffer until no further protein was eluted. The adsorbed protein was eluted with a linear 0.05 to 0.4 M NaCl gradient (total volume = 9 ml of buffer/g of DNA-cellulose). The fractions containing DNA-binding protein 1 were again determined by SDS-slab gel electrophoresis. The peak tubes were pooled and concentrated by the addition of ammonium sulfate to 55% saturation. The precipitate was resuspended in 4 ml of 0.1 M Tris/HCl, pH 7.5, containing 0.1 M NaCl, and dialyzed overnight against 50 volumes of this buffer.

**Step 5: DEAE-Chromatography** — The dialysate was adsorbed onto a Whatman DE52 column (0.9 × 5.0 cm) which had been equilibrated in 10 mM potassium phosphate buffer, pH 6.5. The column was washed extensively with this buffer before eluting DNA-binding protein 2 in 10 mM potassium phosphate buffer, pH 6.5, containing 30 mM NaCl.

**DNA Cellulose**

DNA-cellulose was prepared by the method of Litman (5), using acid-washed cellulose (Whatman CF11) and native calf thymus DNA (Worthington). The product averaged 10 to 12 mg of DNA/g of cellulose.

**Protein Determination**

Protein was measured by the method of Lowry et al. (6), using bovine serum albumin as a standard. Purified protein was quantitated using the extinction coefficients of DNA-binding protein 1 and DNA-binding protein 2.

**Electrophoresis**

SDS-polyacrylamide slab gel electrophoresis was carried out using the system of Laemmli (7) in the apparatus described by Studier (8); the gels were stained according to the procedure of Fairbanks et al. (9). The gels were 1.5-mm thickness. Protein samples were dialyzed exhaustively against 0.1 M sodium citrate, lyophilized, and resuspended in 10 mM sodium phosphate buffer, pH 7, containing 1% SDS and 1% 2-mercaptoethanol. The samples were boiled for 2 min immediately prior to application on the gel. Proteins used as standards to calibrate the gels were: unreduced human IgG (150,000); conalbumin (77,000); human IgG heavy chain (50,000); ovalbumin (43,000); human IgG light chain (25,500); and ribonuclease (14,000).

Analytical disc gel electrophoresis was carried out using the pH 8.7 Tris/glycine system of Saito et al. (10). Immunoelectrophoresis studies were carried out on microscope slides layered with 1% agar in 75 mM barbital buffer, pH 8.6. Electrophoresis was run at 175 V for 90 min in the same buffer.

**Isoelectric Focusing**

Gel electrofocusing was performed according to the method of Wrigley (11) using 6.0% acrylamide gel columns with 1% Ampholine (LKB Produkter). The pH gradient was preformed for 30 min at 400 V. The protein sample in 20% sucrose was applied beneath a layer of 10% sucrose and 1% Ampholine under the basic electrolyte solution. The gels were run for 8 h at 320 V (constant voltage). One gel was sliced into 5 mm segments for measurement of the pH gradient. The slices were suspended in tubes containing 1 ml of deionized water. The tubes were stored at room temperature before measurement of the pH values. Duplicate gels were fixed and stained overnight at 37°C in 3.5% perchloric acid and 0.04% Coomassie blue G (K & K Laboratories) according to the method of Reisner et al. (12). The gels were transferred to a solution of 7.5% acetic acid for 6 h at room temperature before photographing.

**Glycoprotein Stain**

SDS-polyacrylamide slab gels were stained for glycoproteins using the periodic acid-Schiff method of Kapitany and Zbrowa (13).

**Ultracentrifugal Analysis**

Sedimentation equilibrium experiments were performed in a Beckman-Spinco model E analytical ultracentrifuge equipped with a scanner, ultraviolet optics, and a multiplexer. Three samples were centrifuged simultaneously using 19 mm filled Epon double sector cells equipped with sapphire windows. The conditions used for sedimentation equilibrium are essentially the same as previously described (14). The sample was assumed to be at equilibrium when scans taken over the space of several hours showed no more variation than that expected from experimental error. At the end of a run, the speed was increased to 60,000 rpm to deplete the meniscus; after deceleration to the experimental velocity, measurements were made for base-line corrections. The slope of a plot of the natural logarithm of the optical density at 280 nm as a function of the radial distance squared was determined by the method of least squares on a programmable Hewlett-Packard 9100 A calculator. Solvent densities were measured by pycnometry.

**Amino Acid Analysis**

Amino acid analysis was performed with a Beckman model 120 amino acid analyzer using standard techniques. Samples of protein were extensively dialyzed against deionized water. The samples were lyophilized and dried in vacuo at 110°C over P2O5 to a constant weight. Weighed samples were hydrolyzed under reduced pressure in 6 N HCl for 24 and 48 h at 110°C. The hydrolysates were taken to dryness under vacuum and then dissolved in 0.2 M sodium citrate, pH 2.2. Cysteine and half-cystine were determined as cysteic acid after performic acid oxidation according to the method of Hirs (15).

**End Group Analysis**

The NH2-terminal amino acid was determined by dansylation, using the modified method of Weiner et al. (16). Two to three nano-
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moles of protein were suspended in 15 μl of 0.2 m NaHCO₃. A 2.5 mg/ml solution of dansyl-Cl (Regis Chemical Co.) in acetone was prepared and 15 μl was added to the tube containing protein. The solution was mixed and then incubated at 37° for approximately 1 h until the yellow color disappeared. The sample was dried in vacuo over NaOH pellets before hydrolysis in 50 μl of 6 n HCl at 110°. The hydrolyzed sample was dried before being suspended in a minimal volume of 50% aqueous pyridine. Five microliters of this sample were spotted on a polyamide plate (5 × 5 cm). Ascending chromatography was performed (17). The first dimension was run in 1.8% formic acid; the second was run perpendicular to the first in benzene:glacial acetic acid (6:1), and the third dimension was run in the same direction as the second in ethyl acetate:glacial acetic acid: methanol (26:1:1).

Preparation of Antisera

The serum of an adult male was used to isolate the DNA-binding proteins of a normal subject according to the method described earlier (1). The protein mixture was injected subcutaneously into a New Zealand white rabbit, using two injections of 3 mg of protein in complete Freund's adjuvant separated by a 1-month interval. The rabbit was boosted three times over a 6-month period, using injections of 1 mg of protein in incomplete Freund's adjuvant. One week following the second injection, and at 2-week intervals thereafter, 40-ml volumes of blood were collected from the rabbit and clotted. Serum was obtained from the clotted blood following centrifugation and was stored at -20°.

To enhance the antigenicity of DNA-binding protein 2, it was co-polymerized with bovine serum albumin using glutaraldehyde. Rabbits which had been previously sensitized to the albumin received subcutaneous injections of the co-polymerized albumin and DNA-binding protein 2 (0.5 mg of DNA-binding protein 2/injection) in incomplete Freund's adjuvant at 3-week intervals. Serum was obtained as described above.

RESULTS

Purification of DNA-binding Protein 1 — The two major serum DNA-binding proteins can be co-purified through the first two steps of the isolation scheme described under "Experimental Procedures." These steps are essentially the same as described by Brehm et al. (1) for isolating the entire class of DNA-binding proteins in serum. The addition of the 50 mM NaCl wash to the DNA-cellulose column removes contaminating albumin and immunoglobulin G from the DNA-binding fraction. An ammonium sulfate fractionation, the third purification step, separates the two major DNA-binding proteins, DNA-binding protein 1 and DNA-binding protein 2. Approximately 65% of the serum DNA-binding proteins are found with DNA-binding protein 1 in the 0 to 40% precipitate; and 10% with DNA-binding protein 2 in the 55% to saturation precipitate.

The proteins in the 0 to 40% precipitate are further fractionated by recycling through two successive DNA-cellulose columns at pH 6.8 and pH 7.8, using salt gradient elution. The DNA-cellulose chromatography at pH 7.8 produces two protein peaks as seen in Fig. 1. The second peak contains DNA-binding protein 1 plus some minor contaminants. The final purification step, if needed, is Sephadex G-200 chromatography. Beginning with the second DNA-cellulose column, the distribution of DNA-binding protein 1 is followed by SDS-polyacrylamide slab gel electrophoresis. A radioimmunoassay for DNA-binding protein 1 is being developed to be used to quantitate this protein throughout its purification. Approximately 50 mg of protein can be isolated from 250 ml of serum.

Purification of DNA-binding Protein 2 — The 55% to saturation ammonium sulfate fraction contains 0.4% of the serum proteins and consists mainly of DNA-binding protein 2 and albumin that has adsorbed nonspecifically to the DNA-cellulose column. A second DNA-cellulose chromatography to remove this residual albumin is necessary only if the first DNA-cellulose column is not washed with buffer containing 50 mM NaCl before the elution of the DNA-binding proteins. The final purification step, chromatography on DEAE-cellulose, removes all traces of the contaminating albumin. Throughout the purification the distribution of DNA-binding protein 2 is monitored by SDS-polyacrylamide slab gel electrophoresis. Fig. 2 is a composite of the gel patterns seen at each step of the purification. A single protein species is seen after reduction and denaturation. In acrylamide gel electrophoresis of the native protein in a pH 8.7 Tris/glycine system, a single protein band is also visible (Fig. 3). From 250 ml of serum we obtain 8 to 17 mg of purified protein. The purified protein is stored at -20° in buffer containing 50% glycerol (v/v) to minimize the aggregation seen after storage at 4° for extended periods or after repeated freezing and thawing in the absence of glycerol.

Glycoprotein Analysis — DNA-binding protein 1 and DNA-binding protein 2 are glycoproteins. Both contain glucosamine in 6 N HCl hydrolysates for amino acid quantitation. Both proteins give positive periodic acid-Schiff stains. A purified

Approximately 40% of the serum DNA-binding proteins are found with DNA-binding protein 1 in the 0 to 40% precipitate; and 10% with DNA-binding protein 2 in the 55% to saturation precipitate.

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sample of each protein was reduced and denatured in the presence of 1% 2-mercaptoethanol and SDS as described under "Experimental Procedures." Duplicate samples of each protein were subjected to SDS-polyacrylamide slab gel electrophoresis. Each gel was sliced longitudinally and one sample was stained for protein using Coomassie blue R (9) and the other

![Fig. 2 (left). SDS-polyacrylamide slab gel electrophoresis at each step of purification of DNA-binding protein 2 (DBP-2). The gel concentration was 10%. The samples are as follows: 1, QAE-Sephadex eluate (60 µg); 2, DNA-cellulose I eluate (60 µg); 3, 55% to saturation ammonium sulfate fraction (25 µg); 4, DNA-cellulose II eluate (25 µg); 5, DEAE-cellulose, 0.03 M NaCl eluate (10 µg).

Fig. 3 (right). Gel electrophoresis of purified DNA-binding protein 2. The gel conditions are described under "Experimental Procedures." 1, disc gel in pH 8.7 Tris/glycine system (62 µg); 2, SDS-polyacrylamide slab gel with Coomassie blue stain (10 µg); 3, SDS-polyacrylamide slab gel with periodic acid-Schiff stain (10 µg).](image)

for carbohydrate using the periodic acid-Schiff stain method (13). Fig. 3 shows the result for the duplicate samples of DNA-binding protein 2.

**Molecular Weight Determinations**—The molecular weight of DNA-binding protein 1 was determined by ultracentrifugation to be 128,000 ± 4,500 (assumed \( \bar{v} = 0.68 \) g/liter) in the presence of \( 0.1 \) M potassium phosphate buffer, pH 6.8, containing 1 mM 2-mercaptoethanol, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride. These data were obtained using three different concentrations of DNA-binding protein 1 (\( A_{280} = 0.097, 0.184, \) and 0.310) centrifuged at 10,000 and 15,000 rpm on successive days. In each case, the plot of \( \ln A_{280} \) versus the radial distance squared was linear, indicating a homogeneous preparation. The results of the determinations with the most concentrated sample are shown in Fig. 4.

The minimum subunit molecular weight for DNA-binding protein 1 was determined by SDS-polyacrylamide slab gel electrophoresis. Since glycoproteins may exhibit different apparent molecular weights depending on the gel concentration used (18), DNA-binding protein 1 was examined in the denatured but nonreduced state on a 7.5% SDS-polyacrylamide gel, and in the denatured and reduced state on 7.5% and 10% gels. The results from one such gel are shown in Fig. 5. There is no evidence of apparent molecular weight dependence on the gel concentration, nor was there any evidence of interchain disulfide bonds. The average molecular weight obtained from several determinations was 126,800 ± 3,400. This value is in good agreement with the ultracentrifuge data, indicating the absence of oligomers under the chosen buffer conditions for purification and stabilization of DNA-binding protein 1.

The native molecular weight of DNA-binding protein 2 was determined by ultracentrifugation to be 86,700 ± 3,200 (assumed \( \bar{v} = 0.70 \) g/liter) in the presence of \( 0.1 \) M potassium phosphate buffer, pH 6.8, containing 1 mM 2-mercaptoethanol, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride. These data were obtained using three different concentrations of DNA-binding protein 2 (\( A_{280} = 0.16, 0.24, \) and 0.32) centrifuged at 10,000 and 16,000 rpm on successive days. The most concentrated sample appeared to aggregate slightly by the

![Fig. 4. Left, equilibrium centrifugation of DNA-binding protein 1. The protein was centrifuged 2 h at 14,000 rpm; the speed was then decreased to 10,000 rpm and held to equilibrium (+—+). The temperature was 26°C. The 2nd day the speed was increased to 19,000 rpm and held to equilibrium (+—+). The temperature was 22.4°C. The protein sample had a starting \( A_{280} \) of 0.31. Right, equilibrium centrifugation of DNA-binding protein 2. The protein was centrifuged 95 min at 15,000 rpm; the speed was then decreased to 10,000 rpm and held to equilibrium (+—+). The temperature was 21.2°C. The 2nd day the speed was increased to 16,000 rpm and held to equilibrium (+—+). The temperature was 23.4°C. The protein sample had a starting \( A_{280} \) of 0.31. Both proteins were equilibrated in 0.1 M potassium phosphate, pH 6.8, containing 1 mM 2-mercaptoethanol, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride.](image)
second day of the experiment, as seen in Fig. 4, which shows the sedimentation equilibrium plots of this sample at the two different speeds. The aggregation of DNA-binding protein 2 was not unexpected under the conditions of the experiment.

The minimum molecular weight of DNA-binding protein 2 was also measured using SDS-polyacrylamide slab gel electrophoresis. The apparent molecular weight appeared to be dependent on the acrylamide concentration in the gel. The results with three gel concentrations are seen in Fig. 6. The weights found at 10 and 12.5% acrylamide concentrations are similar and approach the apparent minimum molecular weight. The average at these two gel concentrations was calculated to be 85,600 ± 1,600. This value agreed with the sedimentation equilibrium data. Furthermore, a sample of DNA-binding protein 2 analyzed by SDS-polyacrylamide slab gel electrophoresis without prior reduction showed no evidence of interchain disulfide bonds or higher molecular weight oligomers.

Amino Acid Analysis—The results for the amino acid composition of each protein are seen in Table I. The protein recoveries were based on dry weights determined for each sample as described under "Experimental Procedures." Samples of purified DNA-binding protein 1 were hydrolyzed for 24, 48, and 72 h prior to analysis; these analyses were repeated for 24 and 48 h prior to analysis. The amino acid composition was determined as described under "Experimental Procedures." The amino acid content of DNA-binding protein 1 indicates that it is an α₂-globulin.

Extinction Coefficients—A sample of each protein was extensively dialyzed against 0.1 M ammonium acetate buffer, pH 6.8. The protein sample was clarified by centrifugation before obtaining its spectrum. The sample was analyzed for protein by the Lowry (6) method, and another aliquot was dried to a constant weight at 110° over P₂O₅. The actual dry weight determined for DNA-binding protein 1 was 88% of that calculated from the colorimetric assay; a similar experiment gave an 88% value for DNA-binding protein 2. The E cos value at 277 nm for DNA-binding protein 1 was determined to be 11.5.

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<th>Amino acid</th>
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<th>DNA-binding protein 2</th>
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<tr>
<td>Lysine</td>
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* The amino acid composition was determined as described under "Experimental Procedures." Duplicate samples of DNA-binding protein 1 from two different batches of purified protein were hydrolyzed for 24, 48, and 72 h prior to analysis.

† Duplicate samples of DNA-binding protein 2 were hydrolyzed for 24 and 48 h prior to analysis.

‡ Half-cystine was determined as cysteic acid after performic acid oxidation (15).

The E cos value for DNA-binding protein 2 at 278 nm was determined to be 11.7. The extinction coefficients were based on dry weights determined for each protein. There were no absorption peaks in the visible range for either protein.

Isoelectric Focusing—Purified DNA-binding protein 2 yields a single band upon polyacrylamide gel electrophoresis in a pH 8.7 Tris/glycine system or in a system containing SDS. When this same protein is subjected to isoelectric focusing, three major bands are seen. The results for two different preparations of DNA-binding protein 2 are shown in Fig. 7. On the left is a sample of DNA-binding protein 2 isolated from pooled adult human serum (Irvine Scientific Co.); narrow range ampholytes from pH 6 to 8 were used. On the right, this same pattern is seen with DNA-binding protein 2 isolated from the pooled sera of term placentas using narrow range ampholytes from pH 5 to 7. Designating these bands sequentially from the most basic to the most acidic, the bands have isoelectric points of 6.09 ± 0.10, 6.03 ± 0.10, 5.91 ± 0.12. The relative intensity of the three bands varies with the individual preparations of DNA-binding protein 2.

Purified DNA-binding protein 1 was subjected to isoelectric focusing using a system that contained 6 M urea and pH 3.5 to 10 ampholytes. One major band with an isoelectric point of pH 7.01 was seen.

End Group Analysis—The NH₂-terminal amino acid of DNA-binding protein 2 was determined by dansylation of 2 to 3 nmol of DNA-binding protein 2 according to the modified procedure of Weiner et al. (16). Using protein that had been purified from pooled serum samples, two major NH₂-terminal amino acids, aspartate and glycine, were found.

Immunoelectrophoresis of purified DNA-binding protein 1 indicates that it is an α₂-globulin (Fig. 8). DNA-binding protein 1 reacts weakly in double diffu-
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Fig. 7 (left). Isoelectric focusing of DNA-binding protein 2. The samples are as follows: 1, DNA-binding protein 2 (40 µg) purified from pooled adult serum; the gel contained 1% Ampholine (pH 6 to 8). 2, DNA-binding protein 2 (60 µg) purified from pooled cord blood of term placentas. The gel contained 1% Ampholine (pH 5 to 7).

Fig. 8 (right). Immunoelectrophoresis of DNA-binding protein 1. Top, the top well contained 10 µl of whole human serum. Antiserum directed against whole human serum (Behring Diagnostics) was added to the center well immediately following electrophoresis and allowed to develop overnight. Bottom, the bottom well contained 30 µg of purified DNA-binding protein 1. Antiserum directed against human serum DNA-binding proteins (Behring Diagnostics) or α/β-lipoproteins (Miles Labs) or β-lipoproteins (Behring Diagnostics).

Immunoelectrophoresis of purified DNA-binding protein 2, using antisera directed against whole human serum (Behring Diagnostics) or against all of the serum DNA-binding proteins, failed to demonstrate a visible precipitin arc. To enhance the antigenicity of DNA-binding protein 2, the protein was co-polymerized with bovine serum albumin before injection into a rabbit previously sensitized to the albumin. The injections were repeated at 3-week intervals for 10 months. This procedure has resulted in a low titer antiserum that is being used to develop a radioimmunoassay specific for DNA-binding protein 2. However, the antiserum fails to show a precipitin line upon Ouchterlony double-diffusion or counterimmunoelectrophoresis with purified DNA-binding protein 2. When purified DNA-binding protein 2 is subjected to immunoelectrophoresis and simply stained for protein, it appears to migrate as a β-globulin.

Discussion

The two predominant DNA-binding proteins in human serum have been purified. The isolation procedure was so designed that both proteins could be obtained from the same serum sample. The basis of this scheme was the use of DNA-cellulose on a preparative scale early in the isolation procedure to separate the small percentage of DNA-binding proteins from the bulk of the serum proteins. Following the DNA-cellulose chromatography, ammonium sulfate fractionation effectively separated the two proteins from each other. Each protein was further purified by a combination of chromatographic techniques not involving extremes of pH, salt concentration, organic solvent exposure, etc., to minimize loss of the functional activities of the two proteins.

Both proteins have a rather large monomer molecular weight. Using analytical ultracentrifugation and SDS-polyacrylamide gel electrophoresis, DNA-binding protein 1 was calculated to have a minimum molecular weight of 126,000; and DNA-binding protein 2, 86,000. We have no evidence of oligomer formation with DNA-binding protein 1. However, DNA-binding protein 2 aggregates upon repeated freezing and thawing in the absence of glycerol. A comparison of the amino acid content of the two proteins indicates that both are relatively high in proline and cysteine and contain little methionine. The chromatographic behavior of the two proteins on anion exchange columns (QAE-Sephadex, DE52-cellulose) was indicative of the acidic nature of these proteins. The isoelectric focusing experiments were important in providing direct confirmation of this property. Using wide range ampholytes, DNA-binding protein 1 was calculated to have an isoelectric point of pH 7.8. The three species of DNA-binding protein 2 were all calculated to have isoelectric points near pH 6.0 using two different narrow range ampholytes.

The two proteins are not similar in all properties, however. DNA-binding protein 2 appears to be very weakly immunogenic. Antiserum produced against all the serum DNA-binding proteins exhibits a strong precipitin line with DNA-binding protein 1, but none with DNA-binding protein 2 in double diffusion experiments. Because of these results, the protocol for antiserum production was changed in an attempt to raise an antiserum specific for purified DNA-binding protein 2. DNA-binding protein 2 was co-polymerized with bovine serum albumin before injection into a rabbit previously sensitized to the albumin. This protocol has not been successful in produc-
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ing precipitating antibodies against DNA-binding protein 2. However, we have obtained a low titer antiserum that is suitable for use in a radioimmunoassay for DNA-binding protein 2 where the 125I-DNA-binding protein 2-antibody complex is collected on filters after reaction with Sepharose beads that are coupled to goat antirabbit IgG. These results may reflect a strong conservation of the amino acid sequence of DNA-binding protein 2 among mammalian species. We have also tested, by double diffusion, goat antiserum directed against the human serum β-globulins; the results were negative. Perhaps a nonmammalian species may be preferable for the production of antisera for DNA-binding protein 2, as for example in the production of antichromatin antibodies, where chickens proved the most useful (19). Another feasible approach is the use of a protein-DNA complex as the antigen by analogy to the production of antiserum against a mixture of non-histone proteins and DNA as described by Wakabayashi and Hnilica (20) or the production of antichromatin antibodies by Zardi et al. (21).

The combined results of the isoelectric focusing experiments and the end group analyses of DNA-binding protein 2 indicate the presence of polymorphic forms of this protein. The protein has been isolated from pooled adult serum and from pooled cord serum. Upon isoelectric focusing the same three bands of DNA-binding protein 2 were visible, but the relative intensity of each band varied with the starting material. It is now feasible to examine the DNA-binding protein 2 banding patterns from a large number of individual serum samples representing a cross-section of the general population. A protocol for the rapid isolation of the DNA-binding proteins from 1-ml serum samples is available (3, 22). The DNA-binding proteins of each individual can then be examined by multisample slab gel electrophoresis. If precipitating antibodies specific for DNA-binding protein 2 become available, individual serum samples could be used directly to explore the possible polymorphism of DNA-binding protein 2.

This communication has described the physicochemical characterization of two new serum proteins, but has not specified their function other than the general one of being able to bind to DNA. This is not a unique situation in the characterization of plasma proteins, where physical analyses have often preceded functional analyses (23). In this instance, however, the proteins to be characterized were not randomly chosen. Rather, they represented the major species of a new group of proteins selected by their affinity for an important macromolecule, DNA. The roster of known DNA-binding proteins in multicellular organisms has been greatly expanded in the past few years with particular emphasis on the non-histone chromosomal proteins for their role in controlling the process of gene transcription. Several excellent reviews have appeared summarizing the information known about these proteins (24-27). It is only recently that attention has been focused on the DNA-binding proteins in physiological fluids. Studies on the fluctuation in the levels of such proteins in the cerebrospinal fluid (28) and in the serum (3, 22) of diseased individuals have given indirect evidence of their importance. In the former study, Kubinski and Javid demonstrated the ability of cerebrospinal fluid proteins to bind DNA or the synthetic substrate, poly[d(C)] and measured the binding capacity of these proteins from a number of individuals (28). The authors did not determine the physiological function(s) of these proteins that react with nucleic acids. But they did indicate that the data demonstrated significant variations in the binding capacities of the cerebrospinal fluid proteins and that this procedure could be useful in the diagnosis of diseases of the central nervous system. Parsons and his co-workers have characterized a human serum DNA-binding protein which is found in elevated concentrations in the sera of a majority of the individuals tested who had malignant diseases (3, 22). This protein, designated C3DP, shows definite potential as a prognostic indicator in the treatment of malignant disease. Recently, Zardi and his colleagues have presented direct evidence that at least one serum protein has strong structural similarity to human fibroblast chromatin-associated proteins by use of antibodies directed against chromatin (29). Now that homogeneous preparations of the two predominant DNA-binding proteins in serum are available, determination of their specificity and function should be possible. Since these proteins are present at a high concentration in serum and they do bind DNA, they probably perform a significant function.

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REFERENCES


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