Association of Rat C-Reactive Protein and Other Pentraxins with Rat Lipoproteins Containing Apolipoproteins E and A1[†]

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ABSTRACT: C-Reactive protein (CRP) is a member of the pentraxin family of proteins, ubiquitous components of animal serum. This study suggests that, in serum, rat CRP is complexed with lipoprotein and may interact directly with apolipoprotein E. When mixed with diluted rat serum, radiolabeled rat CRP showed a slightly higher sedimentation coefficient (about 15%) than that of the free protein. Elimination of calcium or addition of *O*-phosphorylethanolamine (*O*-PE), a low molecular weight compound that binds tightly to rat CRP in a calcium-dependent manner, abolished this difference. Adsorption of rat serum on a rat CRP affinity gel and elution with PE resulted in the isolation of material containing high levels of apolipoproteins E and A1. The affinity-purified preparation interacted with rat CRP and altered the sedimentation coefficient of the latter to the value observed in whole serum. Conversely, rat CRP increased the sedimentation coefficient of the major component of the affinity-purified material to that of rat CRP in rat serum (about a 1.8-fold increase). When added to the affinity-purified material or to diluted rat serum, human serum amyloid P (SAP) and hamster female protein (FP), two other members of the pentraxin protein family, also had slightly higher sedimentation coefficients. In contrast, human CRP showed no evidence of an interaction in rat serum or with the affinity-purified proteins. This selectivity coincided with the ability of these pentraxins to bind to *O*-PE with high affinity. The sedimentation properties of serum lipoproteins, radiolabeled with [^{3}H]cholesterol, also suggested an interaction with rat CRP. Purified human apolipoprotein E interacted with rat CRP in a calcium-dependent manner that was inhibited by *O*-PE. These results indicated that rat CRP was associated with lipoproteins in serum and that the interaction may be achieved through apolipoprotein E. Association of pentraxins with lipoproteins may suggest possible functions for pentraxins in normal situations and may be important for certain pathological states, where some of the pentraxins as well as some lipoproteins have been implicated in the pathogenesis of amyloidosis.

Hamster female protein (FP),[1] rat C-reactive protein (rat CRP), human C-reactive protein (human CRP), and human serum amyloid P component (SAP) are members of the pentraxin family of serum proteins [reviewed in Baltz et al. (1982), Kolb-Bachofen (1991), and Kilpatrick and Volanakis (1991)], which are characterized by five subunits bound in cyclic symmetry. Two major branches include the CRP-type pentraxins, which have a high affinity for phosphorylcholine (PC), and the SAP-related pentraxins, which bind to carbohydrates such as those found on agarose (Kilpatrick & Volanakis, 1991; Gotschlich & Edelman, 1967; Volanakis & Kaplan, 1971; Anderson et al., 1978; Gotschlich et al., 1982; Pepys et al., 1978). The latter also obtain their name from their association with amyloid deposits (Pepys & Baltz, 1983; Coe & Ross, 1985). SAP was recently shown to bind with high specificity to *O*-phosphorylethanolamine (*O*-PE), while FP and rat CRP displayed a hybrid specificity and interacted equally well with phosphorylcholine (PC) or *O*-PE (Schwalbe et al., 1992). High-affinity binding to *O*-PE may be a useful method of characterizing the pentraxins since this interaction seems to correlate well with the ability of pentraxins to bind to various serum proteins (Schwalbe et al., 1992; also see this study). Protein conformational changes appear to occur upon complexing PC (Dong et al., 1992).

Although the precise functions of the pentraxins are still unknown, their importance is suggested by their extraordinary conservation in evolution [reviewed in Pepys and Baltz (1983)]. Depending on the species, some of the pentraxins undergo rapid alteration in serum levels following most forms of tissue damage [reviewed in Ballou and Kushner (1992)]. Human CRP is normally present in trace amounts but increases by up to 1000-fold during the acute phase response (Baltz et al., 1982; Morley & Kushner, 1992; Skinner & Cohen, 1988). In contrast, serum levels of human SAP are normally high (Pepys & Baltz, 1983; Skinner & Cohen, 1988) and undergo smaller changes during the acute phase response. Rat CRP is a major constituent of normal rat serum and is 2−3-fold higher in acute phase rat serum (Pontet et al., 1981; Nagpurkar & Mookerjea, 1981; DeBeer

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[1] Abbreviations: CRP, C-reactive protein; SAP, serum amyloid P component; FP, hamster female protein; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetate; C4BP, C4b-binding protein; apoE, apolipoprotein E; apoA1, apolipoprotein A1; *O*-PE, *O*-phosphorylethanolamine; PC, phosphorylcholine; HDL, high-density lipoproteins; LDL, low-density lipoproteins; VLDL, very low-density lipoproteins.

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et al., 1982c). In hamsters, pentraxin synthesis is controlled by sex hormones; the serum levels of FP in females are more than 100-fold greater than those in males (Coe, 1977).

Knowledge of the physical state of the pentraxins in serum may help to identify their biological functions. For example, human SAP forms a complex with C4b-binding protein (C4BP; Schwalbe et al., 1990), suggesting a possible role in regulation of complement.

While early studies suggested that human CRP was associated with lipids or lipoproteins (McLeod & Avery, 1941; Wood, 1963; Saito & Hara, 1968), more recent results suggest that interaction is limited to aggregated CRP (DeBeer et al., 1982b; Rowe et al., 1984a; Saxena et al., 1987) or abnormal lipoproteins (Rowe et al., 1984a,b). Rabbit CRP usually shows a complex with low-density lipoproteins (Pontet et al., 1979; Cabana et al., 1982), and affinity adsorption showed CRP-dependent removal of lipoproteins containing apolipoproteins E (apoE) and B (Rowe et al., 1984a). Interaction of rabbit pentraxins with lipoproteins may be of low affinity and is not detected by sucrose density gradient ultracentrifugation [reviewed in Pepys et al. (1985)]. Other studies suggested that rabbit CRP was uncomplexed (DeBeer et al., 1982a,b; Kushner & Somerville, 1970). Interaction of rat CRP and FP with lipoprotein was suggested by their ability to inhibit calcium-dependent heparin–lipoprotein precipitation (Nagpurkar & Mookerjea, 1981; Saxena et al., 1985, 1987b). However, others did not detect an interaction of FP in normal female hamster serum (Coe et al., 1981). In addition, SAP, and probably FP as well, binds to heparin (Schwalbe et al., 1991), which may influence the interpretation of these effects.

Recent evidence shows the presence of apoE (Namba et al., 1991) as well as SAP (Karalia & Grahovac, 1990) in human Alzheimer amyloid deposits. Furthermore, a correlation has been found between the expression of isotype E4 and the development of Alzheimer's disease (Strittmatter et al., 1993a). Pentraxins are components of other amyloid deposits as well. Thus, a direct interaction between these two protein families may provide the basis for a common contribution to amyloid formation.

This study presents the results of continued investigation into the physical state of various pentraxins in serum. In this case, rat serum was the primary experimental medium, and purified pentraxins from several species were investigated. Rat CRP was found to be associated with a component in rat serum that had the characteristics of lipoprotein(s) that were rich in apoE and apoA1. Rat CRP showed some interaction with purified human apoE, which further suggested that this may be an interaction site for rat CRP. The connection of pentraxins and lipoproteins suggests possible sites for pentraxin function. This relationship may also prove a mutual mechanism whereby these proteins might contribute to disease states characterized by amyloid deposits.

MATERIALS AND METHODS

Proteins. Rat CRP was purified with an *O*-phosphorylethanolamine affinity column (12-atom spacer, epoxy activated, from the Sigma Chemical Co.) essentially as described by Coe et al. (1981). Rat serum was purchased from Pel-Freez Biologicals. FP was prepared from female Syrian hamster serum by published procedures (Coe et al., 1981). Highly purified human SAP and human CRP were purchased from Sigma. Human apoE (>95% pure, supplier's estimate) isolated from very low-density lipoprotein and human apoA1 (>98% pure, supplier's estimate) from high-density lipoproteins were purchased from Calbiochem (San Diego). Rat serum was radiolabeled with cholesterol by standard procedures (Foreman et al., 1977). Briefly, a small volume of [^{3}H]cholesterol in ethanol solution (2 μL containing 1 mCi/mL [^{3}H]cholesterol, 53 Ci/mmol, DuPont NEN Research Products) was added to 0.25 mL of rat serum. The mixture was allowed to equilibrate at 37 °C for 60 min and was then stored at 0 °C.

The pentraxins and other proteins were radiolabeled by reductive methylation as outlined by Jentoft and Dearborn (1983). [^{14}C]Formaldehyde was purchased from DuPont NEN. Briefly, proteins (0.15−1.4 mg/mL) in 50 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl were mixed with 20 mM $NaCNBH_3$ (Sigma), followed by the addition of [^{14}C]formaldehyde (50 μCi/mg protein). The reaction mixture was allowed to stand at room temperature for 4 h and then dialyzed against repeated changes of 50 mM Tris buffer (pH 7.5) containing 0.1 M NaCl. The radiolabeled forms of FP and human CRP were further purified by affinity chromatography on *O*-phosphorylethanolamine−Sepharose. The radiolabeled pentraxins (100−200 μg) were applied to the column (0.5 × 2.5 cm) in buffer (50 mM Tris (pH 7.5)/0.1 M NaCl) containing 2 mM calcium. The column was washed to remove unbound materials, and the functional, radiolabeled proteins were eluted with buffer containing 4 mM EDTA. The degree of purity of the protein preparations was determined by the respective sedimentation patterns in sucrose density gradients (see the following).

Sucrose Density Gradient Ultracentrifugation. This procedure was performed in isokinetic gradients (McCarty et al., 1974). A convex gradient was generated by holding the volume in the mixing chamber constant (10 mL/tube). The initial solution in the mixing chamber was the starting buffer (10% (w/v) sucrose), and the reservoir contained 31% (w/v) sucrose. The sucrose that exited the mixing chamber was pumped into centrifuge tubes (14 × 95 mm polyallomer tubes, Beckman Instruments, Inc.). The tubes were stored on ice for 15−30 min, and samples containing the materials to be sedimented (0.3 mL) were applied to the top. The samples were centrifuged at 36 000 rpm for 26 h in a Beckman Model SW40 rotor in a Beckman Model L5-50 preparative ultracentrifuge. The samples were eluted by piercing the bottom of the tube and injecting 31% sucrose. Fraction one therefore represents the top of the gradient. The fractions were collected by drop counting and had a measured volume of 490 μL. Radioactivity was determined in a scintillation counter. The buffer used for sucrose density gradients was 50 mM Tris (pH 7.5) containing 0.1 M NaCl and 0.1% BSA (Sigma). Calcium, *O*-PE, and/or EDTA were added to the buffers as indicated.

Affinity Gel Chromatography. Although several affinity columns were prepared, a typical preparation consisted of 3 mL of Affi-gel 10 (Bio-Rad Laboratories) that was incubated with 83 mg of rat CRP in a total volume of 6.3 mL of 0.1 M MOPS buffer (pH 7.5). The mixture was agitated gently at 4 °C for 16 h and was then washed with 10 mL of cold water. Recovery of rat CRP in this wash indicated that 57 mg of protein was bound to the affinity gel. The column was washed extensively with 0.05 M Tris buffer containing 1.5 mM calcium, 0.1 M NaCl, and 5 mM *O*-PE, followed

by the same buffer without *O*-PE. For affinity purification, rat serum (32 mL) was diluted to 232 mL with Tris buffer containing 1.5 mM $CaCl_2$ and was passed through a column (2.5 × 10 cm) of *O*-PE affinity gel (described earlier). This step removed most of the rat CRP from the serum and improved the results of subsequent steps. The diluted, CRP-deficient serum was then applied to the rat CRP affinity gel column, which was eluted as described in Results.

Amino Acid Sequence. Microsequencing of peptides after gel electrophoresis was carried out with minor modifications of standard methods. The gel and buffers were prepared as described by Laemmli (1970) and used a running gel of 10% acrylamide containing 10% 2-mercaptoethanol. The gel was allowed to polymerize for 24 h before the sample was run. Material isolated by chromatography on the rat CRP affinity gel (0.375 mL of a fraction containing 0.30 mg of protein/mL) was mixed with an equal volume of electrophoresis sample buffer. After incubation at 25 °C for 12 h, the sample was loaded on the acrylamide gel and run at a constant amperage (25 A for stacking gel and 35 A for running gel). The gel was electroblotted to a poly(vinylene difluoride) (PVDF) sequencing membrane (Millipore Corp.) by placing the gel and PVDF membrane between layers of Whatman 3 mm paper in a Bio-Rad transfer blot cell and using 500 mA of constant current at 4 °C for 1 h. The PVDF membrane was stained for protein with Coomassie Blue (1%) in a solution of methanol (40%) and acetic acid (10%) for 10 min. After destaining for 10 min in methanol−acetic acid, the membrane was air-dried. Protein bands that stained with Coomassie Blue were cut and sequenced. Samples electroblotted to PVDF were sequenced directly on the membrane. The stained protein band was cut into 1 × 2 mm pieces and stacked on a precycled Biobrene-treated glass fiber filter in an Applied Biosystems Incorporated 477A pulsed-liquid sequencer with on-line PTH amino acid analysis. Sequencing was performed using reaction program BLOTT-1 (described in User Bulletin 42 from the manufacturer). The BLOTT-1 program was coupled to the conversion program NORMAL-1 and the gradient program NORMAL-1 (both supplied by the manufacturer). PTH amino acids were detected at 269 nm. Data were acquired and analyzed on a Waters Maxima 820 chromatography data station (version 3.31).

Other Methods. Autoradiography of acrylamide gels was carried out after the gels had been stained with Coomassie Blue and soaked in Fluoro-Hance solution (Research Products International Corp.) according to the manufacturer's directions. The gels were dried and exposed to X-ray film for appropriate time intervals to visualize radiolabeled areas. Proteins were quantitated by the Bradford technique (Bradford, 1976) using bovine serum albumin as the standard. Cholesterol was assayed by cholesterol oxidase using the total cholesterol assay kit (Sigma). Phosphorus was quantitated by the method of Chen et al. (1956). The standard buffer consisted of Tris (50 mM, pH 7.5) containing 0.1 M NaCl.

RESULTS

Evidence of a Pentraxin-Binding Material in Rat Serum. Radiolabeled rat CRP, in the presence of diluted rat serum, showed a slightly higher sedimentation coefficient than when *O*-PE was included in the buffer (Figure 1A). Many

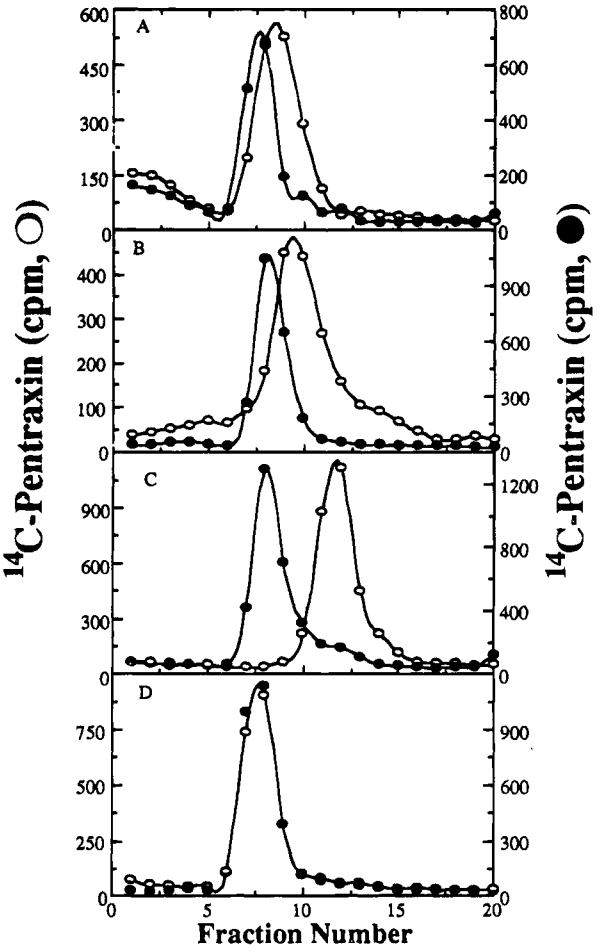

FIGURE 1: Physical state of pentraxins in rat serum. The proteins, added to 0.3 mL of sample containing 0.05 mL of rat serum, were ^{14}C-labeled rat CRP (A, 1.4 μg, 4133 cpm/μg), ^{14}C-labeled FP (B, 0.3 μg, 7783 cpm/μg), ^{14}C-labeled human SAP (C, 0.5 μg, 8900 cpm/μg), and ^{14}C-labeled human CRP (D, 0.33 μg, 10 000 cpm/μg). The two experiments in each panel represent sedimentation in the absence (○) and presence of *O*-PE (●, 500 μM, A−C) or PC (●, 500 μM, D) in the buffers. Calcium (2 mM) was included in all samples and buffers. The yield of radioactivity for the rat CRP samples was about 50%, while the other samples gave a yield of >70%.

auxiliary experiments were conducted throughout this investigation and the results are stated in the text. For example, in the absence of calcium, rat CRP sedimented at the position shown for the sample in Figure 1A that contained *O*-PE. This sedimentation position was also obtained when rat CRP, plus or minus calcium in the buffer, was sedimented without serum (data not shown).

This pattern of behavior was observed for hamster FP or human SAP when they were mixed with rat serum (Figure 1B,C). That is, there was a small calcium-dependent increase in sedimentation coefficient that was abolished when *O*-PE was included in the buffer. The sedimentation change for human SAP was larger (Figure 1C), which may suggest association with a different component, with multiple components, or with multiple copies of the same component. These possibilities were not pursued further. For the purposes of this investigation, the important observation was that all of these proteins appeared to form complexes with other components in rat serum. None of these proteins appeared to exist as free proteins.

In contrast, human CRP showed no calcium-dependent change in its sedimentation coefficient in the presence of

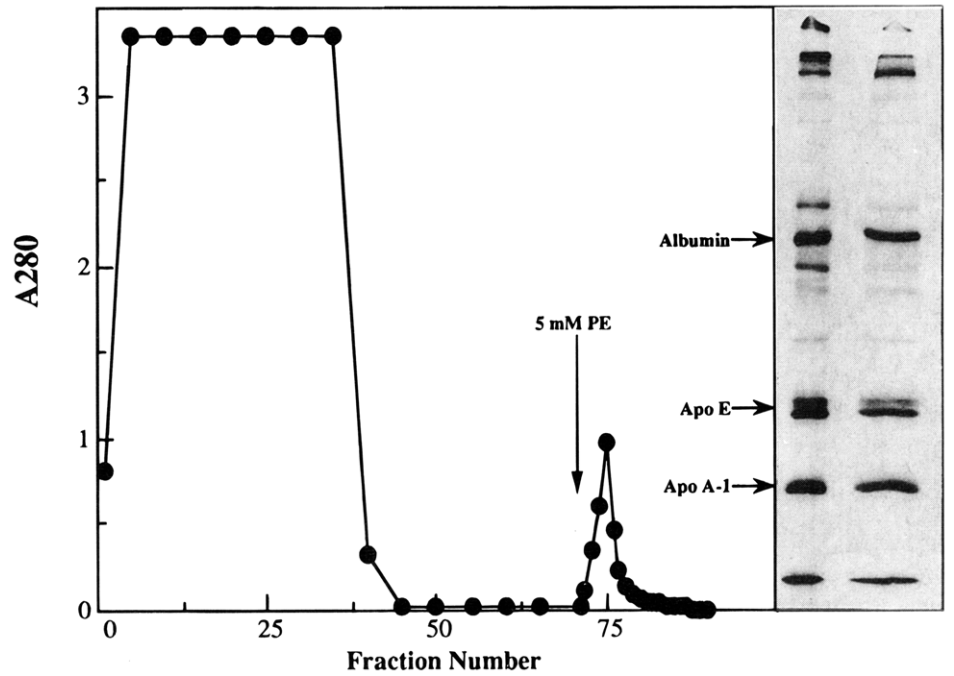

FIGURE 2: Affinity purification. Rat serum (32 mL) was diluted with buffer and depleted of CRP by passage through an affinity gel containing covalently bound *O*-PE, as described in Materials and Methods. This sample was then passed through a column (1 × 4 cm) containing 56 mg of covalently bound rat CRP, and the column was washed with buffer (5 mL per fraction in numbers 1−70). The column was then eluted with buffer containing 5 mM *O*-PE (started at fraction 71), and 1.5 mL fractions were collected. The absorbance at 280 nm is shown. Portions of fractions 75 and 76 were radiolabeled with $[^{14}C]CH_2O$ to obtain a specific activity of 49 900 cpm/μg of protein. Samples containing 2 μg of protein were subjected to gel electrophoresis. The gel was dried and analyzed by autoradiography as described in Materials and Methods. The gels show the results for fractions 75 (gel on the left) and 76 (gel on the right). The origin of the electrophoresis was at the top. The labeled albumin, apoE, and apoA1 were identified in companion samples that were run with non-radiolabeled protein and subjected to sequence analysis as described in Table 1.

Table 1: Amino Acid Sequence of the Affinity-Purified Proteins

A. Protein Band Migrating with a Nominal M_r = 35 000

1 10
Glu(92)[a,b]-Gly(153)-Glu(91)-Leu(112)-Glu(80)-Val(123)-Thr(47)-Asp(?)-Gln(103)-Leu(110)-
11 20
Pro(65)-Gly(106)-Gln(93)-Ser(36)-Asp(?)-Gln(80)-Pro(28)-Trp(7)-Gl u(41)-Gln(<64)-Ala(55)-

B. Protein Band Migrating with a Nominal M_r = 25 000

1 10
Asp(56)[a,c]-Glu(48)-Pro(48)-Gln(35)-Ser(14)-Gln(50)-Trp(11)-Asp(34)-Arg(56)-Val(22)-
11 20
Lys(16)-Asp(<26)-Phe(17)-Ala(23)-Thr(8)-Val(16)-Tyr(10)-Val(16)-A sp(?)-Ala(11)-

[a] The sequence was obtained as described in Materials and Methods. The numbers in parentheses are the yields in picomoles of the amino acid at the cycle indicated. [b] This sequence corresponds with 100% identity to that of rat apolipoprotein E precursor (McLean et al., 1983). [c] The sequence obtained corresponded with 100% identity to that of rat apolipoprotein A1 (Haddad et al., 1986).

rat serum (Figure 1D). The sedimentation position was not altered by the absence of calcium (data not shown) or the addition of PC or *O*-PE (Figure 1D). This result showed that the interactions documented for human SAP, hamster FP, and rat CRP were of high specificity. The latter proteins all share a high-affinity interaction with *O*-PE.

Affinity Purification with Rat CRP. Figure 2 shows the elution profile of rat serum that had been applied to a rat CRP affinity column. CRP-deficient rat serum was diluted and applied to the column. After extensive washes with buffer containing calcium, the column was eluted with buffer containing calcium and *O*-PE. A portion of the protein that eluted with *O*-PE was radiolabeled and subjected to gel electrophoresis. An autoradiogram of the resulting gel showed three major components that were present in all isolations (Figure 2), plus several minor components that varied from one preparation to another. Control experiments run with affinity gel that did not contain rat CRP produced only the component labeled albumin in Figure 2 (data not shown). The amino terminal sequence of this component corresponded to that of rat serum albumin. Thus, serum albumin appeared to be a nonspecific contaminant in this isolation.

The sequence of the major peptide components, M_r = 35 000 and 25 000), showed 100% identity with rat apoE and apoA1, respectively (Table 1). The molecular weights of these peptides also corresponded closely with those of rat apoE (34 000; McLean et al., 1983) and rat apoA1 (24 700; Haddad et al., 1986).

The affinity-purified preparation was assayed for protein, organic phosphate, and cholesterol and gave concentrations of 0.30 mg/mL, 0.14 mM (corresponding to 0.11 mg/mL if all were typical phospholipids of M_r = 775), and 0.162 mg/mL (or 0.42 mM), respectively. Thus, the affinity-purified material appeared to correspond to intact lipoprotein. The staining intensities of various proteins showed that these

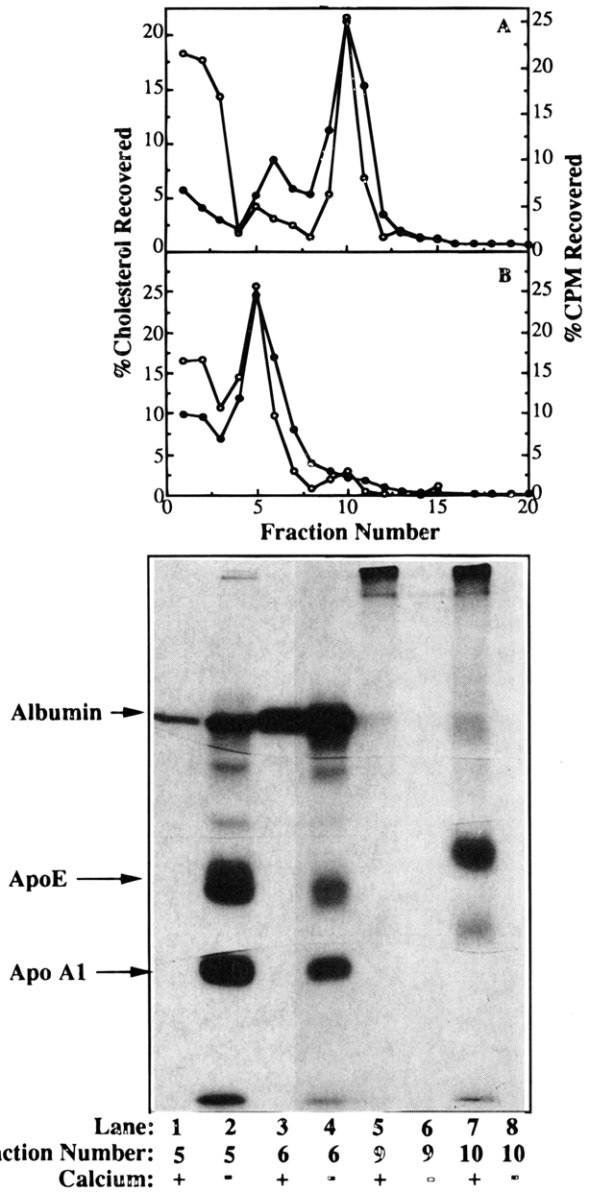

FIGURE 3: Interaction of the affinity-purified material with rat CRP. (A) Samples of the radiolabeled affinity-purified material (15 μg of peptide) described in the legend to Figure 2 were sedimented in a sucrose density gradient in the presence of 315 μg of rat CRP (0.3 mL sample) in buffer containing 1.1 mM $CaCl_2$ but no bovine serum albumin. (B) An identical sample of the radiolabeled material was mixed with 630 μg of rat CRP and sedimented in buffer containing 4 mM EGTA. A portion (0.05 mL) of each fraction was counted for ^{14}C content (●), and the results are plotted as the percent of recovered radioactivity. Total recovery of radioactivity was 41% in panel A and 56% in panel B. Panels A and B also show the cholesterol content of the fractions (○) expressed as the percent of recovered cholesterol in each fraction. The recovery was 40 and 60% for samples in panels A and B, respectively. (C) Fractions 5, 6, 9, and 10 from panels A (+calcium) and B (−calcium) were analyzed by gel electrophoresis and autoradiography as described in the legend to Figure 2.

lipoproteins were highly enriched in apoE and apoA1. The identities of the less abundant components of the lipoproteins were not pursued further in this investigation.

To further show that these peptides were part of the material that interacted with rat CRP, the affinity-purified, radiolabeled sample was subjected to sucrose density gradient ultracentrifugation in the presence of rat CRP (Figure 3A,B). BSA was omitted from the buffers in this experiment in order to allow subsequent gel electrophoresis. Omission of BSA gave lower yields of radioactivity after centrifugation (about 50% of the applied radioactivity) than when BSA was present (see the following). However, the results in Figure 3 show that the major components in the affinity-purified material showed a calcium-dependent shift in sedimentation from a midpoint at about fraction 5 to a midpoint at about fraction 9 (Figure 3A,B). The latter corresponds to the sedimentation position of rat CRP in rat serum (Figure 1A).

Samples from fractions 5, 6, 9, and 10 of the sucrose density gradients shown in Figure 3A,B were subjected to electrophoresis, and the gels were assayed by protein staining and autoradiography. In the presence and absence of calcium, serum albumin sedimented at fractions 5 and 6 (Figure 3C, lanes 1−4). The components identified as apoE and apoA1 sedimented at fractions 5 and 6 in the absence of calcium (Figure 3C, lanes 2 and 4) and were completely shifted to fraction 10 when calcium was present (Figure 3C, lane 7). These results showed calcium-dependent association of rat CRP with the affinity-purified lipoproteins and suggested that serum albumin was a noninteracting contaminant.

ApoE and apoA1 had slightly different migration positions in lane 7, which may result from the presence of precipitated material at the origin in lane 7. Precipitation also may have contributed to lower recoveries of radioactivity in these samples.

The fractions from sucrose density gradient ultracentrifugation were also assayed for cholesterol. The major peak of cholesterol shifted in the same calcium-dependent manner as the radiolabeled peptides (Figure 3A,B).

A number of other experiments were consistent with the conclusion that the affinity-purified material and the material in rat serum that altered the sedimentation behavior of rat CRP were identical. These experiments were similar to those in Figures 1 and 3 and will be outlined without presentation of the data. For example, in the presence of the affinity-purified protein, the sedimentation positions of rat CRP, FP, and human SAP were similar to those shown in Figure 1A−C. The altered sedimentation position was dependent on the presence of calcium, but was reversed by the presence of *O*-PE in the buffer. Conversely, the presence of these three pentraxins altered the sedimentation position of the radiolabeled, affinity-purified material in the manner shown in Figure 3A,B. The buffers used in these experiments contained BSA, and the recovery of radioactive protein was very high (>90% in all cases). Unlike the other pentraxins, but in agreement with the results in Figure 1D, human CRP showed no interaction with the affinity-purified material.

Sedimentation Pattern of Lipoproteins in Rat Serum. Figure 4A shows the sedimentation pattern of [^3H]cholesterol that had been added to rat serum. This method of radiolabeling lipoproteins (Foreman et al., 1977) involves no chemical modification and should monitor the behavior of native lipoproteins, albeit without a precise indication of the amounts of protein involved. That is, the cholesterol concentration will not correlate with the protein content of the lipoproteins. In any event, the sedimentation pattern of cholesterol in the presence of calcium was different from that obtained in the presence of EGTA or calcium plus *O*-PE (Figure 4A). The difference in the two experiments (Figure 4B) provided a clearer indication of the effect of CRP. Radiolabeled cholesterol was shifted out of fractions 1−5 and into fractions 7−11. The excess radioactivity in fractions

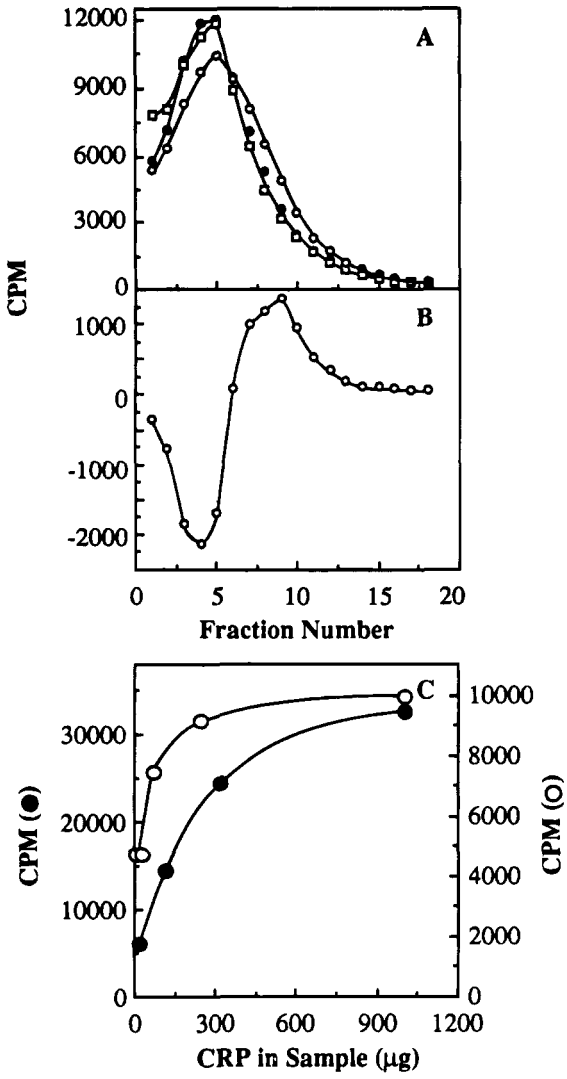

FIGURE 4: Impact of rat CRP on the sedimentation properties of serum lipoproteins and affinity-purified material. (A) Rat serum (20 μL, containing 98 000 cpm of [^{3}H]cholesterol) was diluted to 0.3 mL in standard buffer containing calcium (1.5 mM, ○), EGTA (2.5 mM, □), or calcium plus *O*-PE (1.0 mM, ●). These samples were applied to density gradients and sedimented by the standard method. The yields of radioactivity were identical (less than 1.5% variation) for the three experiments. (B) The radioactivity in fractions from the sample run in the presence of calcium and *O*-PE (A) was subtracted from the radioactivity in fractions of the sample containing only calcium and the difference was plotted. The peak of excess radioactivity in fractions 7−12 corresponded to 5.9 ± 1.1% of the total counts recovered (average and standard deviation for three experiments with two different samples of radiolabeled rat serum). (C) The experiments were run as in panel A (●), except that rat CRP was added to the samples to give the amounts shown. The excess radioactivity in fractions 7−12 was determined by the method described in panel B, and the result is plotted as a function of rat CRP in the sample. The second result shows a similar set of experiments that utilized the affinity-purified lipoproteins described in Figure 2 (4.0 μg of peptide, ○) as the source of lipoprotein. Yields of radioactivity ranged from 80 to 86%.

7−11 of Figure 4B corresponded to 7.0% of the total radioactivity. This excess ranged from 5.7 to 10% in replicate experiments and when different sedimentation runs (experiments minus calcium or plus *O*-PE) were subtracted as background. This amount of radioactivity probably underestimated the amount of lipoprotein that was complexed with rat CRP in whole serum. That is, the serum was diluted 15-fold in the sample applied to the gradient, and the complex had to survive the separation forces created by sedimentation. Thus, the results in Figure 4A,B suggest that a significant amount of the lipoproteins of rat serum was complexed with CRP.

Addition of excess CRP to the serum sample resulted in greater shifts of the radiolabeled lipoproteins. The amount of excess radioactivity in fractions 7−11 (as in Figure 4B) is plotted as a function of the amount of rat CRP added to the serum sample (Figure 4C). The first point of the titration curve represents the endogenous CRP in rat serum. The results indicated that the interaction of rat CRP with lipoproteins was saturable and involved a significant portion of the lipoproteins in rat serum. The fact that the highest levels of rat CRP used in the experiments (Figure 4) resulted in the transfer of only about 50% of the radiolabeled lipoprotein to the CRP peak suggested that the interaction involved only a portion of the lipoproteins. This would be expected if rat CRP bound to one specific peptide component of the lipoproteins, since only the population containing that peptide would show interaction.

The same type of experiment was carried out with the affinity-purified sample. In this case, the results suggested at least two types of interaction. About half of the maximum shift in cholesterol sedimentation was completed with only 10 μg of CRP in the sample. Much more rat CRP was needed to reach the maximum shift of cholesterol to the CRP peak (Figure 4C). Thus, at least a portion of the CRP interaction sites, including most of the sites in the affinity-purified material, appeared to be of adequate affinity to remain in a complex when the CRP concentration of the applied sample was only about 30 μg/mL. The serum concentration of rat CRP is at least 10-fold higher.

Association of Rat CRP with Human apoE. The major peptides in the affinity-purified material were apoE and apoA1 (see before). A preliminary indication of a direct interaction between these peptides and rat CRP was obtained by examining the interaction of CRP with highly purified human apolipoproteins. The commercial preparations of apoE (>95% purity, supplier's estimate) were radiolabeled by reductive methylation and analyzed by ultracentrifugation. The broad sedimentation pattern of human apoE in the presence of *O*-PE suggested two components, which may correspond to monomeric protein (fractions 3−4 of Figure 5A) and dimeric protein (peak at fraction 6). ApoE can self-associate, either by disulfide cross-linking (Weisgraber & Shinto, 1991) or by aggregation at 4 °C, the temperature at which the ultracentrifugation was run (Rall et al., 1986). The presence of serum albumin in all buffers appeared to prevent aggregation and allowed over 80% recovery of radiolabeled apoE after centrifugation. Without *O*-PE, a complex formed with CRP that shifted the majority of apoE to fraction 10 (Figure 5A).

The complex of rat CRP and human apoE that sedimented at fraction 10 (Figure 5A) was reversible. Addition of *O*-PE to material from fraction 10 of an experiment similar to that shown in Figure 5A and recentrifugation in buffer containing *O*-PE produced a sedimentation pattern similar to that shown in Figure 5A for the sample containing *O*-PE. In contrast to the interaction with apoE, no interaction between apoA1 and rat CRP could be detected (Figure 5B).

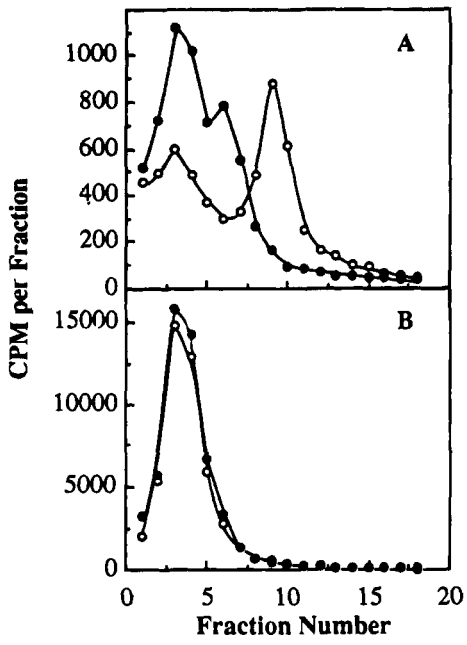

FIGURE 5: Interaction of rat CRP with purified human apoE. (A) Radiolabeled human apoE (8200 cpm, 3780 cpm/ug) was mixed with 500 μg of rat CRP in 0.3 mL of standard buffer containing calcium (1.5 mM, O) or buffer containing calcium plus *O*-PE (1.0 mM, ●). The samples were applied to the sucrose density gradient and centrifuged. Radioactivity in the fractions is shown. Recovery of radioactivity was 84% for the sample containing calcium and 78% for the sample containing both calcium and *O*-PE. (B) Experiments similar to those described in panel A were conducted in buffer containing 1.5 mM calcium (O) or 1.5 mM calcium plus 1 mM *O*-PE (●). Radiolabeled apoA1 (80 500 cpm, 1.35 μg) was used instead of apoE. Recovery of radioactivity was 65 and 60% in the samples with *O*-PE and without *O*-PE, respectively.

DISCUSSION

This study supported the general observation that pentraxins of many types are found in complex with other components of serum. The interaction sites appear to be very species specific, which may correlate with the very different behaviors of pentraxins in different species. For example, human SAP binds to C4BP (Schwalbe et al., 1990) and appears to exert a regulatory impact on complement (de Frutos & Dahlback, 1994). This study showed that rat CRP is bound to lipoproteins and may associate directly with apolipoprotein E. Diverse binding sites may suggest diverse functions in different species. This could, in turn, contribute to the difficulty of determining pentraxin function.

Despite the differences, some common properties of the interaction sites emerged. That is, hamster FP, human SAP, and rat CRP appeared to associate with the same components in rat serum. These pentraxins also display a high affinity for *O*-PE (Schwalbe et al., 1992). Among the mammalian pentraxins, human CRP appears unique with a binding site that is highly specific for phosphorylcholine (Schwalbe et al., 1992). Furthermore, human CRP did not interact to a detectable extent with either human C4BP (Schwalbe et al., 1990) or rat lipoproteins. Human CRP may be the only pentraxin that exists as a free protein in serum.

The current study suggested several levels of heterogeneity with respect to the rat CRP-binding sites in serum. First of all, binding sites may be heterogeneous with respect to affinity. Some sites were filled at low concentrations of CRP, while others required very high levels of CRP (Figure 4C). Consequently, the nature of affinity-purified ligands may vary with the stringency of the isolation procedure. Second, the high-affinity binding interactions may involve a single apolipoprotein that is distributed in several populations of lipoproteins. This was suggested by CRP-dependent depletion of cholesterol-labeled lipoproteins from a broad range of sedimentation positions (fractions 2−6, Figure 4B). Although the CRP−lipoprotein complex sedimented as a narrow peak, this could arise from coincident sedimentation of materials with diverse molecular weights and densities, if these parameters were offsetting with respect to sedimentation velocity. In fact, a surprising property was that the CRP−lipoprotein complex sedimented with a velocity only slightly different from free CRP. It appeared that partial specific volume was a major contributor to the sedimentation velocity of the complexes.

Preliminary studies suggest other levels of heterogeneity as well. For example, the original species used in this investigation was the hamster and its major pentraxin, FP. The sera of over 70 individual hamsters were examined by the addition of radiolabeled FP and centrifugation as for Figure 1.[2] Most individual sera gave sedimentation behaviors similar to those described for the rat (Figure 1). This suggested interaction with a component similar to that detected in rat serum. However, about 20% of these individual sera showed a significant portion of FP bound to a very slow sedimenting material that was found in fractions 1−3 of the sucrose density gradient shown in Figure 1. This would probably represent a lipoprotein of very low density. In a few cases, over 90% of the FP was found in these fractions. Whether these individual variations were specific to the hamster or whether the rat will show similar individual variations is not known. Since the rat serum was pooled from many individuals, a low level of individual heterogeneity might go undetected. Thus, future studies will be needed to determine possible individual as well as species variations in pentraxin behavior. However, knowledge of heterogeneity may assist in future definition of pentraxin function.

Interaction of pentraxins with lipoproteins has been suggested in previous studies, although the results have appeared inconsistent. Interaction between CRP and LDL or VLDL that contain apolipoprotein B or apoE have been reported (Pontet et al., 1979; Cabana et al., 1982). The greatest correlation appeared for apolipoprotein B (Rowe et al., 1984a). In the rabbit, an interaction with HDL was also reported (Pontet et al., 1979), but in less detail. Affinity columns containing rat CRP have been reported to selectively remove the populations of human HDL that contained apoE (Saxena et al., 1987a). Preliminary results in this study showed the interaction of rat CRP with isolated human apoE. In fact, high levels of apoE and apoA1 appeared to be the most unique feature of the affinity-purified material.

Despite the problems posed by the apparent heterogeneity of the lipoproteins that bound rat CRP and the variation in behavior with species and even individual animal, a definite interaction between pentraxins and lipoproteins suggests that they may be involved in modulating lipoprotein behavior under normal or pathological conditions. A new possible example of normal function is suggested in the accompanying paper, which shows that lactic acid is capable of dissociating

[2] R. A. Schwalbe, J. E. Coe, and G. L. Nelsestuen, unpublished data.

pentraxins from serum-binding sites (Evans & Nelsestuen, 1995). Consequently, serum levels of lactic acid may influence lipoprotein metabolism in rat and hamster. Another normal pentraxin function would arise in the acute phase, where pentraxins undergo large changes in concentration (Cabana et al., 1989). These changes, in turn, may contribute to the large changes in lipoprotein content that are observed in the acute phase. Although rat CRP levels change by only a few fold during the acute phase, its initial concentration is quite high and so the quantitative change is large (DeBeer et al., 1982c). The negative regulation of FP in female hamsters during the acute phase could have significant effects on lipoprotein metabolism as well (Coe & Ross, 1983). Pathological effects of pentraxins are suggested by the high levels of FP in the female Syrian hamster, which appear to be correlated with a high incidence of amyloid formation (Coe & Ross, 1983, 1985). Although human SAP is associated primarily with C4BP rather than with lipoprotein, it is possible that SAP interaction with lipoproteins occurs at a low level or under special circumstances. Lipoproteins become constituents of amyloid deposits, including apolipoprotein E (Namba et al., 1991; Strittmatter et al., 1993a,b). In any event, a direct interaction between pentraxins and lipoproteins may suggest a common point for involvement in amyloid formation.

Although unambiguous functions are yet to be identified, this study showed species-specific interaction of pentraxins with lipoproteins. Future clarification of this behavior and its possible involvement in other species may assist in determining pentraxin function.

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