Dissociation of Serum Amyloid P from C4b-Binding Protein and Other Sites by Lactic Acid: Potential Role of Lactic Acid in the Regulation of Pentraxin Function†

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ABSTRACT: Serum amyloid P (SAP) and C-reactive protein (CRP) are two members of the pentraxin family of proteins. These proteins associate with a variety of other materials that are found in serum under normal or pathological circumstances. This study showed that carboxylated compounds, especially lactic acid, were capable of dissociating pentraxins from several macromolecular binding sites. When measured by sucrose density gradient ultracentrifugation, complete dissociation of the complex of hSAP (human SAP) with C4b-binding protein (C4BP) occurred at ≥5 mM lactate. Lactate dissociated the hSAP−membrane complex and prevented hSAP self-association. The only interaction that was not dissociated by 10 mM lactate was the hSAP−heparin complex. The relative efficacies of several dissociating agents were *O*-phosphorylethanolamine > lactate > succinate > carbonate > ϵ-amino-*n*-caproic acid. This suggested that the carboxyl group plus a hydrogen-bonding site on the hydrocarbon chain was important, but a charged amino group was not a contributor to function when the anion was provided by a carboxyl group. The concentration of lactic acid needed to dissociate hSAP from C4BP was dependent on protein concentration in a manner suggesting the cooperative binding of lactate (coefficient = 2) to hSAP. Pure proteins, at concentrations found in normal serum, required about 12 mM lactate for half-dissociation of the hSAP−C4BP complex. Other pentraxins also interacted with lactic acid, but with lower affinities. An important observation was that lactic acid was capable of dissociating rat CRP from lipoproteins in rat serum. Human CRP bound very weakly to lactate, so that lactate probably is not a significant regulator of this pentraxin. Overall, these results suggest that lactate may be a biological regulator of the functions of at least some pentraxins in normal and/or pathological situations.

Serum amyloid P[1] (SAP) is a member of the pentraxin family of proteins, which also includes C-reactive protein (CRP) [for reviews, see Pepys and Baltz (1983) and Skinner and Cohen (1988)]. While the functions of these proteins are not clearly defined, many known properties of these molecules suggest that they may be involved in various aspects of the immune response. For example, pentraxins are species-specific acute phase reactants. In humans, the CRP concentration will increase by up to 1000-fold during the acute phase (Morley & Kushner, 1982; Skinner & Cohen, 1988). While SAP increases by only a few fold, its normal levels are quite high and the actual increases in SAP are substantial.

Many functions of pentraxins are implied by their interaction properties. For example, SAP will interact with a number of intracellular components, such as anionic phospholipids (Schwalbe et al., 1990, 1991), DNA, or chromatin (Breathnach et al., 1989; Butler et al., 1990; Pepys & Butler, 1987) and may have a role following cell damage. SAP also binds to heparin and other sulfated polysaccharides (Hamazaki, 1987, 1989; Nagpurkar & Mookerjea, 1981; Schwalbe et al., 1991), as well as to certain phosphorylated compounds (Schwalbe et al., 1992). CRP binds to chromatin (Du Clos et al., 1991; Robey et al., 1984) and to bacterial polysaccharides that contain phosphorylcholine (Anderson et al., 1978; Coe, 1977; Coe et al., 1981; Gotschlich & Edelman, 1967; Gotschlich et al., 1982; Schwalbe et al., 1992; Volanakis & Kaplan, 1971). Rat CRP appears to be associated with lipoproteins and may associate with apolipoprotein E (Schwalbe et al., 1995). The recent finding that solution phase SAP forms a complex with C4b-binding protein (Schwalbe et al., 1990) suggested that it may impact on C4BP, a known inhibitor of complement (Fujita et al., 1978; Shiraishi & Stroud, 1975). In fact, the SAP−C4BP complex was reported to be less effective as an inhibitor of complement than C4BP alone (de Frutos & Dahlback, 1994). In various pathological states, pentraxins such as human SAP and hamster FP are associated with amyloid deposits (Coe & Ross, 1985; Pepys & Baltz, 1983; Pepys et al., 1979).

Regulation of pentraxin functions might be accomplished by molecules that cause the dissociation of pentraxins from other binding sites. For example, agents that cause dissociation of SAP from C4BP should restore C4BP function and result in reduced complement fixation. Candidates for regulators of SAP include heparin and *O*-phosphorylethanolamine (*O*-PE), which bind to SAP with high affinity and dissociate the SAP−C4BP complex. Unfortunately, the physiological relevance of *O*-PE in serum is questionable, and *O*-PE structure may only mimic the SAP-binding site

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[1] Abbreviations: C4BP, C4b-binding protein; SAP, serum amyloid P component; CRP, C-reactive protein; FP, hamster female Protein; rCRP, rat CRP; SUVs, small unilamellar vesicles; hCRP, human CRP; BSA, bovine serum albumin; hSAP, human SAP; *O*-PE, *O*-phosphorylethanolamine; PhChol, phosphorylcholine; GABA, γ-aminobutyrate; lyso-PC, L-α-lysophosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycolbis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; PS, L-α-phosphatidyl-L-serine; PC, L-α-phosphatidylcholine.

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on biological receptors (Schwalbe et al., 1992). In any event, the identification of relevant molecules that regulate SAP interactions may aid in the eventual determination of SAP function.

In the course of investigating SAP interactions, we have found that lactic acid, at levels that may be encountered, was capable of dissociating SAP from nearly all of its interaction sites. This report describes lactic acid binding and aspects of its mechanism and demonstrates how these properties might serve as regulators of SAP function.

MATERIALS AND METHODS

Materials

Bovine brain phosphatidylserine and egg yolk phosphatidylcholine were purchased from Sigma Chemical Co. and were of the highest purity available (≥98%, supplier's estimate). Human SAP was either purchased from Sigma or purified as described in the following. Human CRP was purchased from Sigma. Rat C-reactive protein was purified as described previously (Coe et al., 1981). Rat serum was from Pel-Freeze Biologicals. ^{14}C-labeled formaldehyde (1 mCi/mL) was purchased from DuPont-NEN. Cryo-poor plasma was obtained from the Memorial Blood Center of Minneapolis. Unless otherwise stated, experiments were performed at 22 ± 2 °C, and all aqueous solutions were buffered with 50 mM Tris (pH 7.5) containing 100 mM NaCl.

Mehods

Protein Purifications. Human SAP was purified by published procedures (Dahlbäck, 1983a,b). Five units (1.3 L) of cryo-poor plasma was centrifuged at 8500*g* for 1 h. Lipid on the surface of the liquid was removed by aspiration, and benzamidine was added to 10 mM. A solution of $BaCl_2$ (1 M, 0.1 vol) was added slowly with stirring. After 2 h, the mixture was centrifuged at 5300*g* for 10 min. The pellet was washed three times by suspension in 0.08 vol of 0.9% NaCl and 5 mM benzamidine followed by centrifugation. Finally, the pellet was dissolved in 0.2 M EDTA (pH 7.4) containing 10 mM benzamidine (150 mL/L starting plasma). This solution was dialyzed extensively against 100 mM sodium phosphate (pH 6.0) containing 10 mM benzamidine. The dialysate was applied to a DEAE-Sephadex column (5 × 14 cm), which was eluted with a 1.2 L gradient of NaCl (0.1−0.7 M) in phosphate buffer. The first peak of protein to be eluted was pooled and dialyzed against 50 mM Tris (pH 7.5) containing 1 mM benzamidine. The solution was made 2 mM in $CaCl_2$ and applied to a heparin−agarose column (2.5 × 8 cm) that had been prepared by published methods (Parikh et al., 1974). A 600 mL gradient of NaCl (0−0.7 M) in the same buffer was used to elute the column. Next, SAP was eluted with a solution of 10 mM EDTA (pH 7.5) containing 1 M NaCl. The purified SAP was analyzed by SDS−polyacrylamide gel electrophoresis. Staining with Coomassie Blue showed a single component that migrated with standard SAP (Sigma).

C4BP was purified essentially as described previously (Dahlbäck, 1983a,b). The $BaCl_2$ precipitation, EGTA solubilization of the pellet, and DEAE-Sephadex column chromatography were as described for hSAP purification. The first peak of protein to be eluted from the DEAE-Sephadex column was pooled and dialyzed against 50 mM Tris (pH 7.5) containing 1 mM benzamidine. The solution was made 2 mM in $CaCl_2$ and applied to a heparin−agarose column. A 600 mL gradient of NaCl (0−0.7 M) in the same buffer was used to elute the column. The major protein peak was pooled and dialyzed against standard buffer. The dialysate was concentrated by pressure dialysis, and solid guanidine hydrochloride was added to a concentration of 3 M. The sample was applied to an S-300 HR column (2.5 x 118 cm) equilibrated in buffer containing 3 M guanidine hydrochloride. The fractions from the major peak of protein were pooled.

Rat CRP and human SAP were radiolabeled by reductive methylation as described (Jentoft et al., 1979; Schwalbe et al., 1992). The protein (1 mg/mL), in a solution of 50 mM phosphate buffer (pH 7.0) containing 100 mM NaCl, was made 20 mM in $NaCNBH_3$. ^{14}C-labeled formaldehyde (50 μCi/mg protein) was added, and the reaction was allowed to proceed at room temperature. After 4 h the reaction solution was dialyzed against buffer to remove unreacted reagents.

Phospholipid Vesicles. Small unilamellar phospholipid vesicles (SUV) were prepared by published methods (Bazzi & Nelsestuen, 1987; Huang, 1969). The appropriate phospholipids were mixed in organic solvent, which was evaporated by a stream of nitrogen. The dried phospholipids (5 mg/mL) were resuspended in 3 mL of buffer. The solution was sonicated with intermittent bursts for a total sonication time of 6 min using a Heat System-Ultrasonics W-385 sonicator. The solution was fractionated by gel filtration chromatography. Fractions containing SUVs were pooled. Lyso-PC was incorporated into preformed vesicles by the addition of a small amount (<1% of the sample volume) of a solution of lyso-PC in ethanol. Phospholipid concentrations were determined using the phosphorus assay (Chen et al., 1956) and assuming a phosphorus to phospholipid weight ratio of 1:25.

Light Scattering. Light scattering at 90° to the incident light and at 320 nm was used as described previously (Nelsestuen & Lim, 1977). A SPEX Fluoromax, with both excitation and emission monochromators set at 320 nm, was used to detect light scattering. For protein interaction with phospholipid vesicles, the components were mixed in the standard buffer. Protein binding to the vesicles was detected by excess light scattering by the solution. Light scattering and protein−membrane binding are related by

$$I_2/I_1 = (M_2/M_1)^2[(dn_2/dc_2)/(dn_1/dc_1)]^2 \qquad (1)$$

I_2 is the light-scattering intensity of the protein−vesicle complex, and I_1 is the light-scattering intensity of the vesicles alone. These values were corrected for light scattering by buffer and free protein and for dilution. M_1 and M_2 are the weight-average molecular weights of the vesicles before and after protein binding, respectively, and $(dn_2/dc_2)/(dn_1/dc_1)$ is the ratio of refractive index increments of the two species (Nelsestuen & Lim, 1977).

For comparative experiments, the light-scattering intensity change was expressed on the relative basis shown in eq 2. The protein and vesicles were mixed, and calcium was added to cause protein−membrane binding. The light-scattering intensity (I_{max}) from this solution was measured. Aliquots of agents that dissociated the complex were added, and

intermediate scattering intensities were measured (I). Finally, excess EGTA was added and the final light-scattering intensity (I_{EGTA}) was obtained. EGTA caused complete dissociation of the interactions studied.

$$\% \text{ binding} = (I - I_{EGTA})/(I_{max} - I_{EGTA})100 \quad (2)$$

Equation 2 was also used to express light scattering of hSAP−C4BP complexes and their dissociation by lactic acid. The light-scattering measurements gave a signal to noise ratio of at least 20:1.

Sucrose Density Gradient Ultracentrifugation. Ultracentrifugation in isokinetic sucrose density gradients was performed as described previously (McCarty et al., 1974). The standard buffer contained 50 mM Tris (pH 7.5), 100 mM NaCl, 0.1% bovine serum albumin, and 1.5−2 mM $CaCl_2$. Lactate was added as indicated. The sucrose density gradient was generated by maintaining a constant volume of buffer in the mixing chamber (10 mL/tube containing 10% (w/v) sucrose) of a gradient maker. The reservoir contained 30% (w/v) sucrose. Gradients were poured into centrifuge tubes (14 × 95 mm polyallomer tubes, Beckman Instruments, Inc.), which were then stored on ice for 15−30 min. Samples (0.4 mL) were mixed in buffer and incubated at 37 °C for 30 min before being applied to the tops of the tubes. The tubes were centrifuged at 36 000 rpm for 26 h in a Beckman Model SW40ti rotor using a Beckman Model L5−50 ultracentrifuge. Fractions (18 drops/fraction) were collected from the top of the tube by pumping a solution of 31% sucrose into the bottom. Liquid scintillation fluid was added and the radioactivity in each fraction was determined with a Beckman LS 5000 TD liquid scintillation counter.

Modeling of Dissociation. The reactions were assumed to consist of two independent and mutually exclusive equilibria:

$$K_{dC4BP} = \frac{[SAP][C4BP]}{[SAP{-}C4BP]} \qquad K_{dLA} = \frac{[SAP][lactate]^n}{[SAP{-}lactate_n]} \quad (3)$$

Since [SAP] appears in both equations and its value is very low due to the high affinity of the interactions, these equations can be combined:

$$\frac{[SAP{-}lactate_n]}{[SAP{-}C4BP]} = \left(\frac{K_{dC4BP}}{K_{dLA}}\right)\left(\frac{[lactate]^n}{[C4BP]}\right) \quad (4)$$

The [lactate] required for half-dissociation of the hSAP−C4BP complex ([SAP−lactate]/[SAP−C4BP] = 1) was determined for a reaction with known concentrations of all components. This value was used to solve eq 4 to obtain the ratio of K_d values for different values of n. Entire dissociation curves were then generated from the K_d ratios and values of n using eq 5. The equation was solved for 20

$$[\text{lactate}] = \sqrt[n]{\left(\frac{\% \text{ free C4BP}}{100 - \% \text{ free C4BP}}\right)\left(\frac{K_{dLA}}{K_{dC4BP}}\right)[C4BP]_{tot}\left(\frac{\% \text{ free C4BP}}{100}\right)} \quad (5)$$

different lactate concentrations, and theoretical curves were drawn through these points. In eq 5, [lactate] refers to the lactate concentration at the proportions of free C4BP (% free C4BP), hSAP-bound (100−% free C4BP) C4BP, and total C4BP ($[C4BP]_{tot}$) in the solutions.

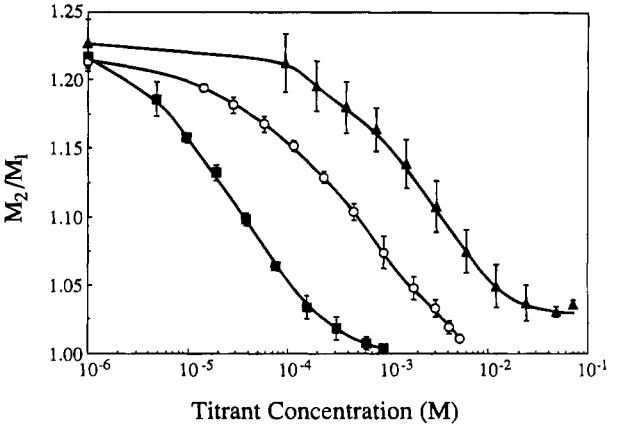

FIGURE 1: Dissociation of hSAP from phospholipid vesicles. Human SAP (5 μg) was mixed with SUVs (13.25 μg, PS/PC, 30:70) in 1 mL of standard buffer containing 2 mM $CaCl_2$. Sequential additions of acetate (▲), lactate (○), and O-PE (■) were made, and the light-scattering intensity of the solution was monitored. Protein−vesicle association is reported as M_2/M_1, calculated from light-scattering intensities by eq 1. The midpoints for dissociation of the protein−vesicle complexes for acetate, lactate, and O-PE were 3.0, 0.40, and 0.035 mM, respectively. The error bars represent the standard deviations of three separate experiments.

Other Methods. SDS−PAGE was performed as described by Laemmli (1970). Protein concentrations were determined by using the dye binding assay described by Bradford (1976) with BSA as the standard. Alternatively, C4BP was quantitated by absorbance at 280 nm using an extinction coefficient for a 1% solution of 14.1 (Perkins et al., 1986).

RESULTS

Dissociation of Human SAP from Phospholipid Vesicles. Human serum amyloid P component (hSAP) associates with phospholipid vesicles in a calcium-dependent manner (Schwalbe et al., 1990, 1991). This complex can be dissociated by competitive ligands such as phosphate esters (Schwalbe et al., 1992). Figure 1 shows this effect, as well as that of several carboxylate compounds. As expected, O-PE was the most effective with half-dissociation at 0.035 mM. Simple carboxylates such as acetate were also capable of dissociating the complex (midpoint of 3.0 mM). Lactic acid was the most effective carboxylate tested with half-dissociation at 0.4 mM. Even bicarbonate dissociated the hSAP−phospholipid interaction with a midpoint of 7 mM (Table 1). While the presence of an amine greatly promoted the efficacy of phosphorylated compounds (e.g., O-PE; Schwalbe et al., 1992), they greatly reduced the efficacy of carboxylates. More than 18 mM GABA or ε-amino-n-caproic acid was required to cause half-dissociation of the hSAP−phospholipid complex (Table 1). Thus, carboxylates were effective in dissociating hSAP from phospholipids, divalent carboxylates such as succinate were no more effective than monovalent carboxylates, and zwitterions were poor substrates. The most effective carboxylate tested was lactic acid, which was interesting because of its presence in serum.

Dissociation of CRP by Lactic Acid. C-Reactive proteins from various species provide a spectrum of interactions: some appear to be more like SAP than others. Two CRP proteins were tested for their interactions with lactic acid.

Table 1: Dissociation of hSAP–Phospholipid Complexes[a]

compound	I_{50}[b] (mM)
O-phosphorylethanolamine	0.035
lactate	0.4
succinate	0.65
bicarbonate/carbonate	7.0
ϵ-amino-*n*-caproic acid	>28
acetate	3
GABA	>18

[a] Dissociation experiments were performed as described in Materials and Methods. [b] The concentration that caused 50% dissociation of the hSAP–phospholipid complex.

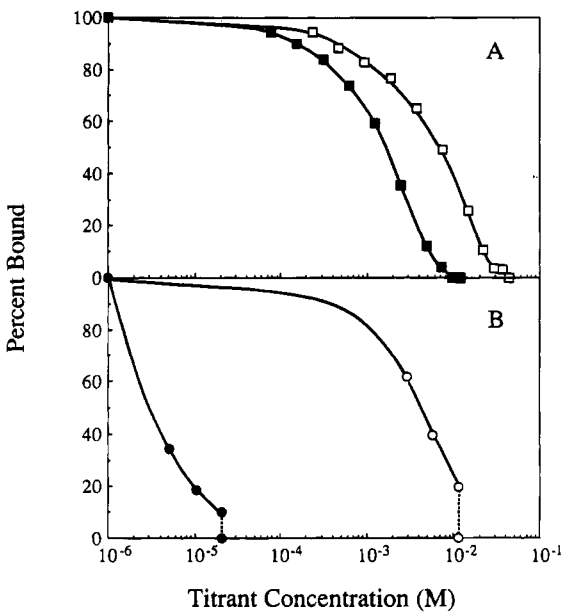

FIGURE 2: Dissociation of rCRP and hCRP from phospholipid vesicles. (A) Lyso-PC (2.2 μg) was mixed with SUVs (30 μg, PS/PC, 30:70) in 2 mL of standard buffer. Rat CRP (37 μg) and $CaCl_2$ (3 mM) were then added. Analysis of relative light-scattering intensities by eq 1 indicated an M_2/M_1 of 1.3. Sequential additions of acetate (□) and lactate (■) were made and light-scattering intensities were measured. The extent of association is represented as percent bound as described by eq 2. The midpoints for dissociation by acetate and lactate were 7.5 and 1.7 mM, respectively. (B) Lyso-PC (5.3 μg) was mixed with SUVs (13.25 μg, PS/PC, 30:70) in 1 mL of standard buffer. Human CRP (1.72 μg) and $CaCl_2$ (2 mM) were then added. Sequential additions of lactate (○) and phosphorylcholine (●) dissociated the complexes, with midpoints of 4.3 mM and 4 μM, respectively. Light-scattering signals are reported as relative changes as described in panel A. Addition of EGTA caused complete dissociation of the complex, as illustrated by the dotted lines.

The first, rat CRP, has greater similarity to SAP than the second protein, human CRP. For example, rat CRP will associate with C4BP while human CRP will not (Schwalbe et al., 1992). The affinity of these CRP proteins for phospholipid vesicles followed this trend. While both required the presence of lysophospholipids for interaction, rat CRP required less of this disrupting agent than did human CRP.

Seven percent (w/w) lyso-PC in PC/PS membranes was adequate to support interaction with rCRP (Figure 2A). Even then, the results suggested that the affinity of rCRP for these modified vesicles was lower than the affinity of hSAP for unmodified phospholipid vesicles. This was suggested by similar amounts of protein binding (M_2/M_1) for SAP and rCRP, despite much higher protein:vesicle ratios for rCRP (Figure 2A) than for hSAP (Figure 1). Acetate and lactate caused dissociation of these rCRP–vesicle complexes with midpoints of 7.5 and 1.7 mM, respectively (Figure 2A). Thus, although the affinity of rCRP for these vesicles was lower, the concentrations of acetate and lactate required to dissociate rCRP were higher than those needed for dissociation of the hSAP–phospholipid complexes (Figure 1). rCRP appeared to have a much lower affinity for lactate than did hSAP. Nevertheless, lactic acid appeared capable of dissociating rat CRP–lipoprotein complexes in rat serum (see the following). Consequently, lactic acid may serve as a regulator of rat CRP function in some situations.

Human CRP (hCRP) binds very poorly to phospholipid vesicles (Schwalbe et al., 1992), and a content of 40% lyso-PC was needed to produce easily measured protein–membrane binding (Figure 2B). This level of lyso-PC approached the amount that destabilizes unilamellar vesicles with conversion to micelles. One explanation for the high requirement for lyso-PC may be that hCRP required substantial distortion of phospholipid bilayers in order to bind. Despite low-affinity interaction, dissociation of the hCRP–vesicle complex required a high concentration of lactate (midpoint 4.3 mM, Figure 2B). In contrast, phosphorylcholine was extremely effective, dissociating the hCRP–vesicle complex with a midpoint of 4 μM (Figure 2B). Overall, in agreement with prior studies, hCRP was the most extreme pentraxin, showing the greatest specificity for phosphorylcholine over lactate. Lactic acid would only be useful in dissociating the lowest affinity interactions of hCRP.

Control experiments tested several properties of these titrations. For example, 2 mM calcium represented a saturating level, so that higher concentrations did not alter the behavior shown in Figures 1 and 2. Modest changes in ionic strength (170 instead of 100 mM NaCl in the buffer) had a small effect (15% dissociation) on these protein–phospholipid complexes.

Effect of Lactate on hSAP Complexes with C4BP and Heparin. Sucrose density gradient centrifugation can be used to detect the interaction of pentraxins with other compounds, such as the complement regulator C4BP (Schwalbe et al., 1990, 1991, 1992). This interaction with C4BP appears to be the major form of hSAP in blood (Schwalbe et al., 1990, 1991, 1992). Heparin and phosphomonoesters can dissociate this complex (Schwalbe et al., 1991, 1992). The effect of lactate on the hSAP–C4BP complex was investigated by using sucrose density gradient centrifugation (Figure 3). Human SAP (labeled with ^{14}C) was mixed with human serum, and the major species sedimented at fraction 13 (Figure 3A) as a 1:1 complex with C4BP, either provided as a pure protein or derived from serum (Figure 3C). When lactate (20 mM) was added to the buffer, all of the hSAP sedimented at the position expected for free hSAP (fraction 9, Figure 3A,B). Lower concentrations of lactate resulted in intermediate sedimentation behavior, suggesting partial dissociation of the hSAP–C4BP complexes. Qualitatively similar behavior was observed at two different concentrations of serum (Figure 3A,B). Mixtures of pure hSAP and C4BP (Figure 3C), at concentrations similar to those estimated to be present in the serum sample (Figure 3A), showed almost identical behavior. These results demonstrated that lactate could affect hSAP interactions even in blood and supported the conclusion that hSAP is bound, almost exclusively, to C4BP in serum (Schwalbe et al., 1990, 1991, 1992).

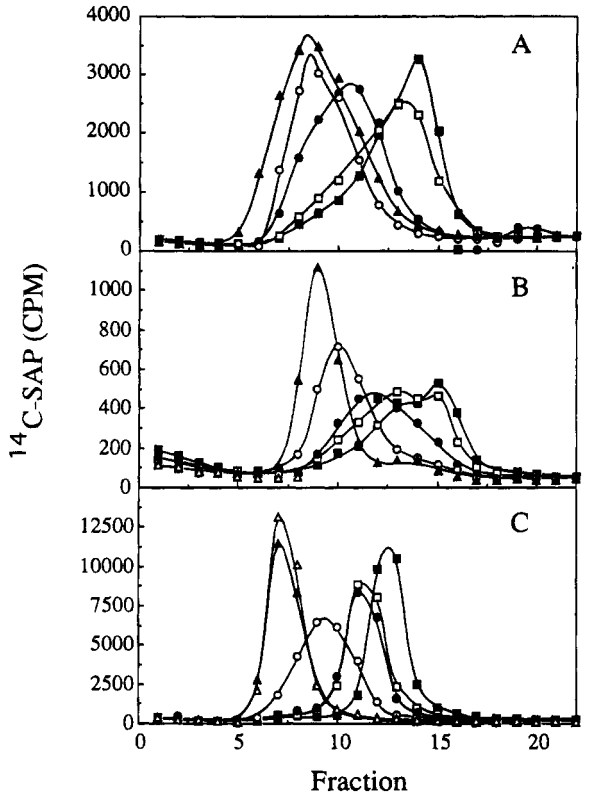

FIGURE 3: Dissociation of serum hSAP−C4BP complex by lactate. (A) [^{14}C]hSAP (1.5 × 10^7 cpm/mg) was added to a 400 μL sample containing 150 μL of human serum. The samples were applied to a tube containing a sucrose density gradient, and the tubes were centrifuged as described in Materials and Methods. Fractions, beginning at the top of the tube, were collected and counted for radioactivity. The concentrations of lactate in the samples and sucrose gradients were 0.6 (■), 1.2 (□), 2.4 (●), 4.8 (○), and 10 mM (▲). (B) The experiments were as described for panel A, except that 50 μL of human serum was used per sample. Lactate concentrations were 0.3 (■), 0.6 (□), 1.2 (●), 2.4 (○), and 10 mM (▲). (C) Pure C4BP (10 μg) and hSAP (3.3 μg) were added to a 400 μL sample of buffer, and ultracentrifugation was carried out as described earlier. Lactate concentrations were 0 (■), 0.6 (□), 1.2 (●), 2.4 (○), 5 (▲), and 20 mM (△). In all three panels, the recovery of radioactivity applied to the tube was about 60%. The sedimentation coefficients in 5−20 mM lactate were the same for all three panels, and the slight off-set of the major peaks was due to slightly different fraction sizes. Calcium (1.5 mM) was present in all samples and buffers.

Heparin and heparan sulfate also interact with hSAP in a calcium-dependent manner (Hamazaki, 1987; Schwalbe et al., 1991). Sucrose density gradient ultracentrifugation was used, and a mixture of [^{14}C]hSAP (0.5 μg) and heparin (5 μg) sedimented as a single major peak at fraction 13 in an experiment similar to the ones shown in Figure 3 (data not shown). This complex appears to contain two SAP molecules (Schwalbe et al., 1991). The presence of 10 mM lactate in the buffers did not change this sedimentation position (data not shown). This implied that the hSAP−heparin complex was of much higher affinity than the hSAP−C4BP complex and agreed with the reported ability of heparin to dissociate the latter complex (Schwalbe et al., 1991).

Human SAP aggregates in the presence of millimolar concentrations of free calcium (Schwalbe et al., 1990) in a manner that is dependent on the concentration of calcium and hSAP (Hamazaki, 1989; Schwalbe et al., 1990). Aggregation can be prevented by hSAP interaction with other components such as phosphomonoesters or vesicles. Lactate also prevented hSAP aggregation (data not shown). This demonstrated that lactate probably interacted with the same sites that bound *O*-PE or C4BP. The X-ray crystal structure showed that the calcium-binding site of hSAP could accommodate either carboxylate (acetate) or phosphate (*O*-PE) (Emsley et al., 1994).

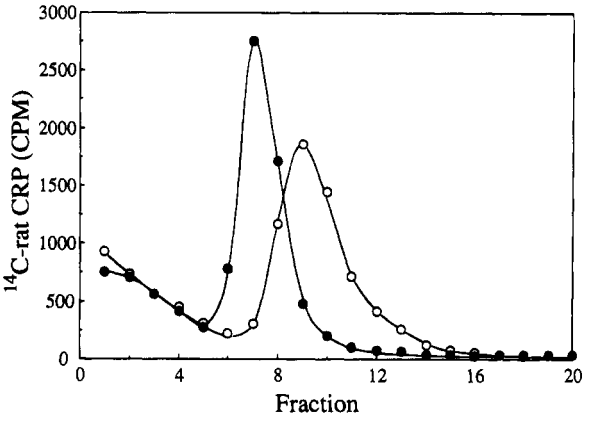

FIGURE 4: Dissociation of rCRP from serum lipoproteins by lactate. ^{14}C-labeled rat CRP (4.02 mCi/g) was added to a 400 μL sample that contained 50 μL of rat serum. Sucrose density ultracentrifugation was carried out as in Figure 3 with the presence (●) or absence (○) of 10 mM lactate in all buffers. Recovery of radioactivity was greater than 65%. Calcium (2 mM) was present in all solutions.

Dissociation of rCRP from Lipoproteins by Lactic Acid. Rat CRP interacts with materials in serum that have been identified as lipoproteins. This interaction produces a small increase in the sedimentation velocity of rCRP in sucrose density gradients (Schwalbe et al., 1995). Lactate (10 mM) dissociated this complex (Figure 4). Thus, despite a lower affinity for rCRP than for hSAP, lactic acid may be able to regulate the state of the lipoprotein−rCRP complexes in rat serum.

Cooperativity of Lactate Binding to hSAP. Lactate and C4BP compete for binding to hSAP. The interaction with C4BP is monovalent (1:1 complex), while several lactate molecules can bind to each hSAP molecule. Intersite communication can produce cooperativity with respect to lactate. The dissociation of hSAP−C4BP by lactate will be greatly influenced by the mechanism of interaction and the concentrations of the proteins. Assumption of a model for these interactions, such as the mutual exclusivity of lactate and C4BP binding (eq 3), allows the design of experiments to determine binding mechanisms. The results can also help test the assumptions. Figure 5 shows the dissociation of hSAP (0.123 μM) from C4BP (0.176 μM) measured by light-scattering intensity. These concentrations are approximately equal to the serum levels of these proteins. The midpoint of dissociation occurred at 11.6 mM lactate. The latter and eq 5 were used to calculate theoretical curves for these concentrations of protein and various cooperativity coefficients. The data best fit the curve with a cooperativity coefficient of 2 (Figure 5A). A similar titration was carried out at a lower protein concentration (Figure 5B). Once again, theoretical curves, obtained from the values in Figure 5A, showed close correlation with a cooperativity coefficient of 2. Both the shift of the curve (midpoint shifted by 1.8-fold for a 3.8-fold change in protein concentration) and the shape

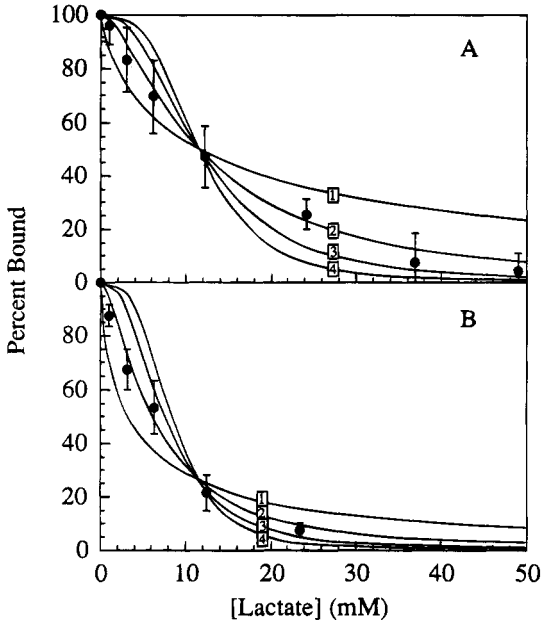

FIGURE 5: Theoretical fit for dissociation of the hSAP−C4BP complex by lactate. (A) Experimental data for lactate dissociation of C4BP (0.176 μM) complexed with hSAP (0.123 μM) are shown by the data points. These are the means and deviations of two separate titrations. The light-scattering results are expressed as percent bound as in eq 2. The ratio of I_i/I_f was 1.43. The solid lines are theoretical curves generated from eq 5, using the ratio of equilibrium constants obtained from the lactate and protein concentrations at half-dissociation of the complex (11.6 mM lactate). Thus, all curves pass through 50% dissociation at 11.6 mM lactate. (B) The data are the means and deviations of two separate experiments conducted with hSAP (0.032 μM) and C4BP (0.046 μM). The solid lines are theoretical curves generated from the values used in panel A, but with the appropriate protein concentrations. In both panels, the boxed numbers represent the n value used to generate that theoretical curve.

correlated with a coefficient of 2 and the ratio of equilibrium constants calculated. In fact, this excellent correlation of curve shape and shift supported the accuracy of the assumptions made in designing this experiment (see Materials and Methods).

Alteration of cooperativity is a potent method of allosteric regulation. However, it is not known whether the cooperativity coefficient for this interaction will change at different solution conditions. Nevertheless, the theoretical results demonstrate the different behavior patterns. The greatest change in lactate requirement, as a function of protein concentration, would occur for a cooperativity coefficient of 1. In this case, the lactate concentration at the midpoint would be directly proportional to protein concentration. The least sensitivity to protein concentration would occur for a cooperativity coefficient of 5. This behavior may be important in the acute phase where serum concentrations of hSAP and C4BP undergo change. Lactate levels needed for dissociation may be altered as a result.

DISCUSSION

Previously, it was shown that the pentraxins interact with phosphorylated compounds with various affinities (Anderson et al., 1978; Coe, 1977; Coe et al., 1981; Gotschlich & Edelman, 1967; Gotschlich et al., 1982; Schwalbe et al., 1992; Volanakis & Kaplan, 1971). Pentraxins can be characterized by their preference for *O*-PE (e.g., hSAP) or PhChol (e.g., hCRP) (Schwalbe et al., 1992). This study found that lactic acid also bound to pentraxins in a calcium-dependent manner. Since lactate is a normal constituent of blood, its concentration may influence the behavior of at least some of the pentraxins *in vivo*.

Human SAP was the most sensitive to lactic acid and was the most probable site of lactic acid regulation. The hSAP−phospholipid interaction was used to compare the relative efficacies of various test compounds. Unlike phosphoryl ligands where the presence of an amino group promoted interaction with human SAP, the presence of an amine group on carboxylated compounds was detrimental to their ability to dissociate hSAP from phospholipids. For example, succinate dissociated the hSAP−phospholipid complex with a midpoint of 650 μM and was significantly more effective than amine-containing compounds such as γ-aminobutyrate (dissociation midpoint $\geq$ 18 mM) and ϵ-amino-n-caproic acid (dissociation midpoint $\geq$ 28 mM). Although less effective than some other carboxylates (dissociation midpoint 7 mM), the concentration of bicarbonate in the serum of a resting human is 15−20 mM, which may be adequate to contribute to the association state of hSAP. The most effective carboxylate compound tested was lactic acid (midpoint for dissociation of hSAP−phospholipid complex was 0.4 mM). This ligand is present at 0.5−2 mM in serum from resting individuals and increases dramatically under some conditions (Chwalbinska-Moneta et al., 1989; Mizock & Falk, 1992). Thus, lactic acid levels may serve to regulate some of the hSAP interactions in blood.

Several methods were used to study the dissociation of the hSAP−C4BP complex. [^{14}C]hSAP added to a sample of human serum sedimented as its complex with C4BP (Schwalbe et al., 1990). The presence of lactate caused a concentration-dependent progressive shift in the sedimentation position toward that of monomeric hSAP. The behavior was similar for two different serum concentrations (Figure 3A,B). The sedimentation patterns and lactic acid requirements were virtually identical when pure proteins were used (Figure 3C). These data demonstrated that lactate altered hSAP binding in serum. The similarity of results for serum and for pure proteins suggested that C4BP was the primary site of hSAP interaction in serum.

More detailed titrations of SAP−C4BP dissociation by lactic acid provided insight into the mechanism of lactic acid interaction with hSAP. For example, each subunit of hSAP should bind one lactic acid molecule, so that cooperativity between lactic acid-binding sites could produce a coefficient (n) of 0−5. The experimental data best fit a cooperativity coefficient of 2. Four other sets of experimental data were generated (data not shown), and in every case, the results were best fit by a value of 2. Cooperative behavior between the subunits of SAP presents the possibility that the cooperativity coefficent could be modulated by other components in serum. Thus, the impact of lactic acid may vary with serum content and conditions.

The lactic acid required to dissociate pure SAP from C4BP suggested that, in the serum of normal, resting individuals, hSAP should be almost completely bound to C4BP. This agrees with experimental observations in diluted serum. However, conditions that raise the lactate concentration in blood should cause dissociation of the complex. High lactate levels could occur systemically or locally. One possible impact could be on the complement system. Since hSAP

interaction with C4BP inhibits the ability of C4BP to inactivate C4b (de Frutos & Dahlback, 1994), high lactate concentrations should decrease complement activity. Tissues with a high degree of anaerobic metabolism, such as solid tumors, may produce high local lactate concentrations, which could lower scrutiny by complement.

Human SAP also binds amyloid deposits in a calcium-dependent manner (Pepys et al., 1979). It has been postulated that hSAP protects amyloid deposits from proteases, thereby contributing to the disease. Thus, it would be interesting to test the effect of lactate on hSAP and amyloid as well.

Blood calcium and hSAP concentrations are about 2 mM and 40 μg/mL, respectively (Pepys et al., 1978). These concentrations cause purified hSAP to aggregate. Phosphomonoesters prevent the aggregation, but are not normally present in the blood at adequate levels. Previously, it was proposed that the aggregation of hSAP in blood was prevented by an unidentified low molecular weight component (Painter et al., 1982). Our studies (data not shown) demonstrated that lactate (2.5 mM) prevented hSAP aggregation (42 μg/mL) in 2 mM $CaCl_2$. Thus, lactate might correspond to the low molecular weight component hypothesized by Painter et al. (1982). Carbonate should prevent hSAP self-association as well. Thus, the blood of a normal rested human, with 1−2 mM lactate and 15−20 mM carbonate, should prevent aggregation of hSAP, even in the absence of macromolecular binding sites. Of course, the association of hSAP with protein ligands such as C4BP also prevents aggregation.

The interaction of lactic acid with other pentraxin proteins was of lower affinity and showed a trend that correlated with other properties. First of all, human SAP bound to phospholipid vesicles without a need for lysolipids, rat CRP bound to vesicles containing low levels of lyso-PC, and human CRP bound poorly, even to vesicles containing high levels of lyso-PC. The ability of lactate to disrupt these complexes was related in the reverse order: the hSAP−phospholipid complex required the least lactic acid for dissociation (midpoint 0.4 mM), the rat CRP−phospholipid complex was intermediate (midpoint 1.7 mM), and the human CRP−phospholipid complex required the highest amount of lactic acid for dissociation (midpoint 4.3 mM). This trend for lactic acid binding paralleled the affinity of these proteins for *O*-PE versus PhChol. That is, hSAP showed the greatest selectivity for *O*-PE (Schwalbe et al., 1992), rat CRP bound both compounds equally well (Schwalbe et al., 1992), and human CRP showed the greatest selectivity for PhChol. This suggested that proteins with the most hSAP-like character bind vesicles readily and are the most sensitive to lactate. The most hCRP-like proteins bind membranes very poorly and are the least sensitive to lactate.

The primary pentraxins of rat and hamster, rCRP and FP, respectively, appear to exist in complex with lipoproteins. Rat CRP binds to lipoproteins that are rich in apoA1 and apoE (Schwalbe et al., 1995). This study showed that lactate dissociated rCRP from these sites (Figure 4) so that high lactate concentrations may alter lipid metabolism, thereby linking carbohydrate and lipoprotein metabolism through serum lactate levels. If serum levels of lactate in the hamster and rat are similar to those in the human, such regulation would appear possible. For example, during periods of anaerobic exercise, the lactate levels in human blood can reach 32 mM (McNaughton & Cedaro, 1991), although 10−14 mM is more common (Åstrand et al., 1986; Bro-Rasmussen et al., 1985; Gastin et al., 1991; Linarsson et al., 1974; Medbo & Burgers, 1990; Scott et al., 1991; Withers et al., 1991). Further studies will be needed to test the possible regulation of lipoprotein metabolism by lactate.

Overall, this study has shown that pentraxins interact with carboxylated compounds including lactate and carbonate, which are normal constituents in blood. The trend of behavior of the various pentraxins linked high-affinity binding to phosphorylethanolamine with binding to phospholipid vesicles and to lactate. The hSAP−C4BP complex was dissociated by lactic acid, so that it is possible that variations in blood lactate levels may impact on complement activity. The rCRP−lipoprotein interaction was also dissociated by lactate, so that, in the rat, blood lactate levels may help regulate lipoprotein metabolism. Testing these possible roles of pentraxins and lactate will require further work.

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