Ca^{2+}-Linked Association of Human Complement $C1\bar{s}$ and $C1\bar{r}$†

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ABSTRACT: The weight-average molecular weight of $C1\bar{r}$, an activated serine protease subcomponent of complement C1, was measured in the presence of widely varying concentrations of Ca^{2+} and the other serine protease subcomponent, $C1\bar{s}$, by utilizing the technique of tracer sedimentation equilibrium. A quantitative model for heteroassociation between the two subcomponents, which takes into account the previously observed Ca^{2+}-dependent self-association of $C1\bar{s}$, was fit to the combined data at each Ca^{2+} concentration. The results indicate that $C1\bar{r}$, which exists as a dimer under all of the conditions explored in this work, can bind up to two molecules of $C1\bar{s}$ at both low and high Ca^{2+} concentrations, but the association constant for binding a single molecule of $C1\bar{s}$ to dimeric $C1\bar{r}$ is estimated to increase on the order of a 1000-fold as [Ca^{2+}] increases from 1 nM to 1.0 mM. Heteroassociation of $C1\bar{r}$ and $C1\bar{s}$ is favored over self-association of $C1\bar{s}$ at all conditions. The results clearly indicate the necessity of taking into account a multiplicity of states of association when attempting to understand the equilibrium average properties of a mixture of the two subcomponents and their binding to C1q in solution.

The first component of complement, C1, is an enzyme complex formed through Ca^{2+}-dependent association of three polypeptide subcomponents, C1q, C1r, and C1s, in the stoichiometry 1:2:2 (Cooper, 1985; Arlaud et al., 1987a; Schumaker et al., 1987). C1r and C1s are highly homologous modular proteins that contain, in addition to the serine protease modules at their C-termini, several additional motifs in their N-terminal regions that are responsible for the various protein–protein interactions involved in assembly of C1. C1s can form Ca^{2+}-dependent dimers (Valet & Cooper, 1974; Tschopp et al., 1980; Rivas et al., 1992). However, in the presence of C1r, which is a stable dimer in the presence or absence of Ca^{2+}, a heterotetramer, C1s–$(C1r)_2$–C1s, is formed (Arlaud et al., 1980a, 1986; Tschopp et al., 1980; Villiers et al., 1985). This tetramer then binds to C1q to form the intact C1 complex (Siegel & Schumaker, 1983; Ingham et al., 1992). C1q possesses no intrinsic catalytic activity, but upon binding of any of several activators to the C1q subcomponent of C1, the homologous C1r and C1s zymogen subcomponents are converted to catalytically active species ($C1\bar{r}$ and $C1\bar{s}$, respectively), triggering the first step of the classical pathway of complement activation (Cooper, 1985).

We have recently characterized the calcium-induced self-association of $C1\bar{s}$ using sedimentation equilibrium methods (Rivas et al., 1992). That work revealed the presence of a high-affinity Ca^{2+} binding site on the $C1\bar{s}$ dimer that was responsible for Ca^{2+}-induced dimerization. In the present work we extend our study to encompass the calcium-linked heteroassociation of $C1\bar{s}$ with $C1\bar{r}$. Chemical and electron microscopic studies have shown that only the monomeric form of C1s binds to $(C1r)_2$ (Arlaud et al., 1980a, 1986; Villiers et al., 1980; Weiss et al., 1986). That is to say, the C1s dimer is disrupted by the interaction with C1r (Busby & Ingham, 1988, 1990). The sites on C1s that are involved in self-association interact instead with two symmetrically disposed sites on the C1r dimer, sites that are homologous with those on C1s. Thus the mixture of C1r and C1s is an example of a two-component system in which self-association and heteroassociation play competing roles. Although the stoichiometry of the C1s–$(C1r)_2$–C1s complex is well established, nothing is known about the equilibrium constants involved or the nature of the linkage between metal binding and protein–protein interactions. We have attempted to obtain this information through careful measurements of the weight-average molecular weight of $C1\bar{r}$ in the presence of a broad range of concentrations of $C1\bar{s}$ and Ca^{2+}.

MODEL FOR EQUILIBRIUM ASSOCIATIONS OF C1R AND C1S

For brevity of notation within equations, we shall denote the component C1r by R and the component C1s by S. Individual molecular species will be denoted in lower case; for example, the monomer of C1s will be denoted by s, and the monomer of C1r, by r.

In accordance with previous findings (Tschopp et al., 1980; Rivas et al., 1992), C1s that is not complexed with C1r is postulated to exist as an equilibrium mixture of monomer and dimer,

$$2s \rightleftarrows s_2$$

with an equilibrium association constant given by

$$K_s = [s_2]/[s]^2 \qquad (1)$$

where brackets indicate the molar concentrations of the indicated species. In accordance with previous findings (Arlaud et al., 1980a; Tschopp et al., 1980) and with results of the present study (see below), C1r is postulated to exist as a dimer either in the absence or in the presence of C1s,

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independent of the concentration of Ca^{2+}. It is assumed that the dimer r_2 may bind either one or two molecules of s,

$$r_2 + s \rightleftarrows r_2s$$

$$r_2s + s \rightleftarrows r_2s_2$$

with the associated stepwise equilibrium constants

$$K_1 = [r_2s]/([r_2][s]) \quad (2)$$

$$K_2 = [r_2s_2]/([r_2s][s]) \quad (3)$$

The two binding sites for s on r_2 are expected to be equivalent by virtue of symmetry. If this is the case, then K_1 and K_2 may be expressed as functions of two other parameters,

$$K_1 = 2K_{rs} \quad (4)$$

$$K_2 = \alpha K_{rs}/2 \quad (5)$$

where K_{rs} denotes the microscopic equilibrium constant for association of a single molecule of s to either site on r_2, and α denotes a unitless interaction factor that represents the possible influence of a single molecule of bound s upon the affinity of r_2 for a second molecule of s. If $\alpha < 1$, then the binding of one molecule of s to r_2 diminishes the affinity of r_2 for a second molecule of s (negative cooperativity); if $\alpha > 1$, then the binding of one s to r_2 enhances the affinity of r_2 for a second molecule of s (positive cooperativity).

The total concentration of C1s, in units of moles of monomer per liter, is given by

$$S_{tot} = [s] + 2[s_2] + [r_2s] + 2[r_2s_2] \quad (6)$$

The total concentration of component C1r, in units of moles of monomer per liter, is given by

$$R_{tot} = 2[r_2] + 2[r_2s] + 2[r_2s_2] \quad (7)$$

Equations 1–7 may be combined and rearranged to yield the following expression

$$b_4[s]^4 + b_3[s]^3 + b_2[s]^2 + b_1[s] + S_{tot} = 0 \quad (8)$$

where

$$b_4 = -2\alpha K_s K_{rs}^2$$

$$b_3 = -\alpha K_{rs}^2 - 4K_sK_{rs}$$

$$b_2 = \alpha K_{rs}^2(S_{tot} - R_{tot}) - 2K_{rs} - 2K_s$$

$$b_1 = K_{rs}(2S_{tot} - R_{tot}) - 1$$

Equation 8 may be solved numerically to yield [s] as a function of K_s, K_{rs}, α, S_{tot}, and R_{tot}. Given the value of [s], the concentrations of all remaining species are calculated using the following equations:

$$[r_2] = R_{tot}/[2(1 + 2K_{rs}[s] + \alpha K_{rs}^2[s]^2)] \quad (9)$$

$$[s_2] = K_s[s]^2 \quad (10)$$

$$[r_2s] = 2K_{rs}[s][r_2] \quad (11)$$

$$[r_2s_2] = \alpha K_{rs}^2[s]^2[r_2] \quad (12)$$

Let the mass fraction of each component existing as the species r_is_j in the equilibrium mixture be denoted by $g_{A,ij}$ where A is either R or S. These are calculated according to the following equations:

$$g_{S,01} = [s]/S_{tot} \quad (13)$$

$$g_{S,02} = 2[s_2]/S_{tot}$$

$$g_{S,21} = [r_2s]/S_{tot}$$

$$g_{S,22} = 2[r_2s_2]/S_{tot}$$

$$g_{R,20} = 2[r_2]/R_{tot}$$

$$g_{R,21} = 2[r_2s]/R_{tot}$$

$$g_{R,22} = 2[r_2s_2]/R_{tot}$$

Let the weight/volume concentration of species X be denoted by $w_x \equiv [X]M_X$, where M_X is the molecular weight of X. Let the fraction of the mass of complex r_is_j that is component R be denoted by $f_{R,ij} = iM_{10}/(iM_{10} + jM_{01})$. Then the weight-average molecular weight of C1r is calculated according to

$$M_{w,R} = \frac{f_{R,20}w_{r_2}M_{r_2} + f_{R,21}w_{r_2s}M_{r_2s} + f_{R,22}w_{r_2s_2}M_{r_2s_2}}{f_{R,20}w_{r_2} + f_{R,21}w_{r_2s} + f_{R,22}w_{r_2s_2}}$$

$$= \frac{M_{r_2}[r_2] + M_{r_2s}[r_2s] + M_{r_2s_2}[r_2s_2]}{[r_2] + [r_2s] + [r_2s_2]} \quad (14)$$

EXPERIMENTAL PROCEDURES

All protein solutions were prepared by dialysis into 25 mM Hepes hydrochloride and 150 mM NaCl, pH 7.4 (Hepes buffer), and equilibrated at the required Ca^{2+} concentration as described in Rivas et al. (1992). All the experiments were performed with the activated forms of human C1s ($C1\bar{s}$) and C1r ($C1\bar{r}$), isolated from Cohn fraction I of human plasma by affinity chromatography on immobilized IgG (Busby & Ingham, 1988). Homogeneity of the preparations was tested by size-exclusion chromatography on Superose 12 (Pharmacia) and by SDS–PAGE on a Pharmacia Phastgel system. The protein concentrations of $C1\bar{s}$ and $C1\bar{r}$ were determined by using values of $E_{280,1\%}$ of 13.7 and 11.6, respectively, with corresponding molecular masses of 80.3 and 86 kDa (Busby & Ingham, 1988).

Tritium labeling of $C1\bar{r}$ was done essentially as described by Caras et al. (1980). Briefly, 250–300 μL of $C1\bar{r}$ in Hepes buffer (0.2–0.3 mg/mL) was incubated at room temperature for 2 h with 8–10 nmol of *N*-succinimidyl (2,3-^{3}H)propionate (DuPont; 0.02 mCi/mg, 5 mCi/mL). The excess of unincorporated label was removed by extensive dialysis against Hepes buffer. A mixture of labeled and cold $C1\bar{r}$ co-eluted after size-exclusion chromatography on a Zorbax G-250 column equilibrated in Hepes buffer. The specific activity ranged from 0.6×10^5 to 1.4×10^5 cpm/μg (0.07–0.16 mol of tritium per mol of $C1\bar{r}$).

Sedimentation equilibrium experiments were carried out in the preparative ultracentrifuge using the general procedures developed by Attri and Minton (1983, 1986), as modified and described by Darawshe et al. (1993). Briefly, polycarbonate centrifuge tubes (7 × 20 mm, Beckman Instruments) containing 70 μL of protein sample (3-mm column height), containing the indicated concentrations of ^{3}H-$C1\bar{r}$, $C1\bar{s}$, and Ca^{2+}, were centrifuged at 5000–6000 rpm in SW-41 or TLS-55 swinging bucket rotors (in Beckman L5–65 and TL-100 ultracentrifuges, respectively) for at least 36 h at 10 °C to assure achievement of sedimentation equilibrium. At the conclusion of centrifugation, the contents of each tube were fractionated onto Beckman Ready Caps using a BRANDEL

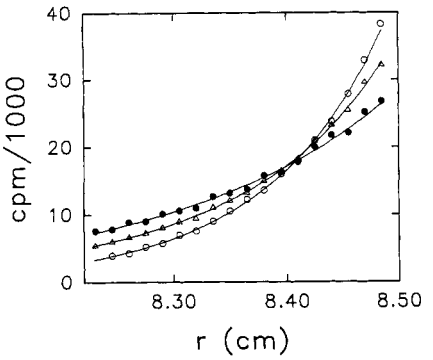

FIGURE 1: Gradients of ^{3}H-C1$\bar{r}$ at sedimentation equilibrium: filled circles, [C1s] = 0; triangles, [C1s] = 1.0 mM; open circles, [C1s] = 3.0 mM. Solid curves represent the respective best fits of equation 15 to each data set.

automated centrifuge tube microfractionator, and the fractions were counted on a scintillation counter (Beckman LS 3801) to obtain the relative concentration of ^{3}H-C1$\bar{r}$ as a function of radial distance at sedimentation equilibrium.

DATA ANALYSIS

The conditions of centrifugation were selected to yield relatively shallow equilibrium concentation gradients of the largest species (r_2s_2) expected to be present. Under such conditions it is anticipated that the weight-average molecular weight of C1r will be approximately uniform throughout the cell, and that the experimentally observed dependence of the concentration of labeled C1r upon radial position r will be well described by the following expression, derived in the Appendix:

$$R_{tot}(r) = R_{tot}(r_f)\exp\left[\frac{M_{w,R}(1-\langle\bar{v}\rangle\rho)\omega^2}{2RT}(r^2-r_f^2)\right] \quad (15)$$

where $\langle\bar{v}\rangle$ is a weighted average of the partial specific volumes of C1r and C1s, calculated as described in the Appendix, ρ is the solvent density, ω is the angular velocity, and r_f is a reference radial position.

The partial specific volumes at 10 °C of C1s (0.716 ± 0.003 mL/g) and C1r (0.710 ± 0.003 mL/g) were calculated from the amino acid and sugar composition (Sim et al., 1977; Mackinnon et al., 1987; Tosi et al., 1987) using 25 °C data and then corrected to 10 °C, as described by Durchschlag (1986).[1] The indicated uncertainties represent 95% confidence limits. These values are quite close to those calculated by Perkins and Nealis (1989) corrected for temperature differences. The value of $\langle\bar{v}\rangle$, calculated from these values using Appendix eqs A6 and A9, is 0.711 mL/g.

Since the amount of radiolabel in each fraction is proportional to R_{tot}, eq 15 was fit to the experimentally observed equilibrium gradient of radiolabel in each sample by the method of nonlinear minimization of χ^2, as described by Hsu and Minton (1991) and Rivas et al. (1992). Equation 15 was found to represent the experimental data to within experimental precision under the conditions of our experiments. In Figure 1 the data and calculated best fit of eq 15 are presented for three solution compositions, corresponding respectively to little or no heteroassociation of C1r and C1s, approximately half-saturation of C1r with C1s, and almost complete saturation of C1r with C1s. The second of these cases represents a "worst-case" situation in which the weight-average molecular weight of C1r is maximally sensitive to concentration changes. The best-fit value of $M_{w,R}$ obtained for each sample is taken to characterize a solution having the loading concentrations of C1r and C1s of that sample. By means of this procedure, tables of S_{tot}, R_{tot}, and $M_{w,R}$ at three different values of [Ca^{2+}] (1 nM, 1 μM, and 1 mM) were constructed. These tables are referred to as the secondary data (the primary data being the actual measured gradients of radiolabel concentration).

Given values of three parameters, log K_s, log K_{rs}, and log α, eq 8–13 may be used to calculate $M_{w,R}$ as a function of S_{tot} at constant R_{tot}. Constraining the value of K_s at each Ca^{2+} concentration to be equal to the previously determined value (Rivas et al., 1992), eq 8–13 were fit to the secondary data at each Ca^{2+} concentration by means of nonlinear minimization of χ^2 (Hsu & Minton, 1991) to obtain best-fit values of log K_{rs} and log α at each Ca^{2+} concentration.

When secondary data obtained from experiments at a single value of R_{tot} (3 μM) were modeled by eq 8–13, it was found that the values of K_{rs} and α could not be individually determined because of cross-correlation between them; i.e., for any given value of K_{rs}, a value of α could be found that would yield a satisfactory fit to the data. We call any pair of values (K_{rs}, α) fitting the data a cognate pair. The method of Hsu and Minton (1991) was employed to establish whether other loading concentrations (R_{tot} and S_{tot}) would allow us to resolve the individual parameter values. We define the discriminator function

$$\Delta M_{w,R}(R_{tot}, S_{tot}) = |M_{w,r}(K_{rs}(1), \alpha(1), R_{tot}, S_{tot}) - M_{w,r}(K_{rs}(2), \alpha(2), R_{tot}, S_{tot})| \quad (16)$$

where [$K_{rs}(1)$, $\alpha(1)$] and [$K_{rs}(2)$, $\alpha(2)$] represent two extremely divergent cognate pairs of parameters. The value of $\Delta M_{w,R}$ is calculated over the experimentally accessible range of values of R_{tot} and S_{tot}, and the resulting surface is examined for the presence and location of maxima. Maxima in $\Delta M_{w,R}$ occur at those loading compositions at which the resulting gradients are calculated to be maximally sensitive to changes in K_{rs} and α and, conversely, where K_{rs} and α are expected to be maximally sensitive to small variations in the experimentally measured gradients (Hsu & Minton, 1991). By means of this procedure, the maximally informative value of R_{tot} was found to be 0.6 μM. A second set of experiments was carried out for this value of R_{tot}, three concentrations of Ca^{2+}, and a variety of values of S_{tot}. The two sets of experiments, with two values of R_{tot}, were globally modeled by eqs 8–13 at each Ca^{2+} concentration, yielding the values of K_{rs} and α presented in the Results section.

RESULTS

C1$\bar{r}$, in the absence of sufficient C1$\bar{s}$, has limited solubility depending on the metal ion environment. C1$\bar{s}$, on the other hand, is easy to handle over a broad range of protein and Ca^{2+} concentrations. The strategy adopted was to examine the weight-average molecular weight of radiolabeled C1$\bar{r}$ as a function of C1$\bar{s}$ concentration at several concentrations of Ca^{2+}. This approach also simplifies the formalism since C1$\bar{r}$ forms a stable dimer that is bivalent toward C1$\bar{s}$, whereas C1$\bar{s}$ is monovalent toward C1$\bar{r}$.

Concentration gradients of ^{3}H-C1$\bar{r}$ (3.0 μM monomer) at sedimentation equilibrium in 1.0 mM Ca^{2+}, alone and in the presence of two different concentrations of C1$\bar{s}$ (1.0 and 3.0 μM), are shown in Figure 1. The best-fit gradients, calculated using eq 15, are also plotted. The calculated gradients correspond to best-fit values of $M_{w,R}$ of 175 ± 9, 254 ± 12,

[1] The partial specific volume of C1s at 10 °C reported in Rivas et al. (1992) is a typographical error.

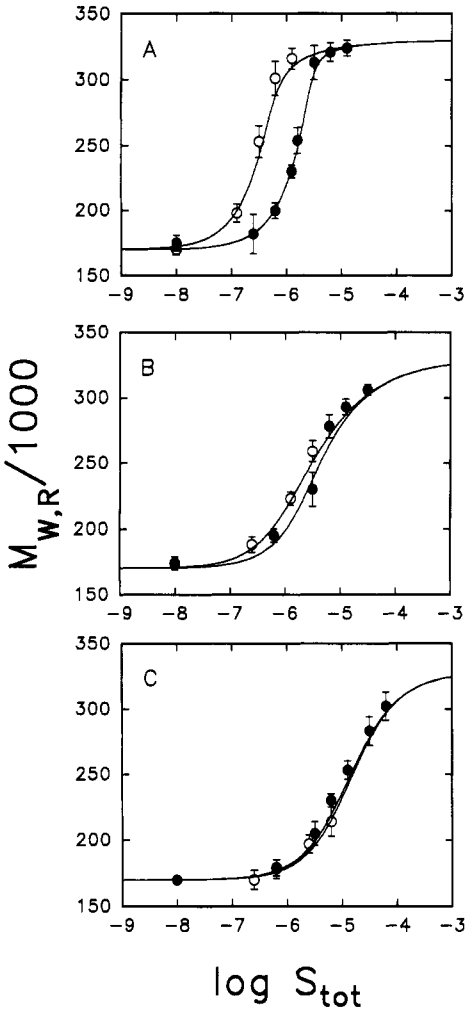

FIGURE 2: Weight-average molecular weight of ^{3}H-C1$\bar{r}$ plotted as a function of log S_{tot} at [Ca^{2+}] = 1 mM (A), 1 μM (B), and 1 nM (C): solid symbols, R_{tot} = 0.6 μM; open symbols, R_{tot} = 3.0 μM. Each symbol represents the mean of values obtained from 3 or 4 replicate experiments, and the error bars indicate ±2 SEM.

and 321 ± 14 kDa, respectively, the first of which corresponds to the literature value for dimeric C1$\bar{r}$, and the last of which is very close to the value of the heterotetramer (Tschopp et al., 1980; Busby & Ingham, 1988). The indicated uncertainties represent 95% confidence limits calculated by taking into account all sources of uncertainty, including uncertainty in $\langle\bar{v}\rangle$. The values of $M_{w,r}$ obtained for ^{3}H-C1$\bar{r}$ centrifuged in the presence of unrelated proteins (ca. 1 μM aldolase or catalase; data not shown) were the same, within experimental error, to that obtained for ^{3}H-C1$\bar{r}$ alone; therefore the interaction with C1$\bar{s}$ was considered to be specific.

Values of $M_{w,R}$ for two values of R_{tot} (0.6 and 3.0 μM monomer), obtained as described above, are plotted in Figure 2 as a function of log S_{tot} for three different concentrations of free Ca^{2+}: 1.0 mM (panel A), 1.0 μM (panel B), and 1.0 nM (panel C). For either concentration of C1$\bar{r}$, the amount of C1$\bar{s}$ required to form the tetramer increases with decreasing [Ca^{2+}]. At 1 mM Ca^{2+}, binding of C1$\bar{s}$ is almost stoichiometric; the separation of the two curves in panel A reflects only the difference in the total concentration of C1$\bar{r}$. At 1 μM Ca^{2+} (panel B) the two data sets appear similar, and higher concentrations of C1$\bar{s}$ are required to saturate C1$\bar{r}$ with C1$\bar{s}$. In the absence of Ca^{2+} (panel C), it is still possible to form r_2s_2, but much higher concentrations of C1$\bar{s}$ are required. Under these conditions, the data for the two different concentrations of C1$\bar{r}$ are essentially superimposable. This

Table 1: Best-Fit Parameter Values and Estimates of Parameter Uncertainty (95% Confidence Limits)

[Ca^{2+}]	log K_s[a]	log K_{rs}	log α	best-fit χ^2	$P(\chi^2)$[b]
1 nM	3.3	4.8 (+0.3, −0.5)	0 (+0.2, −0.2)	9.9	0.43
1 μM	5.4	5.9 (+0.3, −0.6)	0 (+1.0, −0.3)	2.4	0.93
1 mM	6.8	7.5[c]	0[c]	9.1	0.54

[a] Values taken from Rivas et al. (1992). [b] Probability that a value of χ^2 selected from a random distribution characterized by the same number of degrees of freedom as the data set is as large or larger than the best-fit value of χ^2. A value exceeding 0.05 indicates that the best fit is acceptable with a 95% confidence limit. [c] Calculated on the assumption that log $\alpha \sim 0$.

is because the concentration of C1$\bar{s}$ required to have an effect is much greater than either concentration of C1$\bar{r}$.

The solid curves drawn through the data sets in Figure 2 are the calculated best fits of eq 8–13, obtained as described above. At all three Ca^{2+} concentrations the best fit was found to be consistent with the experimental data at a 95% level of confidence as judged by the best-fit value of χ^2 (Press et al., 1986; Hsu & Minton, 1991). The best-fit values of the equilibrium association constants and their respective 95% confidence limits are tabulated in Table 1. The value of the cooperativity parameter, α, was found to be close to unity (i.e., log α was close to 0) at nano- and micromolar concentrations of Ca^{2+}, indicating the absence of cooperativity; the binding of one molecule of s to r_2 does not significantly affect the affinity of the remaining unoccupied site for s. At the highest concentration of Ca^{2+}, 1 mM, the values of K_{rs} and α were so highly correlated that the values of the two parameters could not be individually established. However, if the value of log α is constrained to be 0 as in the other two Ca^{2+} concentrations, then log K_{rs} is well defined by the data, and vice versa. According to this analysis, K_{rs} increases an order of magnitude between 1 nM and 1 μM Ca^{2+} and almost 3 orders upon raising [Ca^{2+}] to 1.0 mM.

DISCUSSION

The present study is the first attempt to characterize in quantitative terms the Ca^{2+}-dependent heteroassociation between C1$\bar{r}$ and C1$\bar{s}$. The application of tracer sedimentation equilibrium methodology allowed us to determine the weight-average molecular weight of C1$\bar{r}$ as a function of the concentrations of C1$\bar{s}$ and Ca^{2+} over a broad range of both. It was possible to account for the combined experimental data at each Ca^{2+} concentration with a simple heteroassociation model that allows for the formation of ternary and quaternary complexes of C1$\bar{r}$ and C1$\bar{s}$, while taking into account the self-association of C1$\bar{s}$ (Rivas et al., 1992). In the context of this model, our results indicate that the formation of r_2s_2 is a Ca^{2+}-dependent process that is non-cooperative at Ca^{2+} concentrations of 1 μM and below and is likely to be non-cooperative at higher concentrations as well. The equilibrium constant for addition of a single monomer of C1s to the C1r dimer, K_{rs}, is low at nanomolar Ca^{2+} concentrations ($K_{rs} = 6 \times 10^4$ M^{-1}) and increases by a factor of ca. 10 at micromolar Ca^{2+} concentrations ($K_{rs} = 7 \times 10^5$ M^{-1}) and by another factor of ca. 40 at millimolar Ca^{2+} concentrations ($K_{rs} = 3 \times 10^7$ M^{-1}). At each Ca^{2+} concentration, the value of K_{rs} exceeds that of K_s to such an extent that heteroassociation of C1$\bar{r}$ and C1$\bar{s}$ predominates over self-association of the latter, even though there appears to be a net loss of one Ca^{2+} binding site for every C1$\bar{s}$ dimer that is consumed in the reaction (Thielens et al., 1990; Rivas et al., 1992).

Although neutron scattering (Perkins & Nealis, 1989) and electron microscopic analyses (Weiss et al., 1986) suggest

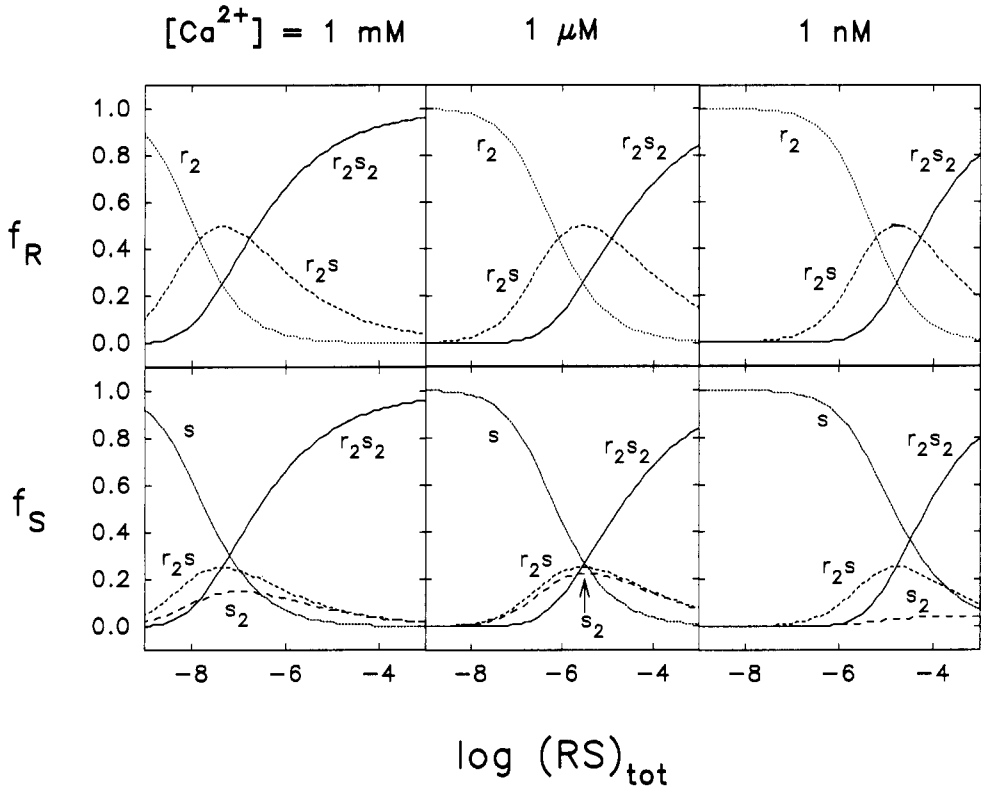

FIGURE 3: Fractional distribution of total C1r̄ and C1s̄ as various species, plotted as a function of $\log(RS)_{tot}$ at the indicated concentrations of $[Ca^{2+}]$.

that the amino-terminal interaction domains of C1r are well separated from the carboxy-terminal catalytic domains, other evidence suggests a possible interaction between them. For example, C1r undergoes spontaneous activation at 37 °C, but only in the absence of Ca^{2+}, even though the only known Ca^{2+} binding sites are located in the amino-terminal domains remote from the bond that is cleaved during activation (Arlaud et al., 1980b; Ziccardi, 1982; Villiers et al., 1983). Furthermore, a derivative of C1r lacking the amino-terminal domains, the so-called γ-B dimer, also undergoes spontaneous activation, but this process is unaffected by Ca^{2+} (Lacroix et al., 1989). Another observation is that the regulatory protein C1-inhibitor, which forms a stable complex with the active sites of C1r̄ and C1s̄, destabilizes the homodimer within the C1s̄–C1r̄–C1r̄–C1s̄ tetramer and renders the heterointeractions between the two proteins independent of Ca^{2+} (Chesne et al., 1982). In other words, perturbation of the carboxy-terminal catalytic region seems to influence the amino-terminal interaction region and vice versa. If the two regions are brought into close proximity through some type of interaction, one might anticipate that binding of C1s to one site on the C1r dimer could influence the binding of a second molecule to the second site. The best-fit value of α was determined in our study to be close to unity at nano- and micromolar Ca^{2+} concentrations, indicating that, at least under those conditions, the two C1s binding sites on the C1r dimer behave independently with respect to binding of C1s. The possibility of some type of cooperative effect at higher Ca^{2+}, while unlikely, can not be ruled out.

Having determined the values of the equilibrium constants in the above model for mixed associations, one can use eqs 8–13 and 15 to calculate, for any values of R_{tot} and S_{tot}, how C1r̄ and C1s̄ are distributed among the various species present in an equilibrium mixture. As an example, in Figure 3 the distributions of C1r̄ and C1s̄ are plotted as functions of their total (equimolar) concentrations at all three Ca^{2+} concentrations. According to these results, only about 50–60% of the C1r and C1s would be in the form of r_2s_2 in a solution having a composition similar to that of blood serum ([C1r̄] = [C1s̄] ~ 0.4 μM; $[Ca^{2+}]$ ~ 1 mM; Cooper, 1985). It should be noted that the calculated compositions plotted in Figure 3 are not likely to reflect the composition of C1r and C1s in blood serum under physiological conditions, as serum ordinarily contains C1r and C1s in largely unactivated (zymogen) form rather than in activated form, and the temperature of our experiments differs considerably from normal blood temperature. Nonetheless, these results do emphasize the probability that both C1r and C1s exist as an equilibrium mixture of molecular species in blood serum, the quantitative nature of which may be revealed by experiments similar to those reported here.

Arlaud et al. (1980a) conducted sedimentation velocity experiments on mixtures of C1r̄ and C1s̄ under conditions similar to those used here and provided evidence for the formation of a trimeric species, r_2s, in the presence of EDTA. The concentrations employed were approximately 15 μM (log = –4.8). As shown in Figures 3, this is very close to the concentration where the r_2s species should be maximized in the absence of Ca^{2+}.

Implications for the Mechanism of Formation of C1. The results presented here afford a possible explanation of a heretofore puzzling observation of Siegel and Schumaker (1983), who measured the dependence of the sedimentation velocity of a trace amount of radiolabeled C1q (denoted by q) upon the concentration of an equimolar mixture of C1r and C1s, through which the C1q was sedimenting. It was assumed that, in this mixture, C1q exists in only two states, namely, the isolated polypeptide q and the complex $q{\cdot}r_2s_2$, according to the equilibrium relationship

$$q + r_2s_2 \rightleftarrows q{\cdot}r_2s_2$$

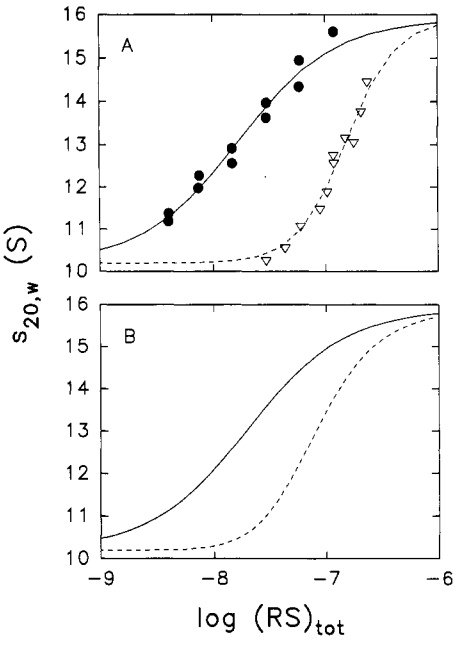

FIGURE 4: Effect of increasing $(RS)_{tot}$ upon the sedimentation coefficient of a trace amount of C1q. Panel A: Data of Siegel and Schumaker (1983). Solid symbols, nonactivated $(RS)_{tot}$; open symbols, activated $(RS)_{tot}$. The curves represent the best fits of an empirical smoothing (Hill) equation to the respective data sets. Panel B: Simulated dependence calculated as described in the text.

with

$$K_{qrs} = [q{\cdot}r_2s_2]/[q][r_2s_2] \quad (17)$$

It follows from this assumption that the fractional change in the weight-average sedimentation velocity of labeled C1q is equal to the equilibrium average fraction of C1q present as the complex, denoted by f_c:

$$s_{20,w} = (1 - f_c)s_{20,w}^{\,q} + f_c s_{20,w}^{\,q{\cdot}r_2s_2} \quad (18)$$

According to eq 17, this fraction is given by

$$f_c = K_{qrs}[r_2s_2]/(1 + K_{qrs}[r_2s_2]) \quad (19)$$

If it is assumed that C1r and C1s in the equimolar mixture exist entirely as the complex, then we may define the total concentration variable $(RS)_{tot} = [r_2s_2]$ and write

$$f_c = K_{qrs}(RS)_{tot}/(1 + K_{qrs}(RS)_{tot}) \quad (20)$$

Equations 18 and 20 adequately describe the dependence of the sedimentation coefficient of C1q upon $(RS)_{tot}$ observed by Siegel and Schumaker for C1q in *nonactivated* C1r/C1s (replotted in Figure 4A). However, in the presence of *activated* C1r/C1s, the observed dependence of the sedimentation coefficient upon $(RS)_{tot}$ is too steep to be described by eqs 18 and 20. Although the shape of the curve seemed to implicate a cooperative binding mechanism, no molecular model was proposed.

We suggest that the difference between the dependence of the weight-average sedimentation coefficient of labeled C1q upon the concentrations of unactivated and activated species is due to the difference between the compositions of the respective solutions. The data of Siegel and Schumaker (1983) for unactivated $(RS)_{tot}$ are consistent with the notion that, in an equimolar solution of unactivated C1r and C1s, essentially all of the protein exists in the form of r_2s_2. In contrast, the present work establishes that the composition of an equimolar mixture of activated C1s and C1r may depend sensitively upon the total protein concentration, as indicated in Figure 3. We make the following simplifying assumptions: (1) Under the conditions of the experiments of Siegel and Schumaker (1983), C1q binds only intact tetramer r_2s_2. We can not rule out the possibility of some interactions between q and s, s_2, r_2, or r_2s, but we assume that the amount of any of these species bound to q is negligibly small relative to the amount of r_2s_2 bound. (2) The equilibrium constant for association of q and r_2s_2, K_{qrs}, is the same for unactivated and activated r_2s_2. Using these approximations, the sedimentation velocity can be calculated as a function of activated $(RS)_{tot}$ ($= R_{tot}/2 = S_{tot}/2$) using eqs 18 and 19, where $[r_2s_2]$ in eq 19 is calculated as a function of $(RS)_{tot}$ by solving eqs 8, 9, and 12 with α assumed equal to 1. The results of this calculation are plotted in Figure 4B. It may be seen that even though no attempt was made to fit the data of Siegel and Schumaker (1983), the simple hypothesis described above reproduces qualitatively the observed difference between the dependence of the sedimentation coefficient of C1q upon the concentrations of unactivated and activated C1r and C1s. The simulated dependence of s_q upon log[activated RS_{tot}] is slightly shallower than that reported by Siegel and Schumaker (1983), but may be steepened by allowing α to exceed unity. Variations in K_{qrs} between activated and nonactivated r_2s_2, on the other hand, would change the relative positions of the two curves in Figure 4B without altering the steepness of either curve.

In summary, the results of the present study and our previous work (Rivas et al., 1992) have permitted the quantitative characterization of equilibrium self-associations and heteroassociations in mixtures of activated C1s and C1r at three broadly different calcium concentrations. They have clearly indicated the necessity of taking into account a multiplicity of states of association when attempting to explain the equilibrium average properties of the solution. In combination with the earlier work of Siegel and Schumaker (1983) the results indirectly provide a hypothesis regarding the nature of the interaction between C1r, C1s, and C1q that may be tested in a future study. Finally, the results directly demonstrate the utility of the technique of tracer sedimentation equilibrium for the study of heteroassociating systems.

APPENDIX

Tracer Sedimentation Equilibrium in an Ideal Solution of Two Solute Components

General Relations. Consider an ideal solution containing two solute *components* A and B that may self-associate or heteroassociate to form molecular *species* a, a_2, a_3, ..., b, b_2, b_3, ..., ab, a_2b_1, a_2b_2, and so forth. Each species is denoted by the general form a_ib_j, where at least one of the two subscripts must be nonzero, and a property of a given species is denoted by subscripting the property variable with the corresponding values of i and j. For example, w_{10}, w_{01}, and w_{11}, respectively, denote the weight/volume concentrations of the species a, b, and ab. At sedimentation equilibrium the gradient of each species will be described by (Hsu & Minton, 1991, Minton, 1994)

$$w_{ij}(r) = w_{ij}(r_{ref}) \exp[\phi_{ij}(r^2 - r_{ref}^2)] \quad (A1)$$

where

$$\phi_{ij} \equiv \frac{M_{ij}(1 - \bar{v}_{ij}\rho)\omega^2}{2RT}$$

where $\bar{v}_{ij}$ is the partial specific volume of a_ib_j, ρ is the density of solvent, ω is the angular velocity of the rotor, R is the molar gas constant, and T is the absolute temperature.

Let us suppose that we have performed an experiment that measures the concentration of component A (and only component A) as a function of radial distance. The total weight/volume concentration of A is given by

$$w_A = \sum_i \sum_j f_{A,ij} w_{ij} \quad \text{(A2)}$$

where $f_{A,ij}$, the fraction of the mass of complex a_ib_j that is component A, is equal to $iM_{10}/(iM_{10} + jM_{01})$. It follows from eqs A1 and A2 that

$$\frac{1}{w_A}\frac{dw_A}{dr} = \frac{\omega^2 r}{RT} M^*_{w,A} \quad \text{(A3)}$$

$M^*_{w,A}$ here denotes the weight-average buoyant molecular weight of A, given by

$$M^*_{w,A} \equiv \frac{\sum_i \sum_j f_{A,ij} w_{ij} M_{ij}(1 - \bar{v}_{ij}\rho)}{\sum_i \sum_j f_{A,ij} w_{ij}} \quad \text{(A4)}$$

Rearrangement of eq A3 and subsequent integration with respect to r yields

$$w_A(r) = w_A(r_{ref}) \exp\left[\frac{M^*_{w,A}\omega^2}{2RT}(r^2 - r_{ref}^2)\right] \quad \text{(A5)}$$

Equation A5 is generally valid so long as the solution behaves ideally with respect to solutes A and B, and the composition of the solution is independent of time. It is not necessary to assume that associations are reversible or that individual species are at chemical equilibrium with respect to each other.

Let us further assume that the partial specific volumes of components A and B are independent of the state of association of each component, an assumption that does not introduce significant error into the analysis of ordinary sedimentation equilibrium experiments carried out on proteins at low to moderate rotor velocities (Hsu & Minton, 1991). This assumption is equivalent to the statement that the partial specific volume of species a_ib_j is a weight-average property of the constituent components:

$$\bar{v}_{ij} = f_{A,ij}\bar{v}_A + f_{B,ij}\bar{v}_B \quad \text{(A6)}$$

where $\bar{v}_A$ and $\bar{v}_B$, respectively, denote the partial specific volumes of pure components A and B. Under these conditions

$$M_{ij}(1 - \bar{v}_{ij}\rho) = iM_{10}(1 - \bar{v}_A\rho) + jM_{01}(1 - \bar{v}_B\rho) \quad \text{(A7)}$$

where M_{10} and M_{01} denote the molecular weights of monomeric A and B, respectively. If, in addition, $\bar{v}_A = \bar{v}_B = \bar{v}$, then eqs A4 and A7 may be combined to yield

$$M^*_{w,A} = \frac{\sum_i \sum_j f_{A,ij} w_{ij} M_{ij}}{\sum_i \sum_j f_{A,ij} w_{ij}}(1 - \bar{v}\rho) \equiv M_{w,A}(1 - \bar{v}\rho) \quad \text{(A8)}$$

Simplifications Applying to the C1r–c1s System. Let C1r be component A and C1s be component B. It is reported in the text that the values of $\bar{v}_A$ and $\bar{v}_B$ are equal to 0.710 ± 0.003 and 0.716 ± 0.003 mL/g, respectively. In the best case, these two values may be taken to be equal within experimental error, in which case eq A8 holds exactly. However, in the worst case $\bar{v}_A$ could be as low as 0.707 mL/g and $\bar{v}_B$ could be as high as 0.719 mL/g. Thus we ask the following general question: Given unequal values of $\bar{v}_A$ and $\bar{v}_B$, is there a (constant) value of $\bar{v}$ such that eq A8 holds *approximately* over the entire range of solution compositions encountered experimentally, and if so, what is the accuracy of the approximation?

To answer this question, we note that when the weight-average buoyant molecular weight of component A is estimated using eq A8 with a constant effective value of $\bar{v}$, the maximum error must be obtained at either one or both extremes of composition: the solution containing pure A and the solution containing a trace of A in an essentially pure solution of B. In the former case, A exists entirely as the species a_k, and in the latter case, A exists almost entirely as the species a_lb_m, where the values of k, l, and m are specified by the association model. That is to say, if we select $\bar{v} = \bar{v}_{k0}$, then $M^*_{w,A}$ calculated according to eq A8 will be most erroneous when A exists primarily as a_lb_m, and conversely, if we select $\bar{v} = \bar{v}_{lm}$, then $M^*_{w,A}$ calculated according to eq A8 will be most erroneous when A exists primarily as a_k. Thus without *a priori* knowledge of the relative abundances of molecular species at intermediate solution compositions, the best guess we can make for the value of $\bar{v}$ that minimizes error in the calculated value of $M^*_{w,A}$ across the entire range of solution compositions is the average

$$\langle\bar{v}\rangle = (\bar{v}_{k0} + \bar{v}_{lm})/2 \quad \text{(A9)}$$

where the values of $\bar{v}_{k0}$ and $\bar{v}_{lm}$ are calculated using the additivity approximation, eq A6.

According to the model of C1r–C1s association presented in the text, $k = 2$, $l = 2$, and $m = 2$. The value of $\langle\bar{v}\rangle$ calculated using eq A9 with the *worst-case* values of $\bar{v}_A$ and $\bar{v}_B$ given above is 0.710 mL/g. The value of $M^*_{w,R}$ calculated using eq A8 with $\bar{v} = \langle\bar{v}\rangle$ is in error by only 1% at the extreme solution compositions and considerably less at intermediate compositions. It follows that, for the purpose of analyzing the gradient of total C1r as a function of radial position, text eq 15 is an excellent approximation to the general relation (eq A5).

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