α-CHYMOTRYPSIN: CHARACTERIZATION OF A SELF-ASSOCIATING SYSTEM IN THE ANALYTICAL ULTRACENTRIFUGE

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Introduction

We have studied the dimerization of α-chymotrypsin as an example of a typical self-associating system in order to show how such a reaction may be characterized. α-Chymotrypsin has also been suggested as a model system to ensure the proper operation of the analytical ultracentrifuge. An ideal model system should be a well-characterized association where a single equilibrium reaction is being observed with little effect from solution conditions such as pH, ionic strength and temperature. Such a system probably does not exist. However, α-chymotrypsin has been well-characterized and has been used to test the consistancy of instrument measurements over time.[1] At about pH 4, α-chymotrypsin exhibits a monomer-dimer equilibrium, but the association constant can vary with different lots and different buffer conditions.[2-4] Therefore, it is a good idea to have a standard lot number and buffer for repeated examination. In 0.01 M acetate, 0.2 M KCl, pH 4.4 at 20°C, the dimerization constant has been reported to vary about two-fold between lots of α-chymotrypsin purchased from Worthington Biochemicals with a maximum of 44×10^3 L/mol.[4] The variation appears to be a function of lot number and not of experimental error since the same lot showed less than 10% variation over a period of four years.

Materials and Methods

A commercial sample of α-chymotrypsin (Worthington Biochemical Corp., lot # 37K093) was evaluated for self-associative behavior by sedimentation equilibrium using a Beckman Coulter Proteomelab XL-A analytical ultracentrifuge from Beckman. The protein was run at three concentrations (0.2, 0.4 and 0.6 mg/mL) in 10 mM NaOAc, 0.2 M NaF, pH 4.0 at 283 nm and 20°C without further purification. The literature value of 0.736 mL/g was used as the partial specific volume.[5] A buffer density of 1.001 g/mL was also used in the calculation. Sedimentation equilibrium data were evaluated using a nonlinear least-squares curve-fitting algorithm[6] contained in the Proteomelab XL-A Data Analysis Software. The self-association model shown in equation 1 permits analysis of either a single ideal species or up to four associating species, depending on which parameters in the equation are allowed to vary during convergence. Data were analyzed both as single and multiple data files.

$$
\begin{aligned}
A_{r,\text{total}} = {} & e^{\left[\ln(A_{\text{monomer},r_0}) + \frac{(1-\bar{v}\rho)\omega^2}{2RT} M(r^2 - r_0^2) - BM(A_{\text{total},r} - A_{\text{total},r_0})\right]} \\
& + e^{\left[\ln(A_{\text{monomer},r_0}) + \ln(K_{a,2}) + \frac{(1-\bar{v}\rho)\omega^2}{2RT} n_2 M(r^2 - r_0^2) - BM(A_{\text{total},r} - A_{\text{total},r_0})\right]} \\
& + e^{\left[\ln(A_{\text{monomer},r_0}) + \ln(K_{a,3}) + \frac{(1-\bar{v}\rho)\omega^2}{2RT} n_3 M(r^2 - r_0^2) - BM(A_{\text{total},r} - A_{\text{total},r_0})\right]} \\
& + e^{\left[\ln(A_{\text{monomer},r_0}) + \ln(K_{a,4}) + \frac{(1-\bar{v}\rho)\omega^2}{2RT} n_4 M(r^2 - r_0^2) - BM(A_{\text{total},r} - A_{\text{total},r_0})\right]} + E
\end{aligned}
\qquad (1)
$$

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where A_r = absorbance at radius r
$A_{monomer,r_0}$ = absorbance of the monomer at the reference radius r_0
M = monomer molecular weight
n_2 = stoichiometry for species 2
$K_{a,2}$ = association constant for the monomer-*n*-mer equilibrium of species 2
n_3 = stoichiometry for species 3
$K_{a,3}$ = association constant for the monomer-*n*-mer equilibrium of species 3
n_4 = stoichiometry for species 4
$K_{a,4}$ = association constant for the monomer-*n*-mer equilibrium of species 4
E = baseline offset
B = second virial coefficient for nonideality

The association constant was converted to units of M^{-1} using an extinction coefficient $E^{1\%}_{280}$ of 20.4,[5] and assuming a value of twice that for the dimer; *i.e.,*

$$K_{conc} = K_{abs} \times \frac{\varepsilon l}{2} \qquad (2)$$

where K_{conc} = The association constant in M^{-1}
K_{abs} = The association constant in terms of absorbance (estimated directly from a best-fit curve of a monomer-dimer self-associating system)
ε = The extinction coefficient in L/mol-cm [44,064 L/mol-cm for α-chymotrypsin monomer, given an absorptivity (*a*) of 2.04 L/g-cm and a monomer molecular weight of 21,600 g/mol[5]]
l = The pathlength in cm (1.2 for a 12-mm centerpiece)

Results and Discussion

A stepwise approach used to determine the self-associative behavior of α-chymotrypsin is presented here. A general approach to more complicated systems is beyond the scope of this paper and is dealt with in a separate publication.[7]

Step 1. Transforming an equilibrium gradient (absorbance *vs.* radius) into a plot of $M_{w,app}$ *vs.* absorbance provides information about the associative order of the system. The transformation involves moving a segment of data points (typically 10–40) across the radial path one data point at a time and calculating $M_{w,app}$ from the slope of a ln*(A) vs.* r^2 plot of this subset. A plot of $M_{w,app}$ vs. absorbance (taken as the midpoint of each segment) creates a series of connecting lines whose slope is proportional to the molecular weight. The shape of the plot can provide an estimate of self-associative behavior with respect to concentration. This assumes that the sample obeys the Beer-Lambert Law where absorbance and concentration are proportional.

For material behaving as a single ideal species, $M_{w,app}$ does not vary with absorbance. The plot for an associating system, on the other hand, curves upward with increasing absorbance. Dividing the molecular weight at the maximum absorbance by the molecular weight at the minimum absorbance (*i.e.*, the monomer molecular weight, M_1) provides a first approximation of the associative order of the system. In the case of α-chymotrypsin (Figure 1a), the material appears to be associating as a monomer-dimer, although assembly to a higher order aggregate is possible. At this point it is not possible to discriminate between the two.

In general, a system can be made to assemble more fully by running it at higher concentrations. It should be noted, however, that systems will begin to exhibit nonideality (from excessive crowding or charge effects) when pushed to higher concentrations and that this can obscure the highest associative state.

A second, more qualitative method that can provide information about the homogeneity of the system is simply a plot of ln *(A) vs.* r^2 as mentioned above. This plot yields a straight line with a slope proportional to the molecular weight. For a single ideal species, the line remains straight over the entire radial path. For an associating system, however, the line will deflect upward, due to the presence of higher molecular weight aggregates redistributing to the cell bottom. The apparent linear nature of the ln *(A) vs.* r^2 plot for chymotrypsin illustrates how this type of technique can be misleading (see Figure 1b). Deviations of less than 10% can be difficult to perceive with the naked eye.

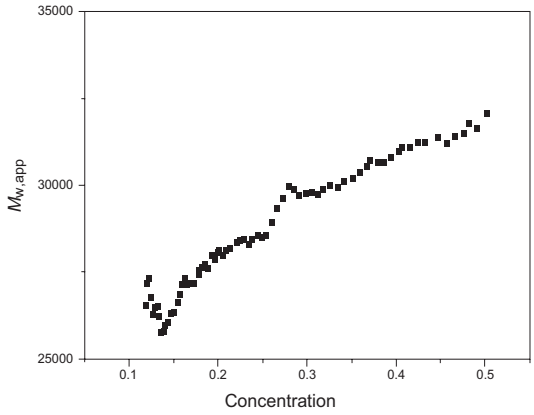

Figure 1a. $M_{w,app}$ *vs.* concentration plot of α-chymotrypsin at pH 4.0. The appearance of the gradient increasing to the next multiple of monomer molecular weight (21,600 g/mol, Ref. 5) suggests the dimer as a likely associative state.

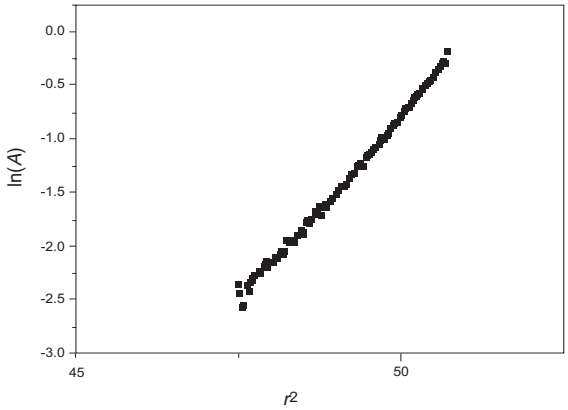

Figure 1b. The equilibrium gradient depicted as a plot of ln*(A) vs.* r^2. The apparent linear nature of this plot suggests an ideal species, which runs contrary to the earlier evidence. Due to the inherent insensitivity of this approach, this type of diagnostic is more common in detecting the absence of homogeneity; i.e., deviations from a straight line indicate that a sample is definitely not behaving ideally.

Step 2**.** The data are fit to a single ideal species model. In addition to getting an estimate of the apparent weight-average molecular weight ($M_{w,app}$), the pattern from the residuals (the points off the best-fit curve) can provide insight into the behavior of the system. Figure 2 shows a residual pattern for α-chymotrypsin consistent with an associating system and a $M_{w,app}$ (30,012) > M_1 (21,600). This information also helps to confirm the plot of $M_{w,app}$ *vs.* absorbance.

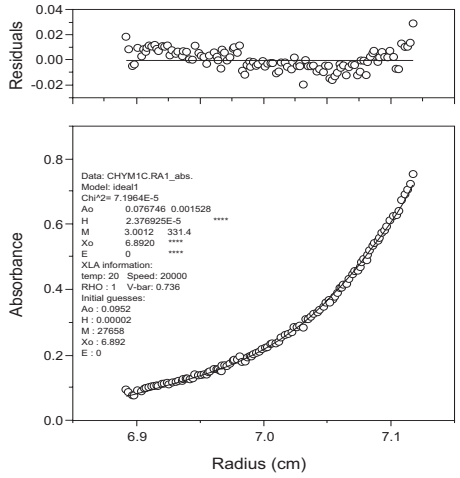

Figure 2a. The equilibrium fit results of α-chymotrypsin modeled as a single ideal species. The fitted parameter for the weight-average molecular weight ($M_{w,app}$), estimated at 30,012 g/mol, was found to be higher than the monomer molecular weight, suggesting aggregation. The residuals from the best-fit curve reveal a systematic pattern indicative of an aggregating system.

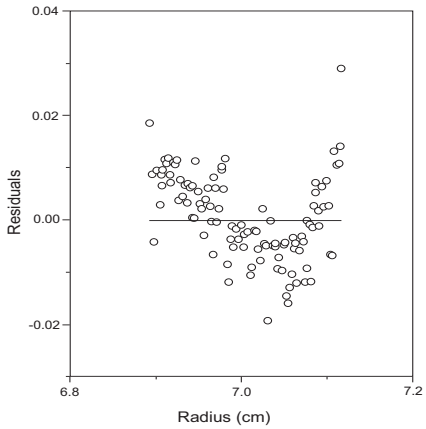

Figure 2b. The residuals plot scaled to make the pattern indicating association more recognizable.

Step 3. The data are fit to a more complex self-associating model. In addition to identifying the stoichiometry, the association constant of the system can also be estimated. This step is also used to test the reversibility of the system. A reversible self-associating system should yield the same association constant independent of rotor speed or initial concentration. The more complex models are usually evaluated with multiple data files (assuming individual data files have been pretested for any aberrant behavior). In addition to facilitating convergence on a global least-squares minimum, multiple data files carry the advantage of collectively spanning the associative range of the system.

One of the caveats of dealing with more complex models, however, is that the larger number of parameters that are used to describe the model may cause problems during a fit. For example, if too many parameters are allowed to vary at one time, the statistical significance of the fitted values can be compromised for the sake of a fit. For this reason, the number of parameters allowed to vary during a fit should be kept to a minimum. As a general rule, the monomer molecular weight and the stoichiometry are typically constrained to their known (or suspected) values during a fit. Estimates of M_1 can be made on the analytical ultracentrifuge under denaturing conditions. The other parameters are then determined by successively allowing each one to vary over a series of iterative fits.

α-Chymotrypsin was modeled as a monomer-dimer system, as shown in Figure 3a. In this first example, the term for the baseline offset *(E)* was constrained at zero. The baseline offset term corrects for any residual components that contribute to the absorbance of the system. This term is usually measured by overspeeding the equilibrium run and reading the absorbance of the depleted region of the gradient (the meniscus-depletion method). As shown in Figure 3a, two of the files have a pronounced slope to them. The baseline correction term is included in Figure 3b, and the fit is seen to improve dramatically. The fit is evaluated on the basis of the randomness of the residuals, the magnitude of the residuals (expressed in terms of standard deviations, *i.e.*, the average absorbance collected at each radial position), by the relative tightness of the confidence limits, by the goodness of fit statistic and by checking some of the fit parameters for physical significance. The fitted values for the baseline offset in this example were confirmed by meniscus depletion at 45,000 rpm for about 6 hours.

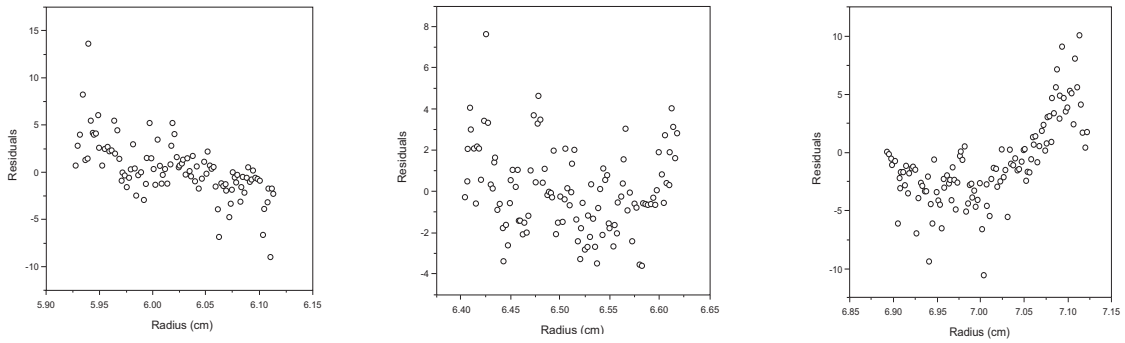

Figure 3a. Multiple data files (three concentrations) showing the deviation from a best-fit curve of a monomer-dimer associative model. In this example, the baseline offset term (E) was constrained at zero and the residual pattern is skewed for two of the data files

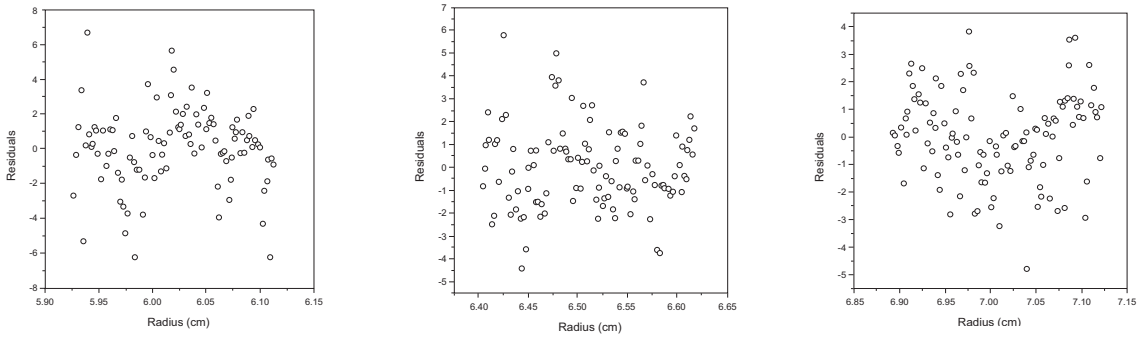

Figure 3b. The same example with the baseline offset term allowed to vary. In this case, the residuals for all three files are shown to afford a random scatter, indicating a good fit to the model.

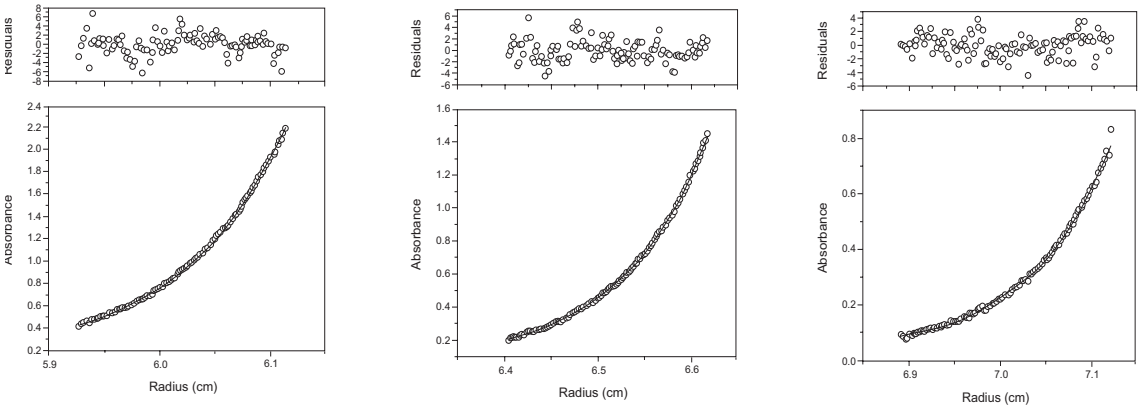

Figure 3c. The best-fit curve and the residual plot for each of the three files from the same monomer-dimer fit of Figure 3b.

The values estimated for the association constant are presented in Table 1. The literature values for the association constant are shown to vary from lot to lot. This has been attributed to incomplete participation of the monomer in the equilibrium (incompetent monomer); with further purification, there is an increase in the association constant and more consistent readings.[4] At this point the correct model appears to be a monomer-dimer system, although higher order models should also be evaluated.

Step 4. α-Chymotrypsin was modeled as a monomer-trimer system, and as shown in Figure 4, the fit is markedly worse than for the simple dimerization. The residuals are nonrandom and the magnitude of the residuals is high. The way in which the data dips away from the best-fit curve indicates that fits to higher order systems will result in even worse fits.

The conclusion based on this brief exercise is that α-chymotrypsin at pH 4.0 behaves as a reversible monomer-dimer self-associating system.

Table 1. The Monomer-Dimer Self-Association Constants for α-Chymotrypsin[1]

Concentrations/ Buffer System	K_{abs} [2]	*95% Confidence Limits* K_{abs}	K_{conc} [3] *(X 10^{-3} L/mol)*	K_{lit} [4]
0.2, 0.4, and 0.6 mg/mL; 10 mM NaOAc, 0.2 M NaF, pH 4.0	2.56	2.17–3.03	67.3	44.4 35.7 14.9 27.4

1. The sedimentation equilibrium gradients were curve-fit using multiple data files and the Proteomelab XL-A Data Analysis Software.

2. The association constant as estimated from a best-fit curve in terms of absorbance units using $\bar{\nu}$ = 0.736 mL/g and ρ = 1.001 g/mL.
3. The association constant converted into units of M^{-1} using ε = 44,064 L/mol-cm.
4. Different literature values for the association constant (determined at pH 4.4), reflecting lot-to-lot variation.

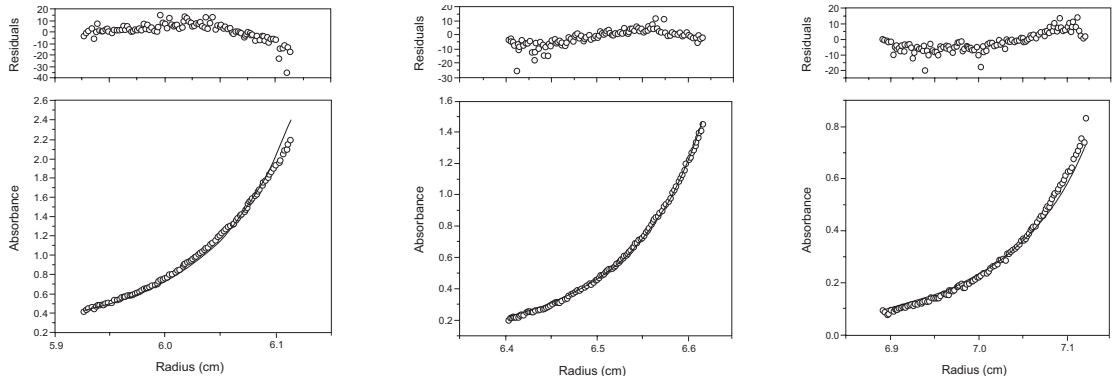

Figure 4. Shows a relatively poor fit when the same three data files are fit to a monomer-trimer self-associating system under similar fit conditions

References

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CENT-1319APP12.15-A