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Thermal Denaturations of Staphylococcal Nuclease Wild-Type and Mutants Monitored by Fluorescence and Circular Dichroism are Similar: Lack of Evidence for Other Than a Two State Thermal Denaturation

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Abstract

It is unclear whether the thermal denaturation of staphylococcal nuclease is a two state, three state, or variable two state process. The thermal denaturation of wild-type staphylococcal nuclease was followed by tryptophan fluorescence and circular dichroism signal at 222 nm, forty-two and fourteen times respectively. Analysis of this data using a simple two state model gave melting temperatures of 53.0 ± 0.4°C (fluorescence) and 52.7 ± 0.6°C (CD) and van't Hoff enthalpies of 82.4 ± 2.6 kcal/mol and 88.6 ± 4.2 kcal/mol. Ninety-seven mutants also had these parameters determined by both fluorescence and CD. The average difference between the melting temperatures was 1.05 ± 0.75° and the average difference between van't Hoff enthalpies was 1.6 ± 4.8 kcal/mol. These very similar results for the two spectroscopic probes of structure are discussed in the context of the different models that have been proposed for nuclease denaturation. It is concluded, for most nuclease variants, that the errors introduced by a two state assumption are negligible and either virtually all helical structure is lost in any initial unfolding event or any intermediate must have low stability.

Keywords

Thermal unfolding; residual structure; melting temperature; van't Hoff enthalpy

Introduction

The thermodynamic parameters of protein stability can be determined by solvent and thermal denaturation experiments. Typically, however, only one or the other technique is used. We were interested in comparing the solvent and thermal denaturation of staphylococcal nuclease, a model protein used by ourselves and others for studies of mutational effects upon protein stability. There is evidence that at least some mutants of nuclease, in particular the V66W mutant, unfold during solvent denaturation in three states, rather than the two state model normally used for data analysis [1-13]. Maki *et al.* showed that a proline-minus variant of nuclease could have no more than 5% of an equilibrium intermediate at any urea concentration, while a F76W mutant of this proline-minus variant has nearly 50% of the protein populating an equilibrium intermediate at moderate urea concentrations. Bolen's group has argued convincingly [13,14] that neither a simple two or three state model fully explains the data for

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wild-type nuclease and the majority of mutants that have been studied. They generally favor the variable two state model proposed by Shortle [15,16], wherein the character of the denatured state changes with mutation, denaturant concentration, or temperature. However, they point out several disagreements between this model and experimental data [12-14].

Similar questions can be raised about whether a two, three, or variable two state model is more appropriate for analysis of thermal denaturation of nuclease. Carra and Privalov, proponents of the three state model for solvent denaturation [17], have concluded that thermal denaturation of wild-type nuclease is two state [17,18]. On the other hand, Eftink's group has shown that V66W mutant with tryptophan or tryptophan analogues, appears to be better fit by a three state thermal unfolding model [7]. Therefore, as a necessary first step before comparing the thermal and solvent denaturation behavior of many staphylococcal nuclease mutants, we present here data comparing the thermal denaturation of staphylococcal nuclease and 97 mutants monitored by two different probes of structure, tryptophan fluorescence and the circular dichroism (CD) helical signal at 222 nm.

Tryptophan fluorescence is a fairly local probe of structure, monitoring the degree of solvent exposure of the single tryptophan found in staphylococcal nuclease at position 140. (Figure 1) The CD signal at 222 nm, on the other hand, monitors the amount of helix found throughout the protein. The physical model for three state denaturation in nuclease is that the C-terminal helix, which ends with tryptophan 140, unfolds in the first transition. Carra and Privalov propose that the other helices unfold in this transition as well, leaving only the beta barrel structure, an idea supported by some hydrogen exchange data [16,19,20]. The remaining structure then breaks down cooperatively in a subsequent step. The putative intermediate is indistinguishable by tryptophan fluorescence from the more fully unfolded form as the 140 side chain is fully exposed in both. The loss of all helical content would not allow CD to distinguish between the partially and fully denatured states. While it seem certain that the C-terminal helix breaks down easily, it seems less clear whether the other helices would in fact break down in any initial unfolding step. NMR [21-25], CD [26], and mass spectrometry [27,28] study of denatured nuclease have identified residual helical structure in both of the first two helices, which are not proximal to the C-terminal helix in primary or, for the case of the first helix, tertiary structure. Further, Bolen's group has shown that the denatured state becomes more compact at lower concentrations of denaturants [13,14] and compactness is commonly held to lead to an increase in secondary structural content. If a similar trend were true for high and low temperatures, mutants denaturing at lower temperatures might have a more compact denatured state with a higher helical content that breaks down at higher temperature. Thus, one would predict that the two probes of structure, fluorescence and CD, might give different results if a three state denaturation or a variable two state model more closely reflects the reality of the thermal denaturation than does a simple two state model.

Theory

A more detailed theoretical analysis bears this out, but points out important limitations on what the two probes of structure can differentiate. In past work staphylococcal nuclease denaturation behavior has been analyzed by a two state model. The apparent equilibrium constant, K_{app}, for the two state reaction *native state* Ý *denatured state* is calculated using the equation:

$$K_{app} = \frac{[N]}{[D]} = \frac{I_N - I}{I - I_D} \quad 1)$$

where [N] and [D] are the concentration of the native and denatured states, respectively, I_N is the fluorescence or circular dichroism of the native state, I_D the fluorescence or circular dichroism of the denatured state, and I is the fluorescence or circular dichroism intensity at a

given temperature. In practice, both I_N and I_D for both fluorescence and CD decrease as temperature increases in a generally linear fashion.

Let us suppose that the denaturation behavior of nuclease is, instead, a three-state equilibrium.

$$N \overset{K_1}{\acute{Y}} D_1 \overset{K_2}{\acute{Y}} D_2$$

The equilibriums, defined by K_1 and K_2, in the absence of evidence to the contrary, will be assumed to be two-state and [N], [D_1] and [D_2] are the concentrations of protein in, respectively, the N, D_1 and D_2 states. We adopt the nomenclature of D_1 and D_2 for the partially unfolded and the more completely unfolded state, respectively, to emphasize that the intermediate state is not completely folded, is presumably enzymatically inactive, and to avoid some of the connotations associated with the term “intermediate.” Therefore:

$$K_1 = \frac{[D_1]}{[N]} \tag{2}$$

and

$$K_2 = \frac{[D_2]}{[D_1]} \tag{3}$$

The overall, total equilibrium, K_T, for *native state* Ý *denatured states* is then given by

$$K_T = \frac{[D_1] + [D_2]}{[N]} = \frac{[D_1]}{[N]} + \frac{[D_2]}{[N]} = \frac{[D_1]}{[N]} + \frac{[D_2]}{[D_1]}\frac{[D_1]}{[N]} = K_1(1 + K_2) \tag{4}$$

The fraction of the protein in each state can be expressed terms of these equilibrium constants as well.

$$\frac{[N]}{[N] + [D_1] + [D_2]} = \frac{1}{1 + K_1 + K_1K_2} = \frac{1}{1 + K_T} \tag{5}$$

$$\frac{[D_1]}{[N] + [D_1] + [D_2]} = \frac{1}{\frac{1}{K_1} + 1 + K_2} = \frac{K_1}{1 + K_T} \tag{6}$$

$$\frac{[D_2]}{[N] + [D_1] + [D_2]} = \frac{1}{\frac{1}{K_1K_2} + \frac{1}{K_2} + 1} = \frac{K_1K_2}{1 + K_T} \tag{7}$$

The overall intensity, I, of any spectral probe of the protein is related to the fraction of the protein in a given state and the intensity of the protein in that state, I_N, I_{D_1}, I_{D_2}, respectively representing the intensity of the N, D_1, and D_2 states.

$$I = \frac{I_N}{1 + K_T} + \frac{I_{D_1}K_1}{1 + K_T} + \frac{I_{D_2}K_1K_2}{1 + K_T} \tag{8}$$

Rearranging we find that

$$I(1 + K_T) = I_N + (I_{D_1} - I_{D_2})K_1 + I_{D_2}K_T \tag{9}$$

$$K_T = \frac{I_N - I + \left(I_{D_1} - I_{D_2}\right)K_1}{I - I_{D_2}} \quad 10)$$

The physical evidence indicates that the fluorescence intensity of tryptophan 140 (W140) is identical in both the D_1 and D_2 states,[1] in other words I_{D_1} is equal to I_{D_2}. Therefore, the overall equilibrium between the native state and the denatured states, K_T, at a particular temperature, in equation 10 simplifies to the relationship

$$K_T = \frac{I_N^{140} - I^{140}}{I^{140} - I_D^{140}} \quad 11)$$

where I^{140} is the measured fraction weighted intensity of W140, I_N^{140} is the intensity of W140 in the native state and I_D^{140} is its intensity (assumed to be equal) in either the D_1 or D_2 state. This is just a restatement of equation 1 and thus the fluorescence intensity of W140 gives an accurate measurement of the overall equilibrium constant between the native states and the denatured state(s) at a given temperature, regardless of whether the relationship between these states is best described by a two state or three state model. This is also true for CD if all helical signal is lost in the first transition.

A variable two-state model is similar to the two state treatment. The most significant difference is that the character of the denatured state changes with temperature, but in a second order manner rather than the first order manner of the three state model. In the case of fluorescence monitoring of staphylococcal nuclease thermal denaturation, the value of I_D is not affected by the character of the denatured state since tryptophan 140 is apparently fully solvent exposed in any denatured state. However, the possibility exists that residual helix exists in the denatured state, particularly at lower temperature. This helix would break down as the temperature increases, but in a non-cooperative fashion. How this would be reflected in the denaturation curve is hard to model since many assumptions about precisely how the helix breaks down have to be made. Qualitatively, however, Bolen's data [13,14] show a rapid expansion in denatured state size at low denaturant concentrations followed by a much slower one at high denaturant concentrations. If a similar pattern holds for temperature, then one might expect for mutants which denature at lower temperatures a rapid, but non linear loss of CD signal due to denatured state changes to be overlaid upon a native state denaturation, leading to disagreement between CD and fluorescence curves. For mutants that denature at higher temperatures, the slower loss of any smaller amount of helix left would be blended into the normal linear loss of CD signal with temperature in I_D and there would not be any significant difference between fluorescence and CD determined T_m.

On the other hand, the three state denaturation can be well modeled. Suppose the CD signal is different for each of the native, D_1 and D_2 states. While I_N and I_{D_2} are easily measured from the native and denatured baselines, I_{D_1} is much harder to determine. The value of K_1 is also unknown and therefore a solution of equation 11 to determine K_T is not possible. Alternatively, if either K_1 or I_{D_1} is known, then the other could be determined using the K_T values as determined by fluorescence, but we, unfortunately, do not know either. Nevertheless, we can use this relationship to estimate important parameters of any possible three state denaturation. Rearranging equation 9 in a different way we find that the intensity of any spectral probe can be described as follows.

$$I = \frac{I_N + I_{D_2}K_T + \left(I_{D_1} - I_{D_2}\right)K_1}{\left(1 + K_T\right)} \quad 12)$$

If one compares CD and fluorescence data, examination of equation 12 shows that the key term in the disagreement between the two techniques is the value of $K_1/(1 + K_T)$, if one assumes there is a significant difference in the values of I_{D_1} and I_{D_2} in the CD. Such an assumption seems reasonable if the physical model for the first denaturation step is just the loss of the C-terminal helix. The second transition would be the breakdown of the beta barrel and the long and short helix it contains. We estimate that in this case the loss of helical intensity would be roughly equal in each transition. It may be more realistic to assume that much more helical intensity is lost in the first transition than the second, a point we shall return to later. For now though, the question is whether $K_1/(1 + K_T)$ is of appropriate value to be detectable. The larger K_1 and/or the smaller K_T, the larger the difference in the intensity. However, recall from equation 5 that K_T is equal to $K_1(1 + K_2)$. Further, K_1 and K_2 must both generally trend upwards or down together as the temperature is raised or lowered. In other words, it is a difficult condition to fulfill that K_1 is large and K_T small.

The practical effect of this is most clear at the temperature of overall melting, when K_T is 1. At this temperature $K_1 = 1/(1 + K_2)$. Near the overall melting temperature, K_1 must be comparable to or less than K_2, otherwise the D_1 state is never significantly populated and the reaction is really more simply described as a two state unfolding mechanism. However, the value of $K_1/(1 + K_T)$ is maximized the closer to equality that K_1 and K_2 are. This happens the closer the value of the melting temperature of the first transition is to the second. In other words, if there is a large difference in the melting temperatures for the two transitions, there will be less difference between the transition as monitored by CD and fluorescence. If the two transitions are similar in melting temperature, i.e. both D_1 and D_2 are well populated near the overall melting temperature, where K_T is 1, there will be a larger difference in intensity. This translates into a difference in the melting temperature measured by the two techniques when analyzed by the two state model.

Experimental

Therefore we followed thermal denaturations of wild-type staphylococcal nuclease and 97 mutants by fluorescence and CD. Mutagenesis and protein expression and purification were carried out as described elsewhere [29]. Each mutant, at protein concentration of approximately 50 g/ml in a buffer consisting of 25 mM phosphate, 100 mM NaCl, pH 7.0, was subjected to a thermal denaturation in our Aviv ATF-101 fluorometer (296 nm excitation, 325 nm emission), as previously described in detail [30,31]. Thermal denaturations using circular dichroism (CD) at 222 nm as a probe of structure were performed using a Jasco 700 spectropolarimeter equipped with a Peltier thermocontrol unit in the same buffer but at protein concentrations of approximately 80 g/ml in a standard 1 cm cuvette with stirring. Thermal ramps were generally performed from 20 to 70°C at a rate of 50°C/hr for CD and 60°C/hr for fluorescence. Less stable mutants were started at lower temperatures. Temperatures in both instruments were calibrated throughout the range to the same NIST traceable thermometer. One difference of note is that temperatures in the fluorometer are measured directly in the solution by a Teflon coated thermocouple, while those in the CD instrument are measured from a thermocouple in the wall of the cuvette holder, since the CD software was not capable of recording an additional temperature input. The temperature of the solution in the cuvette presumably lags behind that of the wall, but we have not attempted to apply a correction factor for this effect.

Analysis of the data yields two thermodynamic parameters of unfolding; the temperature at which half of the protein molecules are unfolded (T_m) and the van't Hoff enthalpy of the unfolding reaction (ΔH_{vH}) at the midpoint temperature. The data was processed by first doing linear fits of the native and denatured baselines. Tryptophan fluorescence is not actually linear with temperature, but as shown by Eftink [32,33], the assumption of baseline linearity usually

introduces negligible error. However, it is possible this assumption or other differences in fitting of the baselines of fluorescence versus CD data are responsible for some of the slight systematic differences discussed later between T_m and ΔH_{vH} values returned by the two probes of structure. Native baselines for wild-type nuclease were usually fit over a ten degree range starting at 25 to 28°C and denatured baselines over a 8-9 degree range, starting at 66 to 67°C. Similar ranges were used for mutants, but starting points were adjusted appropriately. To find the T_m, temperature in Kelvin was fit over the range of $\ln K_{app} < 2$ and > -2 as a linear function of $\ln K_{app}$, calculated by a two state model. A second order least squares fit of $\ln K_{app}$, again over the range of $\ln K_{app} < 2$ and > -2, was made to 1/T to determine the van't Hoff enthalpy of the unfolding reaction.

Results and Discussion

Average values for wild-type

In order to determine the error in each of these values we have repeatedly followed the thermal denaturation of wild-type nuclease by fluorescence and CD; forty-two and fourteen times respectively. The fluorescence denaturations have taken place over a period of twelve years and have been performed by a number of different individuals using multiple preparations of wild-type staphylococcal nuclease. The denaturations that were monitored by CD took place over only an eighteen month period, were largely performed by one individual and, although multiple protein preparations were used, the number was not as great. The average value of the melting temperature as determined by fluorescence was 53.0°C with a standard deviation of 0.4°C and 52.7 ± 0.6°C when determined by CD. The average van't Hoff enthalpy value determined by fluorescence was 82.4 ± 2.6 kcal/mol and 88.6 ± 4.2 kcal/mol when determined by CD. For the fluorescence data, it appears that variations between protein preparation, presumably related to the level of purity, are the largest source of variation in the value of T_m and ΔH_{vH}. This potential source of variation is not discernable in the noisier CD data. The higher melting temperature for wild-type staphylococcal nuclease measured by fluorescence is statistically insignificant, but is the opposite of what would be expected if a three state denaturation were observed by CD but not by fluorescence. The difference in van't Hoff enthalpies, 6.2 kcal/mol, is slightly greater than the square root of the sum of the squared standard deviations, 4.9, but here again the higher value for the CD data is opposite what one would expect if the CD transition were broader than that measured by the fluorescence probe.

Comparison of mutants

Wild-type was examined multiple times, but mutants were typically measured only once unless the initial experimental run was obviously flawed, which if it occurred was most commonly due to noise at high temperatures caused by gas bubbles forming on the cuvette wall in the beam path. Mutants with fluorescence midpoint temperatures that were less than 35°C were excluded from this study as these mutants are too unstable to give a true native baseline. The values for T_m and ΔH_{vH} for each mutant are available in a table in the supplementary material.

Even though data obtained from a CD thermal denaturation is inherently more noisy than tryptophan fluorescence, there is a very strong correlation ($r^2 = 0.9787$, Figure 2) between the midpoint temperatures of staphylococcal nuclease mutants calculated by both techniques using a two-state model of analysis. Also, the slope of the regression line is 1.012, negligibly different from a 1:1 dependence. Similarly, the agreement between the van't Hoff enthalpies is good ($r^2 = 0.8441$, Figure 3) and again the slope of the regression line is very close to one (0.8452), although there are several prominent outliers.

In regard to the variable two state model, we suggested above that the Bolen data [13,14] showing more compact denatured states at low denaturant concentrations might, by analogy,

mean that denatured states were more compact at lower temperatures. If this compactness leads in turn to more helical structure, one might expect to see a greater divergence in CD and fluorescence values for T_m and ΔH_{vH} for mutants that denature at lower temperatures than those that denature at higher temperatures. In fact, Figure 2 does not show such a general trend. Two outliers in Figure 3 do have quite significantly higher fluorescence ΔH_{vH} values but given the larger error in ΔH_{vH} and the number of other mutants not showing this behavior, it does not constitute a statistically significant trend.

The strong correlation in midpoint temperatures and van't Hoff enthalpies evaluated from two different probes suggests that either wild-type nuclease and mutants thermally unfold by a two state mechanism or, at the very least, if unfolding is better reflected by a variable two state or three state model, that the errors introduced by calculating thermodynamic parameters based on two-state assumptions are very low.

An examination of the differences in T_m and ΔH_{vH} measured by the two probes of structure offers further support for this supposition. These two parameters are plotted against one another in Figure 4. First, note that differences in T_m and ΔH_{vH} are poorly correlated with one another. This is significant because it suggests that these differences are due to random experimental error, rather than any systematic error introduced by incorrect modeling and data analysis.

Further, a three state and possibly a variable two state denaturation would tend to lead to higher T_m values in a two state analysis of CD data, since the second transition, observable by CD but not by fluorescence, would occur at a higher temperature. Similarly, a broader transition generally equates to lower ΔH_{vH} values from the CD data. The difference in T_m for each these variant forms of nuclease is on average is fairly small, 1.05°C with a standard deviation of 0.75. Still, this implies that there is a systematic difference between the two values with some statistical significance. However the fluorescence data generally gives the higher value for T_m, which is not what we expect if the two state model used for analysis is deeply flawed. As noted above, we calibrated the steady state temperatures on both instruments over the range in question and the differences in the way sample temperatures are measured would tend to result in higher recorded temperatures for the CD. We have been unable to isolate the reason for this difference but believe, because it has a sign opposite from that expected from modeling error, that it is an experimental systematic error. On the other hand, this raises the possibility that this systematic experimental error is disguising another systematic error (of opposed sign) arising from the incorrect assumption of the two state model.

We doubt this for two reasons. First, this would mean that any experimental systematic error was in fact larger by the amount of the cancelled analytical systematic error. We believe that the measures we have taken to calibrate the instruments make an even larger systematic experimental error very unlikely to have evaded our notice. Second, any systematic error in temperature will have a minor impact on the van't Hoff enthalpy, defined as $-R(d(\ln K_{app})/d(1/T))$, and T is, at the smallest, 308°K. A one degree systematic error in T changes a 60 kcal/mol value of ΔH_{vH} at this temperature by a negligible 0.2 kcal/mol. That this is the case is supported by the poor correlation of differences in T_m and ΔH_{vH} noted above. In fact, the average value for the difference in the van't Hoff enthalpies for wild-type and each mutant is −1.6 kcal/mol with a standard deviation of 4.8. Not only is the average difference statistically insignificant, the sign indicates that the CD van't Hoff enthalpy is higher on average than the fluorescence van't Hoff enthalpy, in the opposite direction of that expected if the CD were recording a broader transition. In short, after detailed examination of the data, there is no experimental evidence in this body of work that the use of a two state model for staphylococcal nuclease thermal denaturation monitored by CD introduces measurable or meaningful error relative to the fluorescence data. This, as the discussion above describes, means that only

relatively low levels of the intermediate D_1 can accumulate and/or that most helix is lost in the initial breakdown of structure.

This is a qualitative statement, but we can approach it more quantitatively for the three state model. As shown in equation 5, the overall equilibrium, K_T is equal to $K_1(1 + K_2)$. Each equilibrium can be related to the free energy difference for that transition by the equation $\Delta G = -RTlnK$. In turn, the Gibbs-Helmholtz equation expresses how each ΔG changes as a function of temperature, T, and the constants, ΔH_m, the enthalpy of unfolding at the melting temperature, T_m, and $\Delta C_{p,}$ the difference in heat capacity between the states in question. Expressing K_T in this fashion leads to a rather complex equation for which no single solution is possible, but we examined the range of ΔH_m, T_m, and ΔC_p values for each of the two transitions that reproduce the experimentally measured ΔH_m and T_m values for the overall transition of wild-type. The CD and fluorescence monitored denaturations were modeled as described above and we first assumed that half the overall CD helical signal was lost in each of the two transitions or, in other words, that I_{D1} was equal to $(I_{D2} + I_N)/2$. The simulated CD intensity generated by an underlying three state model was then analyzed with a two state assumption and the difference between T_m in the modeled fluorescence and CD data was allowed to be as much as 1.5 degrees, a difference that we would be able to detect. The range of values of ΔH_m, T_m, and ΔC_p for each of the two transitions that meet these restrictions proves to be quite limited. As the discussion above makes clear, the most important parameter is the difference between the two transition T_m values. We found that this difference had to be, generally speaking, 10°K or greater and, notably, that the T_m for the second transition had to be lower than that of the first, otherwise we found a large difference in the apparent T_m returned by two state analysis of the simulated fluorescence and CD data. If this condition is met, then the required values for ΔH_m and ΔC_p for the second transition were low, under 25 kcal/mol and 0.7 kcal/(mol·K), anything higher would be readily detected. Higher values for ΔH_m and ΔC_p of the second transition are possible only if the T_m for the second transition is low, in other words, if the denaturation goes straight from N to D_2 at higher temperature without populating D_1. In terms of the population of D_1, any accumulation greater than 20% of total protein would be readily detectable and, depending on the values of the other parameters, in most cases, much less than this would be detected. If two-thirds of the CD signal is lost in the first transition, i.e., I_{D1} was equal to $(I_{D2} + I_N)/3$, then the T_m for the two transitions could be roughly equal with values for ΔH_m and ΔC_p for the second transition similar to those cited above, and as much as 40% of the protein could accumulate in the D_1 state, before we could be reasonably certain of detecting it. If I_{D1} is equal to $(I_{D2} + I_N)/4$, the values for the second transition ΔH_m and ΔC_p can be comparable to the first transition and the T_m for the second transition higher approximately 15 degrees, with nearly 80% of the protein accumulating in the D_1 state, before detection is likely.

Conclusions

Fluorescence data returns values for melting temperature and van't Hoff enthalpy of the overall denaturation of staphylococcal nuclease that are not dependent upon whether a two state, variable two state, or three state thermal denaturation is occurring, although in the latter two cases the character of the denatured state is assumed to vary from mutant to mutant. Comparison of the CD and fluorescence data for staphylococcal nuclease indicate that if a three state thermal denaturation is occurring, with comparable amounts of CD signal lost in each transition, the transition between the two denatured states has a significantly lower unfolding temperature than the transition between the native state and the first denatured state. In addition, the values of ΔH_m and ΔC_p for any postulated transition between the two denatured states must be quite low if as little as 20% of the protein ever populates the D_1 state. However, if three-quarters or more of the CD signal is lost in a first transition, it would be very difficult to detect any difference between CD and fluorescence determined values, and we can not rule out a multi-

state denaturation. Lastly, we find no evidence that mutants with lower T_m values have more compact denatured states with significantly greater amounts of helix. The results presented here do not rule out the possibility that some mutants of staphylococcal nuclease thermally denature by other than a two state mechanism, indeed, we regard that as likely. However, for wild-type and the mutants examined here, a two state model for thermal denaturation fits the data well and, within the bounds of our assumptions, a three state denaturation mechanism appears unlikely. In future communications, we will present data that further defines the possible ranges of thermodynamic parameters of different models of denaturation of nuclease and its mutants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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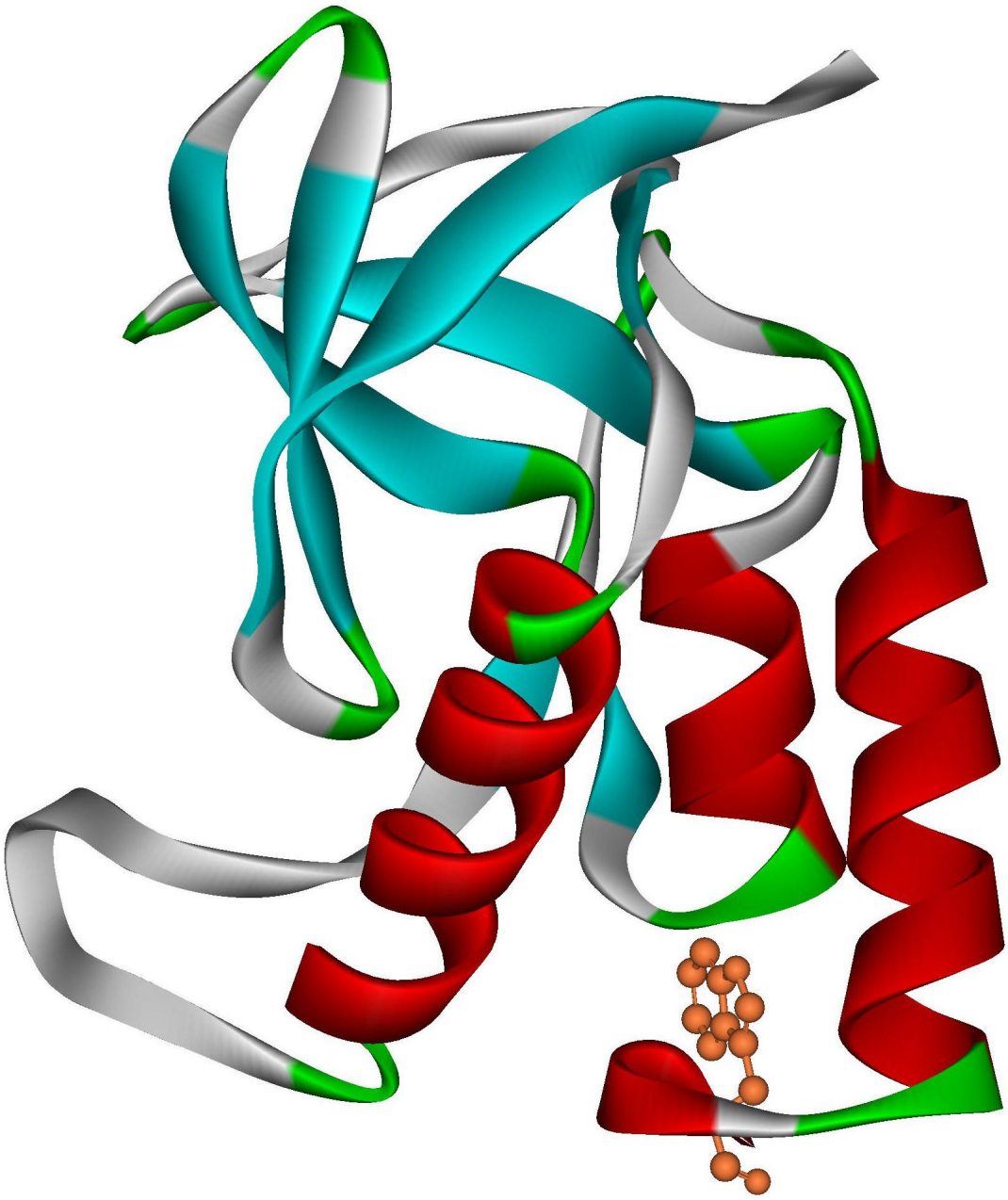

Figure 1.
Ribbon diagram of staphylococcal nuclease (PDB 1EY0) with tryptophan 140 shown in orange. Helical secondary structure is shown in red.

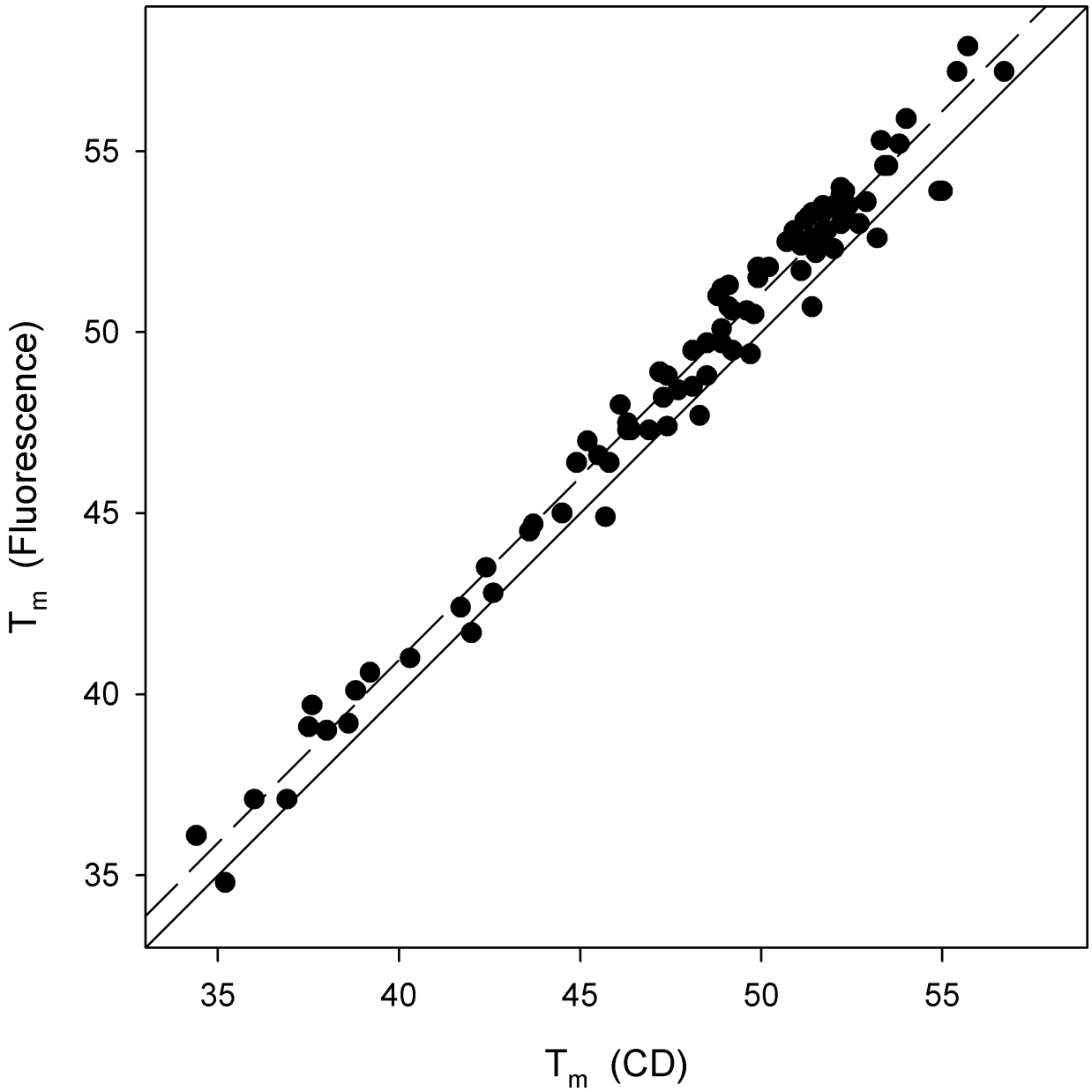

Figure 2.
Plot of midpoint temperature in units of °C calculated from fluorescence thermal denaturations as a function of midpoint temperature calculated from circular dichroism thermal denaturations. The solid line is that upon which all points would fall if there were perfect correspondence in the two values. The dashed line is the result of a least square fit. The slope of this regression line is 1.012, the Y-intercept is 0.463, and the value of r^2 is 0.9787.

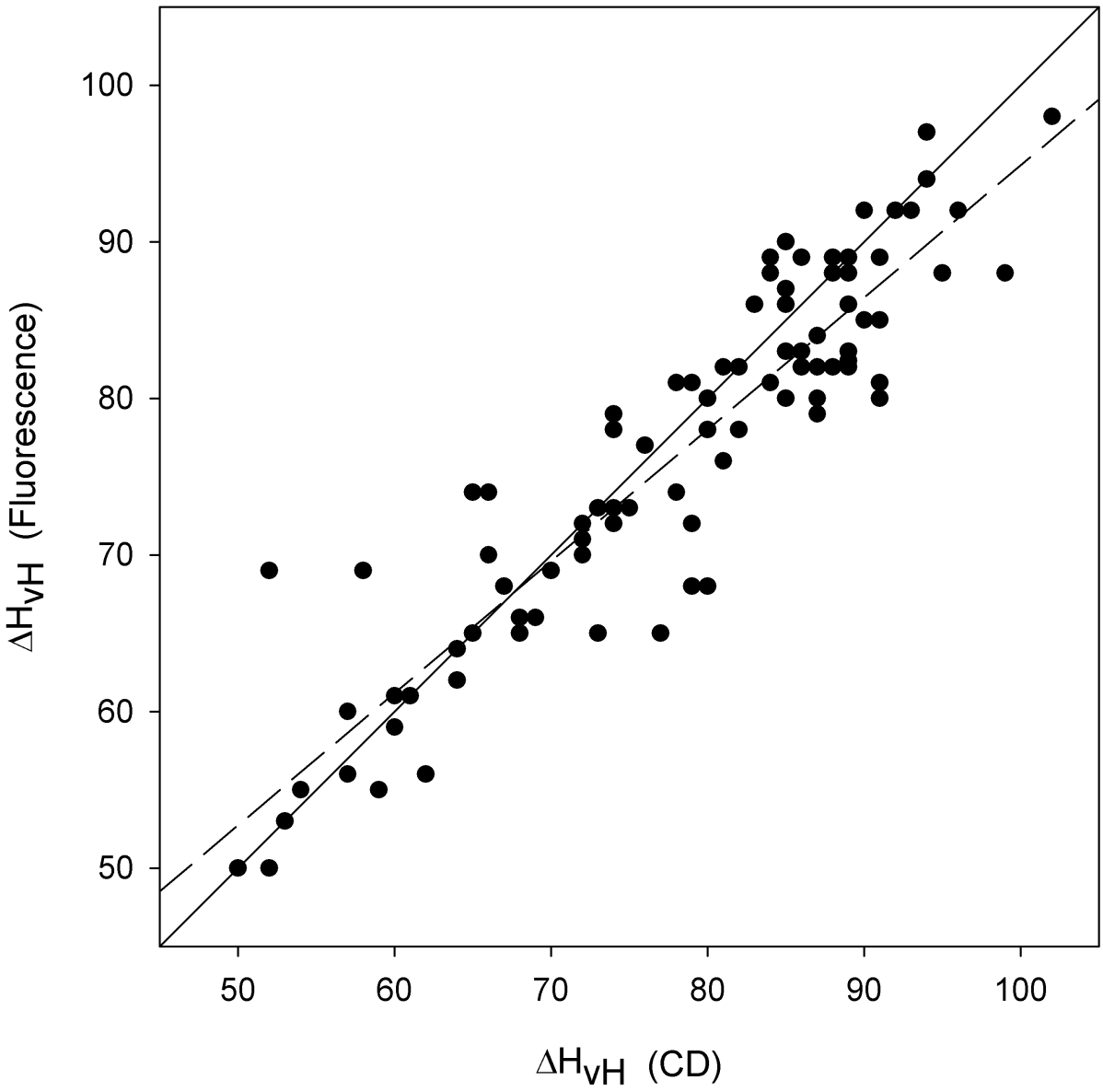

Figure 3.
Plot of van't Hoff enthalpies in units of kcal/mol calculated from fluorescence thermal denaturations versus those calculated from circular dichroism thermal denaturations. The solid line is that upon which all points would fall if there were perfect correspondence in the two values. The dashed line is the result of a least square fit. The slope of this regression line is 0.8441, the Y-intercept is 10.436, and the value of r^2 is 0.8441.

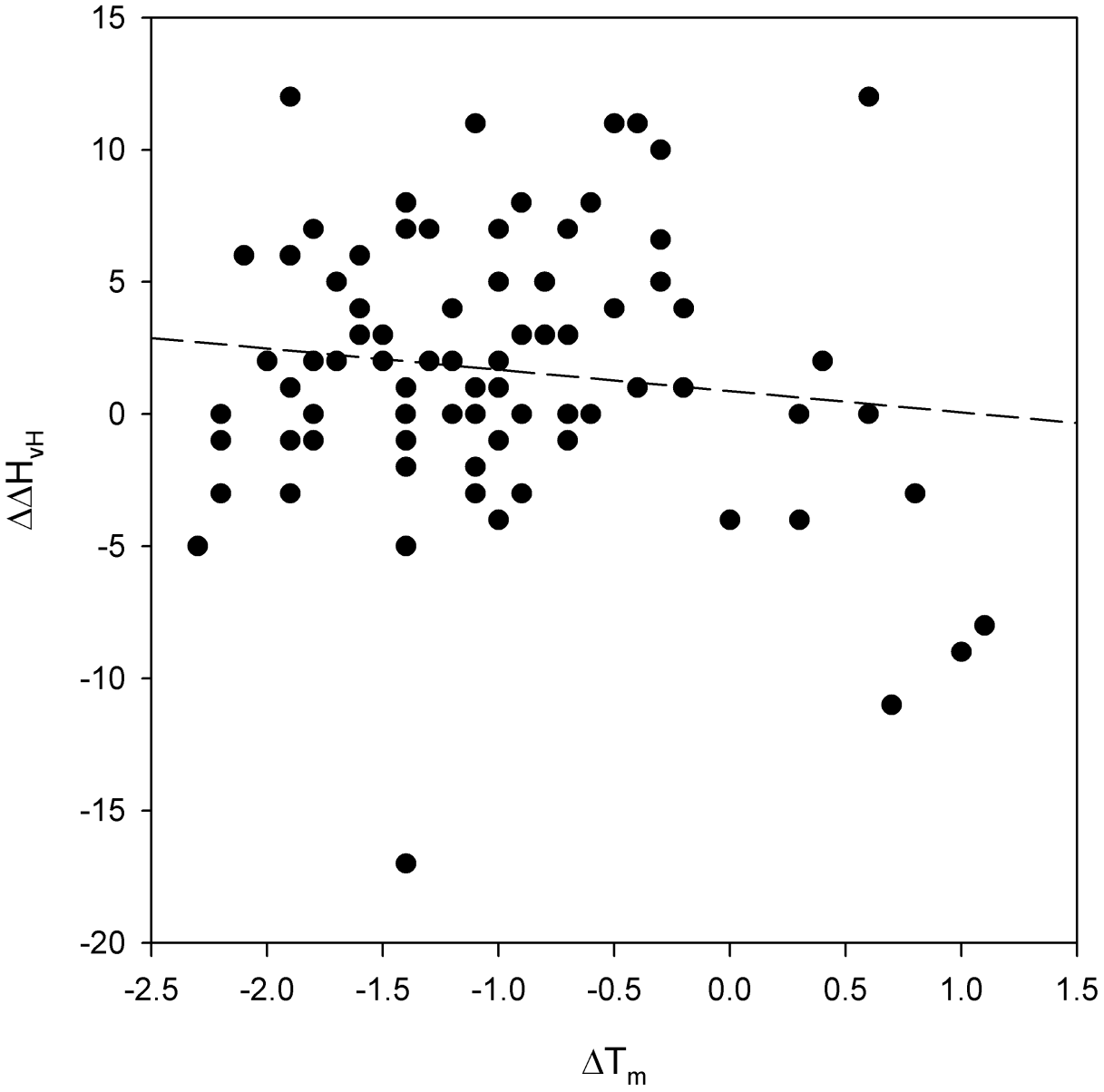

Figure 4.
Plot of the difference in midpoint temperatures from fluorescence and circular dichroism thermal denaturations (in units of °C) versus the difference in van't Hoff enthalpies for the same denaturations (in units of kcal/mol). The dashed line is the result of a least square fit. The slope of this regression line is −0.88, the Y-intercept is 0.73, and the value of r^2 is 0.019.