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# EFFECTS OF CHYMOTRYPSIN UNFOLDING ON STRUCTURE AND FUNCTION:CHANGES IN INTRINSIC FLUORESCENCE AND ENZYME ACTIVITY DO NOT ALWAYS TELL THE SAME STORY

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**Abstract**

**We have probed the effects of chymotrypsin unfolding by simultaneously monitoring enzyme esterase activity (hydrolyzing *p*-NPA) and intrinsic tryptophan fluorescence. We found that for thermal denaturation, fluorescence changes were slightly more sensitive to heat than they were to activity changes, as judged by a lower unfolding temperature, entropy, and enthalpy. Below $T_U$, esterase activity increased with temperature, giving $E_a$ = 8 ± 3 kcal/mol; the temperature for optimal activity was $T_{max}$ = 36.4 ± 1.5 °C. For guanidine hydrochloride (GuHCl) denaturation carried out at 20 – 37 °C, fluorescence and activity decreases matched quite well. For salt denaturation, fluorescence decreased above 1.5 M NaCl, whereas esterase activity rose linearly with NaCl, up to 2 M NaCl, then decreased. The effects of NaCl on chymotrypsin showed dramatic differences between changes reported by intrinsic fluorescence vs. esterase activity, in contrast to thermal and GuHCl denaturation, where fluorescence and activity changes coincided fairly well.**

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## Introduction

The relationship between structure and function has been an intense area of biochemical study for nearly a century. The literature on protein structure, folding, and denaturation is immense, as is the literature on the effects of structural changes on protein function. However, a search of the literature shows that the impacts of denaturation on both structure and function are not often addressed together in a single study, and when they are, results are almost always analyzed qualitatively. For example, we found only 27 published papers that assayed both structural changes and activity decline resulting from denaturation; about half of these papers reported some comparisons between structure and activity[1-14], however nine did not.[15-23] Only seven authors fit their results to a biophysical model of denaturation.[12,19,21-25] Accordingly, we have assayed both the structure and activity of chymotrypsin exposed to denaturation by heat, salt, and guanidine hydrochloride (GuHCl), analyzed our results quantitatively using accepted biophysical models, and assessed the correlation between effects on structure and activity.

Protein folding and unfolding can be characterized by the experimental methods listed in Table 1. Note that as a protein unfolds, it loses structure in reverse level order: As denaturing conditions go from mild to extreme, quaternary structure is lost first (subunit dissociation), followed by loss of tertiary, then secondary structure (helix/sheet/coil), and under the most extreme conditions, primary structure (polypeptide chain fragmentation). Thus, if the active site of an enzyme requires the optimal placement of amino acid side chains in a specific tertiary structure, but activity is insensitive to secondary or tertiary structures elsewhere in the protein, then modest structural changes that impact enzyme activity could be detected by a well-placed fluorophore, chromophore, or spin label, but remain undetected by FTIR or far-UV circular dichroism. Conversely, a fluorophore, chromophore, or spin label located far from the active site could register a structural change that does not affect enzyme activity. Differences between structural changes reported by a site-directed spin label vs. circular dichroism (global structure) have been explored.[4]

Although native structure at the active site is required for optimal enzyme activity, one cannot predict *a priori* whether partial unfolding of an enzyme would impact the active site more, less, or to the same extent as the rest of the protein. Therefore, using chymotrypsin as our model enzyme, we set out to test how well denaturation-induced structural changes detected by intrinsic fluorescence[26,16] tracked with decreases in enzyme activity. Chymotrypsin has three tryptophan residues near the active site (trp 207, 215, and 237[16]), however, the linkage between its composite intrinsic fluorescence emission and enzyme activity at the active site has not been explored in a rigorous, quantitative manner.

Protein unfolding (a.k.a., denaturation) can be instigated by changes in temperature (proteins unfold at high temperature,

**Table 1**: Experimental methods used to probe protein folding and unfolding

| method | Level of structure probed | location |
|---|---|---|
| Differential scanning calorimetry | All levels | Whole protein |
| 2D-NMR | All levels | Whole protein |
| FTIR | Secondary structure | Whole protein |
| Circular dichroism, 200-230 nm | Secondary structure | Whole protein |
| Circular dichroism, 280-300 nm | Tertiary structure | near trp/tyr/phe residues |
| absorbance, UV (280 nm) | Tertiary structure (usually) | near trp/tyr/phe residues |
| absorbance, visible | Tertiary structure | Near chromophore |
| Fluorescence, intrinsic | Tertiary structure | near trp/tyr/phe residues |
| Fluorescence, extrinsic | Tertiary structure | Near attached extrinsic flurophore |
| EPR | Tertiary structure | Near attached extrinsic spin label |

and also at low temperature) and by the addition of various solutes, e.g.,chaotropic agents (urea, GuHCl, alcohols), acids, bases, salts, detergents, and organic liquids (both polar/water miscible, and nonpolar/immiscible). In Table 2 we list the protein structure stabilizing forces that are weakened by each type of denaturant. We chose to study heat, GuHCl, and salt (i.e., NaCl) because they weaken different stabilizing forces; in addition, biophysical modeling has advanced the furthest for denaturation by heat and chaotropic agents.

GuHCl and NaCl are both chloride salts, so conceivably, it could be hard to separate the chaotropic and salt-induced effects of GuHCl. This, however, has not been observed to be the case, because the denaturation effect of GuHCl stems from the $GuH^+$ cation and not the $Cl^-$ anion. GuHCl denaturation data are always fit to the same biophysical model as urea (a non-salt chaotrope; see for example ref. [27]), and as we show here, differ dramatically from NaCl-induced denaturation.

Chymotrypsin is an esterase/peptidase whose catalytic mechanism was delineated in the 1960s by Blow *et al*.[28] Most undergraduate biochemistry textbooks feature it prominently in their discussion of catalytic mechanism. Suffice it to say here that, like all serine proteases, chymotrypsin has an active site catalytic triad in which histidine base-catalyzes (i.e., deptrotonates) a serine hydroxyl, which then nucleophilically attacks the substrate carbonyl carbon, catalyzing hydrolysis (the scheme is depicted in the Appendix). Chymotrypsin activity is easily assayed using substrates like *p*-nitrophenyl acetate, which is hydrolyzed to yield the chromophore *p*-nitrophenoxide.

We chose to characterize changes in enzyme structure by following the intrinsic fluorescence of chymotrypsin's tryptophan residues. Fluorescence emission generally varies inversely with solvent polarity and is also quenched (and red-shifted) by water.[29,30] Thus, protein denaturation induced by the addition of organic co-solvents, which is accompanied by a decrease in solvent polarity, is characterized by an increase in intrinsic fluorescence.[1,30] On the other hand, other types of denaturation leave solvent polarity unchanged and expose buried tryptophan side chains to water, thus lowering intrinsic fluorescence[32,33,25,21], as we observed for chymotrypsin.

## Materials and Methods

All reagents were purchased from SigmaAldrich and used without further purification. α-Chymotrypsin (80 mg) was dissolved in 100 mL of 10 mM HEPES buffer, pH 6.5, and used fresh daily. The substrate *p*-nitrophenyl acetate (pNPA) was dissolved in buffer (10 mM HEPES, pH 6.5) to make a 3.0 mM stock solution. *p*-nitrophenoxide ($pNP^-$) production was measured using a Beckman UV 5240 at 405 nm, molar absorptivity = 18,800 $M^{-1}cm^{-1}$. Guanidine hydrochloride (GuHCl) and NaCl were dissolved in buffer to give highly concentrated stock solutions (≈ 6 M). Samples containing 5.0 mL of enzyme stock solution and variable amounts of GuHCl or NaCl and buffer were mixed in 10.00 mL volumetric flasks and incubated for 15 minutes to establish conformational equilibrium. Sample cells were prepared by mixing 1.0 mL of pNPA stock with 2.0 mL of enzyme/GuHCl stock, giving a final enzyme concentration of 0.13 mg/mL. Unless otherwise specified, sample cells were temperature controlled at 37.0°C using a NESLAB RTE-210 circulating water bath. Thermal denaturation was accomplished by heating the enzyme solution for 15 minutes at the specified temperature. Chymotrypsin intrinsic fluorescence from tryptophan side chains was measured with a Varian SF-330, excitation at 290 nm and emission at 335 nm. Data were fit via nonlinear regression using Kaleidagraph software.

**Table 2:** Protein stabilizing forces weakened by denaturants.

| denaturant | weakened force |
|---|---|
| heat | H-bonds |
| urea, GuHCl | H-bonds, hydrophobic force, hydration shell |
| acids/bases | salt bridges, H-bonds |
| salt | salt bridges, hydration shell |
| detergents | hydrophobic force |
| organic co-solvent | hydrophobic force |

*Biophysical models of denaturation*

For thermal denaturation, the parameter $T_U$ can be defined as the temperature at which the equilibrium features a 50-50 mixture of native and unfolded protein ($P_N$ and $P_U$, respectively; $T_U$ is sometimes erroneously called a "melting" temperature, however, because proteins are not solids they do not "melt".). Thus, at $T << T_U$, $[P_N]_{eq} \approx [P]_{total}$, and at $T >> T_U$, $[P_U]_{eq} \approx [P]_{total}$. Consider a spectroscopic parameter whose intensity ($I$) is proportional to the concentration of native protein, so it asymptotically approaches a maximum value, $I_{max}$ at $T << T_U$, and likewise approaches $I_{min}$ at $T >> T_U$. Using standard thermodynamic equations, it can be shown[26,35,41,47] that $I_T$ varies with $T$ according to:

$$\text{Equation 1:} \qquad I_T = I_{min} + \frac{I_{max} - I_{min}}{1+exp\left[\frac{\Delta S°_U}{R}\left(1-\frac{T_U}{T}\right)\right]}$$

According to this equation, the decline of $I$ with $T$ will be sigmoidal, centered at $T_U$; the slope of the decline depends on $\Delta S°_U$. A higher value of $T_U$ signifies a more thermally stable protein, i.e., more resistant to thermal denaturation; furthermore, $T_U = \Delta H°_U/\Delta S°_U$ [26,25,42,48], so $\Delta H°_U$ can be calculated from best-fit values of $T_U$ and $\Delta S°_U$. A higher value of $\Delta S°_U$ means a steeper decline with temperature (still centered around $T_U$), hence a more cooperative unfolding process.

In the derivation of Equation 1 we assume that $\Delta S°_U$ is essentially temperature-independent over the 20 – 60 °C range studied here, i.e. the change in heat capacity upon unfolding is negligible ($\Delta C°_{P,U} \approx 0$). However, using Privalov and Gill's value of $\Delta C°_{P,U}$ = 3.0 kcal/mol/K[34], we find that $\Delta S°_U$ would actually increase substantially over this temperature range (by over 380 cal/mol/K). Thus it is advisable to account for the temperature dependence of $\Delta S°_U = \Delta S°_{U,298} + \Delta C°_{P,U} \cdot \ln(T/298)$:

$$\text{Equation 2:} \quad I_T = I_{min} + \frac{I_{max} - I_{min}}{1+exp\left[\frac{1}{R}\left(\Delta S°_{U,298} + \Delta C°_{P,U} \cdot ln\left(\frac{T}{298}\right)\right)\left(1-\frac{T_U}{T}\right)\right]}$$

Similarly, for the dependence of enzyme activity ($\nu_0$) on tem-

perature, we can derive[35,36]

$$\text{Equation 3:}\quad v_0 = \frac{A_{app}\cdot exp\,(-\frac{E_a}{RT})}{1+exp\,[\frac{\Delta S°_U}{R}\left(1-\frac{T_U}{T}\right)]} = \frac{A_{app}\cdot exp\,(-\frac{E_a}{RT})}{1+exp\,[\frac{1}{R}\left(\Delta S°_{U,298}+\Delta C°_{P,U}\cdot ln\,(\frac{T}{298})\right)\left(1-\frac{T_U}{T}\right)]}$$

The change in $\nu_0$ with $T$ differs from spectroscopic intensity in two ways: $\nu_0$ falls to zero for $T >> T_U$, and due to the influence of activation energy, at $T << T_U$, $\nu_0$ generally rises with $T$ before it starts to decline as $T$ approaches $T_U$. This means that enzymes have an optimal temperature, $T_{max}$, where the temperature is high enough for $\nu_0$ to benefit from activation, but low enough to avoid substantial denaturation. Thus, fitting of $\nu_0$ vs. $T$ data gives four parameters, including $E_{a,app}$, $\Delta S°_U$, and $T_U$.

For protein unfolding induced by the addition of chaotropic denaturants, spectroscopic changes are given by[26,36,42,48]

$$\text{Equation 4:}\qquad I_{den} = I_{min} + \frac{I_{max} - I_{min}}{1+exp\,[\frac{\Delta G°_{U,wtr}}{RT}\left(\frac{[denaturant]}{C_{50}}-1\right)]}$$

Similar to thermal denaturation (Equation 1), the decline of $I$ with increasing denaturant is sigmoidal, centered at $C_{50}$ (the denaturant concentration yielding 50% inhibition/unfolding): higher $C_{50}$ means more resistance to denaturation. $\Delta G°_{U,wtr}$ is the free energy of the unfolding equilibrium in the absence of denaturant. $\Delta G°_{U,wtr}$ also gives the slope of the sigmoidal decline, so a higher value of $\Delta G°_{U,wtr}$ means both that the protein is more stable in the absence of denaturant, and also that denaturant-induced unfolding will be more cooperative. Finally, the sensitivity (m) of the protein to denaturant-induced unfolding (in units of kcal/mol/M) can be calculated from[26,36,42,48] $\Delta G°_{U,wtr}/C_{50}=m*$.

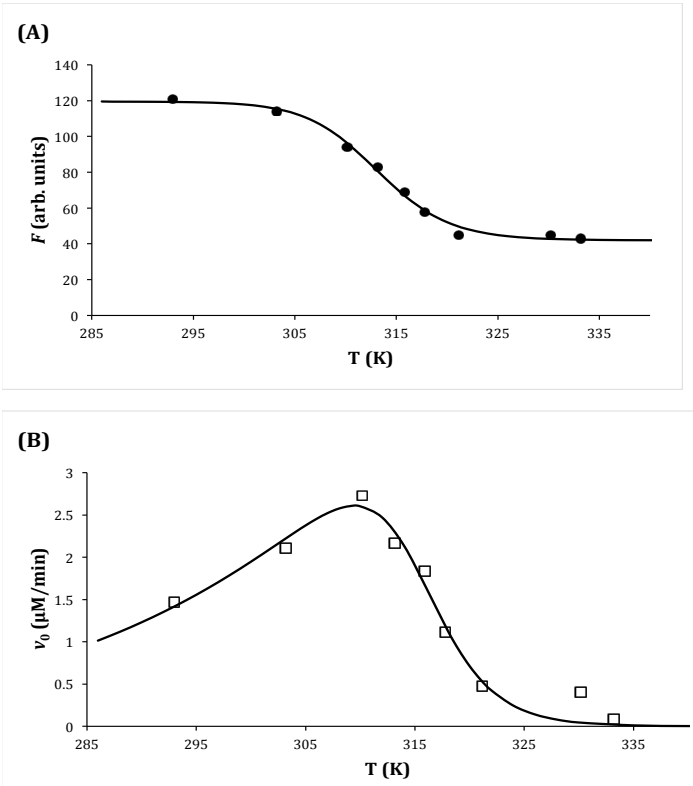


**Figure 1:** Thermal denaturation of chymotrypsin followed by changes in (A) intrinsic fluorescence intensity ($F$), and (B) enzyme activity ($v_0$). Fluorescence data (A) are fit to Equation 1; best-fit values are: $I_{min}$ = 42.7 ± 1.7 a.u.; $I_{max}$ = 125 ± 6 a.u.; $\Delta S°_{U,298}$ = 104 ± 28 cal/mol/K; $\Delta C°_{P,U}$ = 1.2 kcal/mol/K; $T_U$ = 312.7 ± 0.7 K = 39.6 ± 0.7 °C; $R^2$ = 0.996. Enzyme activity data (B) are fit to Equation 3; best-fit values are: $A_{app}$ = (3 ± 17) x $10^7$ μM/min; $E_{a,app}$ = 8 ± 3 kcal/mol; $\Delta S°_U$ = 150 ± 70 cal/mol/K; $T_U$ = 315.0 ± 2.0 K = 41.9 ± 2.0 °C; $R^2$ = 0.964.

*In the literature, $m$ is generally interpreted as a measure of unfolding cooperativity, but since $\Delta G°_{U,wtr}$ is proportional to $m$, both are directly related to cooperativity.

Similarly, for the dependence of enzyme activity ($\nu_0$) on chaotropic denaturant, we can derive[26,35,36,42,48]

$$\text{Equation 5:}\qquad v_0 = \frac{v_{0,wtr}}{1+exp\,[\frac{\Delta G°_{U,wtr}}{RT}\left(\frac{[denaturant]}{C_{50}}-1\right)]}$$

Equation 5 shows that $\nu_0$ declines sigmoidally to zero as denaturant is added, with a midpoint of $C_{50}$ and a slope that depends on $\Delta G°_{U,wtr}$.

Using Equations 1-5, we can compare directly the fitted parameters obtained from following structural changes (spectroscopic $I$ vs. $T$ and $I$ vs. [denaturant]) and enzyme activity changes ($\nu_0$ vs. $T$ and $\nu_0$ vs. [denaturant]) that result from denaturation.

## Results

*Thermal denaturation of chymotrypsin*

The intrinsic fluorescence intensity ($F$) of chymotrypsin ($\lambda_{exc}$ = 290 nm, $\lambda_{em}$ = 335 nm) decreased sigmoidally with increasing temperature, as shown in Figure 1A. Enzyme activity rose with temperature, reaching a maximum value at 36.4 ± 1.5°C, then declined (Figure 1B). Best-fit values are listed in Table 3.

*Guanidine hydrochloride denaturation of chymotrypsin*

Guanidine hydrochloride denaturation of chymotrypsin was assayed as above by intrinsic fluorescence and enzyme activity, at 20 – 37°C. In order to compare directly the two types of data, results are normalized to give the fraction of native enzyme, $f_N$, at each [GuHCl] using Equation 6.

$$\text{Equation 6:}\qquad f_N = \frac{y - y_U}{y_N - y_U} = \frac{y - y_{min}}{y_{max} - y_{min}}$$

**Table 3**: Best-fit values for fluorescence and activity vs. temperature.

| Fit parameter | Fluorescence (Figure 1A) | Activity (Figure 1B) | Activity** Lozano [11], Fig. 1B |
|---|---|---|---|
| $\Delta S°_{U,298}$ (cal/mol/K) | 104 ± 28 | 150 ± 70 | 580 ± 70 |
| $T_U$ (°C) | 39.6 ± 0.7 | 41.9 ± 1.5 | 41.4 ± 0.2 |
| $\Delta H°_{U,298}$ (kcal/mol)* | 33 ± 9 | 46 ± 21 | --- |
| $\Delta G°_{U,298}$ (kcal/mol) | 2 (± 12)*** | 2 (± 29)*** | --- |
| $E_{a,app}$ (kcal/mol) | --- | 8 ± 3 | 7.0 ± 1.2 |
| $T_{max}$ (°C) | --- | 36.4 ± 1.5 | 37.9 ± 2.0 |
| $R^2$ | 0.993 | 0.967 | 0.995 |

* Calculated from $\Delta H°_U = T_U\Delta S°_U$;.

** Substrate was *N*-acetyl-L-tyrosine ethyl ester

*** The uncertainties in $\Delta G°_{U,298}$ of 12 and 29 kcal/mol seem large, but they result from propagating the uncertainties of $\Delta H°_{U,298}$ and $T\Delta S°_{U,298}$. For fluorescence measurements these errors are 9 and 8 kcal/mol, respectively, and for activity, 21 and 21 kcal/mol, respectively. Essentially, the uncertainty in $\Delta G°_{U,298}$ is not particularly large, but rather, the value of $\Delta G°_{U,298}$ turns out to be small (i.e., 2 kcal/mol).

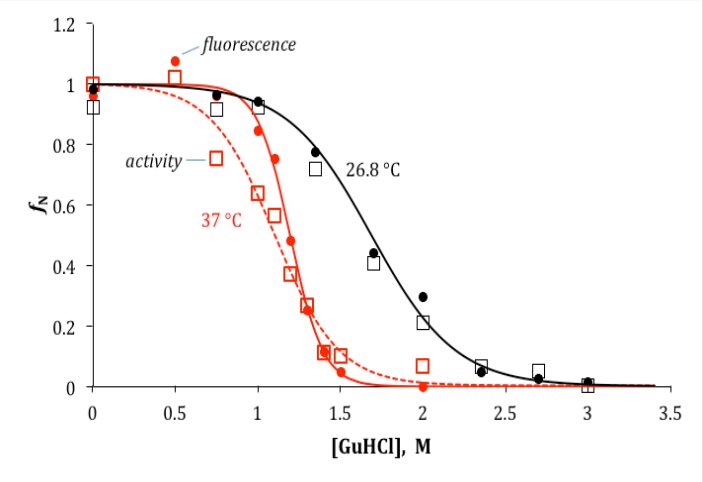


**Figure 2:** Guanidine hydrochloride denaturation of chymotrypsin at 26.8 (black) and 37.0 °C (red) assayed by changes in intrinsic fluorescence intensity (filled circles), and enzyme activity (open squares). Fluorescence data are fit to Equation 4 (solid lines); activity data are fit to Equation 5 (dashed line). Best-fit values are listed in Table 4.

Asymptotic $y$ values are $y_{max} = y_N$ for the fully native enzyme in the absence of GuHCl, and $y_{min} = y_U$ for the fully unfolded enzyme in the presence of excess GuHCl; they are determined by fitting raw data to Equation 4 (fluorescence) and Equation 5 (activity). In Figure 2 we plot $f_N$, calculated from fluorescence and activity data, vs. [GuHCl] at 26.8 and 37°C.

Note that at 26.8°C, a single curve can be fit to both fluorescence and activity data. The same was true at 19.8 and 32.2°C (data not shown). At 37°C, $C_{50}$ was about the same for fluorescence and activity data, however activity decline began to manifest at lower GuHCl concentrations, e.g., 0.75 M vs. 1 M. Best-fit values for all data sets, 20 – 37°C, are listed in Table 4, and plotted in Figure 4 in the Discussion.

*Salt denaturation of chymotrypsin*

Raising ionic strength by adding salt should weaken intra-protein salt bridges by electrostatic screening. At least one salt bridge in chymotrypsin, between $asp_{194}$-$COO^{-}$ and the N-terminal $ile_{16}$-$NH_3^+$, is known to enhance substrate binding by maintaining the binding cleft in an unblocked conformation (ref. [37], pp. 411-413). Furthermore, the strength of this salt bridge is rather modest (-3 kcal/mol, ref. [37], p. 306). Thus, adding salt is expected to weaken salt bridges, weaken the native conformation of the protein, and inhibit enzyme activity.

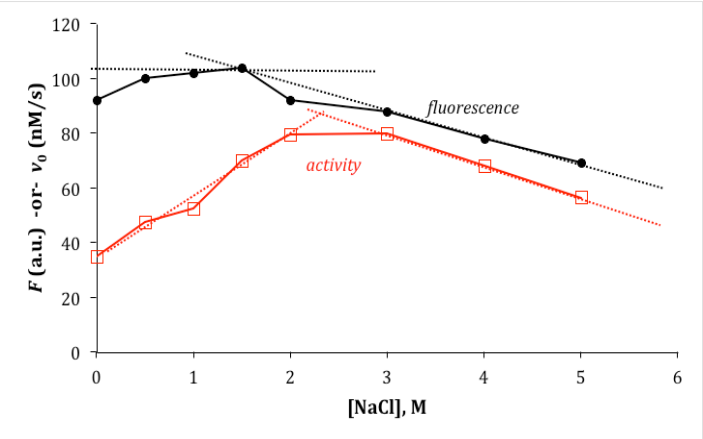


**Figure 3:** Effect of NaCl on chymotrypsin intrinsic fluorescence (filled black circles) and esterase activity (open red squares) at 37 °C.

What we found was a more complicated, biphasic effect, as shown in Figure 3. Fluorescence rose slightly (9%) from 0 to 0.5 M NaCl, then remained roughly constant up to 1.5 M NaCl. Above 1.5 M NaCl, $F$ declined linearly by ≈10 a.u. per molar NaCl; at 5 M NaCl, F was 25% below its value in the absence of salt. Enzyme activity rose dramatically, by 22 nM/s per molar NaCl up to 2 M, then above 3 M NaCl, activity declined by 12 nM/s per molar NaCl. Even at 5 M NaCl, $\nu_0$ was 60% higher than in the absence of salt.

## Discussion

*Thermal Denaturation:* For heat denaturation of chymotrypsin at pH 4.3, Privalov[37,33] reported $\Delta S°_U$ = 187 cal/mol/K and $\Delta H°_U$ = 66 kcal/mol. Our best-fit results (at pH 6.5) are lower, but the difference is less than the fitting uncertainty and thus may not be statistically significant. This is a bit surprising, as low pH is known to weaken protein structure.[38,34,39,26] Our $E_a$ of 8 kcal/mol compared well with published values of 6.5 and 7 kcal/mol (for hydrolysis of benzoyl- and N-acetyl-L-tyrosine ethyl ester substrates[39,11]). This $E_a$ along with the $T_U$ of 40 – 42 °C shows that chymotrypsin has evolved to function optimally at $T_{max}$ around 37 – 40°C (Figure 1B).

$\Delta S°_U$ and $T_U$ values from fluorescence data were slightly lower than values from activity data, with the differences a bit higher than the uncertainty. Thus, although one cannot draw this conclusion with too much statistical confidence, it seems as if tryptophan fluorescence is slightly more sensitive to heat denaturation than is the enzyme's active site.

*Guanidine Hydrochloride Denaturation:* Our best-fit value of $C_{50}$(GuHCl) = 1.7 M (at 26.8 °C/pH 6.5) agreed fairly well with the 1.8 M value reported by Greene and Pace[27] at 25°C/pH 4.3, and the 1.45 M value reported by Morrisett and Broomfield[4] at 27 °C/pH 3. Our value of $\Delta G°_{U,wtr}$ = 3.7 ± 0.5 kcal/mol is somewhat lower than the 7.5 ± 0.5 kcal/mol value reported[27] at pH 4.3, and the 5.5 ± 1.2 kcal/mol value reported[4] at pH 3; however, the dif-

**Table 4:** For Guanidine hydrochloride denaturation of chymotrypsin, best-fit parameters for $\Delta G°_{U,wtr}$ and $C_{50}$ from data that were gathered at 20 – 37 °C.

| T | Assay | $\Delta G°_{U,wtr}$ (kcal/mol) | $C_{50}$ (M) | $m$* (kcal/mol/M) |
|---|---|---|---|---|
| 19.8 °C | activity | 4.88 ± 0.20 | 1.558 ± 0.010 | 3.13 ± 0.13 |
| 19.8 °C | fluorescence | 4.19 ± 0.22 | 1.472 ± 0.013 | 2.84 ± 0.15 |
| 26.8 °C | activity | 2.7 ± 0.6 | 1.74 ± 0.09 | 1.5 ± 0.3 |
| 26.8 °C | fluorescence | 3.7 ± 0.5 | 1.68 ± 0.04 | 2.2 ± 0.3 |
| 32.2 °C | activity | 3.60 ± 0.22 | 1.773 ± 0.020 | 2.03 ± 0.12 |
| 32.2 °C | fluorescence | 3.03 ± 0.21 | 1.664 ± 0.025 | 1.82 ± 0.13 |
| 37 °C | activity | 3.5 ± 0.6 | 1.10 ± 0.04 | 3.2 ± 0.6 |
| 37 °C | fluorescence | 7.4 ± 0.9 | 1.195 ± 0.015 | 6.2 ± 0.8 |

* $m$ is the proportionality factor for the linear dependence of unfolding free energy on [GuHCl], calculated from $\Delta G°_{U,wtr}/C_{50}$.

ference may not be statistically significant, as it is less than twice the uncertainty.

Comparing $\Delta G°_{U,wtr}$ from fluorescence and activity data at 20 – 37°C (Figure 4A), we see the expected decrease with temperature, although the trend is rather noisy ($R^2$ = 0.4); the folded protein is less stable at higher temperatures. From the slope of the linear regression we get $\Delta S°_{U,wtr}$ = 70 ± 40 cal/mol/K. Note that this value is lower than those derived from the thermal denaturation data (104 ± 28 and 150 ± 70 cal/mol/K). Furthermore, for thermal denaturation, $\Delta G°_{U}$ = 0 at $T_U \approx$ 41°C; however, for GuHCl denaturation at 41°C, we can estimate from (Figure 4A) that $\Delta G°_{U,wtr} \approx$ + 2.8 kcal/mol. This difference is not unexpected as the two denaturation processes (heat vs GuHCl) undoubtedly follow different pathways because heat mainly weakens hydrogen bonds while GuHCl weakens hydrophobic forces and, to a lesser extent, hydrogen bonds. For example, it has been shown that whereas heat is effective at denaturing both α-helical and β-sheet proteins, GuHCl denatures β-sheet proteins (e.g., chymotrypsin) effectively, but not α-helical proteins.[41] From the slope and the intercept of the linear regression in (Figure 4A) we can calculate $\Delta H°_{U,wtr}$ = 25 ± 11 cal/mol/K, and $\Delta G°_{U,wtr,25°C}$ = 4.1 ± 1.5 kcal/mol.

Note that below 37°C, $\Delta G°_{U,wtr}$ from fluorescence and activity data match fairly well (Figure 4A); this can also be seen in the similar dependence of fluorescence and activity data on [GuHCl] (Figure 2 and ref. [42]). In contrast, at 37°C, $\Delta G°_{U,wtr}$ obtained from fluorescence data is more than twice the value from activity data (Figure 4A). We note in passing that $T_{max}$ for enzyme activity is close to 37°C (Figure 1B); it is unclear whether there is a connection between these two effects, but clearly, something very interesting is happening around 37°C that bears further study.

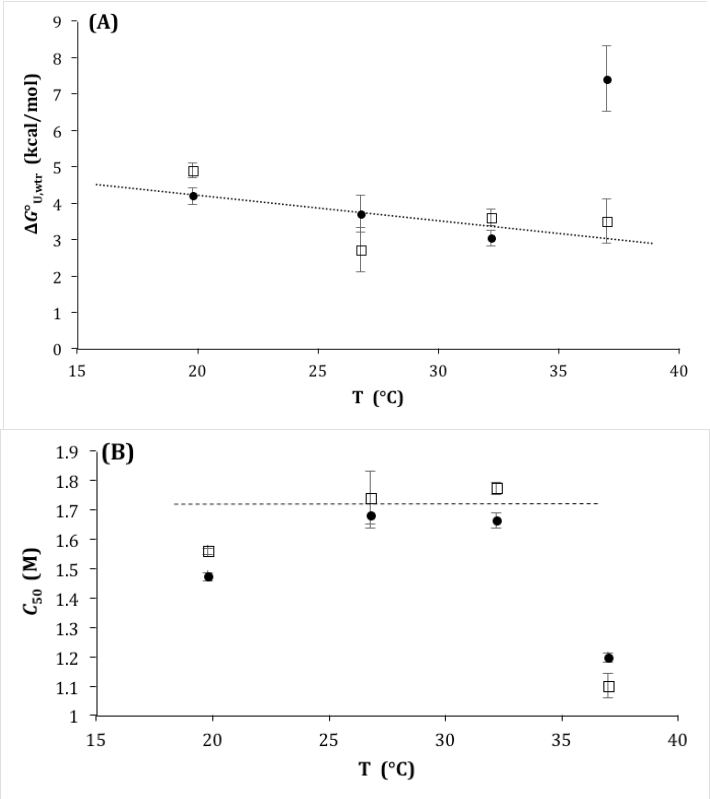


**Figure 4:** Best-fit values of $\Delta G°_{U,wtr}$ (A) and $C_{50}$ (B) from fitting chymotrypsin-GuHCl denaturation; fluorescence data (filled circles) and esterase activity data (open squares). Linear regression (A, dotted line) gives: slope = -70 ± 40 cal/mol/K; intercept = 5.9 ± 1.1 kcal/mol; $R^2$ = 0.4.

GuHCl $C_{50}$ best-fit values from fluorescence and activity data at 20 – 37°C match fairly well (Figure 4B). Additionally, these values have a rather curious temperature dependence. Heating from 19.8 to 26.8°C makes chymotrypsin more resistant to GuHCl: $C_{50}$ rises from 1.5 to 1.7 M. Heating to 32.2°C leaves $C_{50}$ unchanged, but further heating to 37°C dramatically increases GuHCl sensitivity: $C_{50}$ falls from 1.7 to 1.1 M. This temperature trend is quite distinct from that for $\Delta G°_{U,wtr}$, but that is not surprising as these two parameters report on different physical aspects of the GuHCl denaturation process. Further study of both the low and high temperature effects on $C_{50}$ are underway.

To close our guanidine hydrochloride denaturation discussion, we note that chymotrypsin esterase activity did not decline to zero at high [GuHCl]; rather, it reached an asymptotic low activity of about 2 – 3 nM/s (data not shown). It has been noted in the literature that urea and guanidinium chloride do not completely denature most proteins: Some residual structure remains even at high denaturant concentrations.[3,26,43] Apparently, this level of residual structure in chymotrypsin is sufficient to maintain a low level of esterase activity.

*Salt Denaturation:* We have found sodium chloride to be only a mild denaturant of chymotrypsin. Whereas GuHCl caused declines of 45-50 a.u. from $F_{max}$ to $F_{min}$ (data not shown), even at 5 M, NaCl caused a decline of only 35 a.u. ($F_{max}$ = 104 a.u., $F_{5M}$ = 69 a.u.), with no sign of reaching an asymptotic $F_{min}$.

NaCl altered intrinsic fluorescence and esterase activity in dramatically different ways (Figure 3). Fluorescence is roughly constant up to 1.5 M NaCl, after which it falls moderately (and linearly). On the other hand, esterase activity rises over 2-fold from 0 – 2 M NaCl, then falls moderately (and linearly) above 3 M. Thus, in terms of the impact of enzyme unfolding, tryptophan intrinsic fluorescence is clearly more sensitive to ionic strength than is esterase activity: Unfolding-induced structural changes reported by tryptophan above 1.5 M NaCl do not begin to ramify into the active site until > 3 M NaCl.

Although reports of high-salt (> 2 M) denaturation of chymotrypsin are scarce in the literature, the rise in chymotrypsin activity from 0 – 2 M NaCl (and KCl) was reported as far back as 1966.[44,45] These authors found that salt-activation increased $k_{cat}$ and also decreased $K_m$ (tighter substrate binding), and the activation applied mainly to the acylation step[44], as opposed to the deacylation step (see *Appendix*). The authors also imputed the activity increase as being due to salt-induced changes in the conformation of the enzyme, however, our fluorescence results show that these changes are not registered by chymotrypsin's tryptophan fluorescence. Thus, the changes seem to be restricted to the active site.

## Summary

We used measurements of changes in esterase activity and intrinsic fluorescence upon denaturation to probe the unfolding of α-chymotrypsin. For thermal denaturation, tryptophan fluorescence showed thermal unfolding beginning at slightly lower

temperature, and with slightly lower $\Delta S°_{U}$, compared to esterase activity. However, the differences were not much larger than the statistical uncertainty. In general, for both thermal unfolding and GuHCl denaturation, intrinsic fluorescence and esterase activity followed the same trends; changes detected by tryptophan fluorescence generally matched those detected by inhibition at the active site (except for GuHCl denaturation at 37°C). On the other hand, changes induced by added NaCl have very different effects on chymotrypsin intrinsic fluorescence and esterase activity. Below 1.5 M, NaCl had almost no effect on fluorescence, but increased esterase activity. Fluorescence fell above 1.5 M NaCl, but activity only began to fall above 3 M NaCl. The increased sensitivity of intrinsic fluorescence to unfolding that was only hinted at in thermal denaturation was quite clear in salt denaturation.

In the future we plan to make more low temperature measurements, in order to compare the activation energy from an Arrhenius plot to the fitted value from Equation 5. We also plan to make more measurements around 37°C in order to explore the difference in $\Delta G°_{U,wtr}$ derived from fluorescence vs. activity data. Finally, we plan to explore the effects of denaturation induced by detergent, acid, and organic liquid co-solvents (e.g., ethanol, methanol, dioxane, acetonitrile, THF, DMSO, DMF) on chymotrypsin intrinsic fluorescence and esterase activity.

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Appendix I: Catalytic Mechanism of Chymotrypsin