Covalent Bond Changes as a Driving Force in Enzyme Catalysis†

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ABSTRACT: A procedure is developed for assessing covalent and noncovalent aspects of the acylation of α-chymotrypsin (α-Ct) by the substrate trifluoroethyl furoate (S) to form furoyl–chymotrypsin (F-Ct) and trifluoroethanol (P_1). The free energy change (–4.31 kcal/mol) for the acylation at pH 7, α-Ct + S ⇌ F-Ct + P_1, contains contributions from covalent bond changes as well as noncovalent changes. The noncovalent changes are considered to be manifested in the structures of α-Ct and F-Ct, and a noncovalent free energy difference between α-Ct and F-Ct has been evaluated from the difference in unfolding free energy changes for these proteins. The unfolding free energy changes demonstrate that F-Ct is 1.6 kcal/mol less stable than α-Ct at pH 7. Thus, despite an overall favorable free energy change for acyl–enzyme formation (–4.31 kcal/mol), covalent linkage of the furoyl moiety to the active site is thermodynamically destabilizing to the enzyme. The consequence of this unfavorable effect of the furoyl moiety on the noncovalent free energy is that "binding" of the covalently linked furoyl moiety cannot be the driving force for acylation. The source of free energy driving acylation is the covalent bond-breaking and bond-making involved in transesterification of the furoyl group to the enzyme. Since the (noncovalent) Michaelis complex between α-Ct and substrate is significantly more stable (thermodynamically) than F-Ct, a substantial amount of noncovalent free energy must be given up on forming F-Ct. The destabilization residing in F-Ct is consistent with the possibility that energy transduction occurs when the Michaelis complex is converted to F-Ct and that destabilization is relieved on reaching the next activated complex.

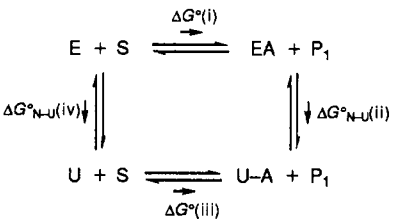
Many theories of enzyme action have as a basic tenet the idea that enzymes have the ability to manage energy and/or transduce energy (Eyring et al., 1954; Jencks, 1975; Lumry, 1959, 1991). Experimentally, it has been extremely difficult to investigate questions of energy management or transduction in enzymes, so very few experiments have been performed for the purpose of directly establishing whether enzymes make use of free energy to do useful work in catalysis. Little is also known about the nature of free energy sources which might become available to an enzyme, the magnitudes of the free energy sources, and how free energy might be manipulated by an enzyme to do useful work in catalysis. While progress has been modest in this area, the situation is more reflective of the lack of tools to investigate the problems than it is in the degree of interest in the issues.

We have been developing thermodynamic-based methods for the purpose of evaluating the interplay between covalent and noncovalent free energy terms that arise during the course of α-chymotrypsin-catalyzed hydrolysis of the nonspecific ester substrate trifluoroethyl furoate (TFEF).[1] A description of the catalytic sequence is given in eq 1 with E and S representing enzyme and substrate, respectively, EA the covalent furoyl–chymotrypsin intermediate, and P_1 and P_2 the alcohol and acid products of TFEF hydrolysis, trifluoroethanol and furoic

$$E + S \rightleftharpoons ES \overset{P_1}{\rightleftharpoons} EA \overset{H_2O}{\rightleftharpoons} EP \rightleftharpoons E + P_2 \quad (1)$$

acid. The EA species is of central interest in this system, and the free energy change for the acylation phase of the catalytic sequence can be described by combining the events of Michaelis complex formation and its conversion to acyl–enzyme into a single composite reaction as illustrated by reaction (i) of

Scheme I



Scheme I. The free energy change, $\Delta G°$(i), can be obtained by conventional means, and it is clear that this composite reaction contains free energy contributions from covalent bond-breaking and bond-making as well as noncovalent contributions from several sources, including interactions between the acyl moiety and active site residues, conformational rearrangements in accommodating the acyl moiety in the active site, and changes in linkages of solvent components between E and EA as a consequence of acylation. These noncovalent free energy contributions are manifested in the structure of EA and, therefore, affect the noncovalent structural integrity of EA.

In the approach illustrated in Scheme I, information about the structural integrities of E and EA is obtained by evaluating their unfolding free energy changes (reactions ii and iv). The differences between these two unfolding free energy changes, however, provide a means for evaluating the magnitude of the noncovalent free energy change which takes place on conversion of E to EA. Reaction iii, then, represents only the covalent bond-making and bond-breaking events of acylation and has none of the noncovalent aspects of catalysis embodied

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[1] Abbreviations: F-Ct, furoyl-α-chymotrypsin; α-CT, α-chymotrypsin; TFEF, trifluoroethyl furoate; TFE, trifluoroethanol; ATrEE, *N*-acetyl-L-tryptophan ethyl ester; α-Ct·TFEF, Michaelis complex between α-Ct and TFEF; CD, circular dichroism; NMR, nuclear magnetic resonance; EA, acyl–enzyme intermediate.

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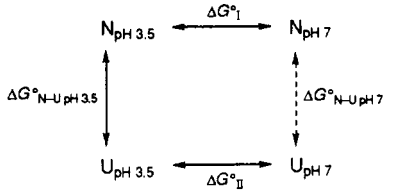
Scheme II



in reaction i. Scheme I implies that some aspects of the interplay between covalent and noncovalent free energy contributions in such an enzyme-catalyzed reaction may be observed through consideration of the thermodynamic cycle.

While Scheme I presents a strategy for studying free energy changes in catalysis, there are practical problems which prevent the experiments from being carried out in a direct manner near the catalytically optimum pH of 7. Any experimental attempt to unfold α-chymotrypsin in the neutral pH range would result in severe autolysis, making it impossible to directly obtain reversible unfolding of the enzyme (E) at this pH (Martin & Frazier, 1963). Likewise, unfolding of furoyl–chymotrypsin (EA) would likely be very difficult experimentally since the furoyl moiety is expected to hydrolyze to a significant extent during unfolding of EA at pH 7 (Rossi & Bernhard, 1971). In the acid pH range, however, E and EA can be reversibly unfolded without occurrence of deacylation or autolysis (Bernhard & Lau, 1971; Martin, 1964; Martin & Frazier, 1963; Rossi & Bernhard, 1971).

Obviously, a direct approach to unfolding free energy measurements at pH 7 is unrealistic. But, due to the fact that reversible unfolding of E and EA is possible at low pH, reversible unfolding free energy changes for α-chymotrypsin and furyol–chymotrypsin can be obtained at pH 7 in a less direct manner (Bolen & Santoro, 1988). Scheme II provides such a strategy for evaluating reversible unfolding free energy changes by use of a thermodynamic cycle. The scheme suggests making reversible unfolding free energy measurements at low pH ($\Delta G°_{N\text{-}U\ pH\ 3.5}$) where deacylation and/or autolysis is not a problem, in addition to making reversible titration free energy measurements on the native ($\Delta G°_{I}$) and unfolded ($\Delta G°_{II}$) forms of E and EA. From these quantities, the unfolding free energy changes for E and EA at pH 7 (dashed reaction in Scheme II) can be calculated by completion of the cycle. This scheme avoids autolysis as an experimental problem since native and unfolded forms of the enzyme are never present at the same time. Similarly, deacylation can be markedly reduced by avoiding the denaturation event in the neutral pH range (Rossi & Bernhard, 1971). The work described here presents the results of these measurements.

EXPERIMENTAL PROCEDURES

Proteins and Chemicals. α-Chymotrypsin, salt free and three times recrystallized from Worthington (CDI), was found to be 98% active or better as judged by V_m measurements using *N*-acetyl-L-tryptophan ethyl ester (ATrEE) (Sigma Chemical Co.) as a substrate with the k_{cat} value of 26.9 s^{-1} reported by Zerner et al. (1964) as the standard. The concentration of α-CT was determined at 280 nm by using a molar extinction coefficient of 50 000 cm^{-1} M^{-1}.

Furoylimidazole (mp 48–49 °C) was prepared by the method of Caplow and Jencks (1962) from reaction of furoyl chloride (Fluka Chemie AG) and recrystallized imidazole. Furoic acid (98%) from Aldrich Chemical Co. was purified by recrystallization from water/methanol at 5 °C and trifluoroethyl furoate (TFEF) was prepared by the method of Inward and Jencks (1965). The density of TFEF at 20 °C was measured to be 1.371 g/mL with a DMA 602 Density Meter from Anton Paar USA, Inc., and this value was used in preparing solutions of TFEF at the concentrations given.

Furoyl–chymotrypsin (F-Ct) was prepared by adding 40 μL of 10 mg/mL furoylimidazole, dissolved in acetonitrile, to 3 mL of 0.03 M citrate buffer at pH 6.0 containing 30 mg of α-Ct. The reaction was allowed to proceed for 5 min prior to adjusting the pH to 3.5, and the products were then dialyzed overnight against a pH 3.5 HCl solution at 4 °C. About 2–3% residual activity of α-Ct was usually detected in the F-Ct preparations by use of ATrEE as a substrate. Deacylation of F-Ct at pH 7.0 to produce furoic acid and α-Ct resulted in an isosbestic point at 250 nm from which a molar extinction coefficient of 27 760 ± 70 cm^{-1} M^{-1} was determined for F-Ct. Slow deacylation of F-Ct (a few percent per day) was observed even at pH 3.5, and only freshly prepared F-Ct was used in the experiments reported here.

Solutions of 8–10 M ultrapure urea (from Schwartz Mann Co.) were treated with a mixed bed ion exchange resin (AG501-X8, Bio-Rad Laboratories) and then filtered immediately before use. Ultrapure guanidinium chloride (GdnHCl) from Amresco was used without further purification.

Unfolding Measurements. Urea-induced unfolding of α-Ct or F-Ct was monitored by following the change in absorbance at 293 nm as a function of urea concentration. Freshly prepared stock solutions of F-Ct and α-Ct were dialyzed against a HCl solution at pH 3.5, 4 °C, prior to use. A volume of 1.40 mL of F-Ct or α-Ct in pH 3.50 β-alanine buffer (0.05 M) containing 0.174 M NaCl was delivered into a 1.0 × 1.0 cm quartz cuvette, containing a teflon-coated stir bar and fitted with a screw cap. The protein was then titrated with a urea solution containing about 8–9 M urea in the presence of 0.174 M NaCl and 0.05 M β-alanine. Aliquots of between 50 and 200 μL of the urea solution were added with a PB600 dispenser (Hamilton Co.) equipped with a 2.5-mL Hamilton syringe. The approach to equilibrium was followed over time, typically requiring from 10 to 45 min within the unfolding transition range. The absorbance at 293 nm was recorded before adding each aliquot of urea. The contribution of urea to the absorbance at 293 nm was subtracted from the recorded data, and the correction for sample dilution was made. The initial protein concentrations were about 0.3–0.4 mg/mL. Since dilution of urea solution results in decrease of the pH of the solution, the pH of the urea stock solution was adjusted to a pH of 3.65 so that the pH of the mixture of urea and protein at the midpoint of unfolding transition equals 3.50. With this procedure, the final pH of the entire unfolding transition was held within the limits of 3.50 ± 0.03.

Reversibility was shown by both spectral and activity measurements. α-Ct was unfolded in 5.8 M urea and then diluted to the nondenaturing concentration of 1.9 M and allowed to stand for 45 min. The enzyme was then added to an assay solution containing ATrEE substrate and initial velocity measured. The activity of refolded α-Ct was 97% or better of the control which consisted of α-Ct exposed only to the 1.9 M nondenaturing concentration of urea prior to assay.

Urea-induced unfolding of α-Ct as well as F-Ct was also monitored by circular dichroism (CD) at 230 nm. Titration of either α-Ct or F-Ct with urea was performed in exactly the same manner described above but with an Aviv 62DS Circular Dichroism spectropolarimeter equipped with a magnetically stirred 1.0 × 1.0 cm cuvette thermostated at 25.0 ± 0.1 °C. Initial protein concentrations ranged from 0.14 to 0.06 mg/mL. In separate experiments it was observed

that the ellipticity at 230 nm (θ_{230}) was a linear function of the protein concentration up to at least 0.17 mg/mL of protein.

Potentiometric Titrations. Potentiometric titrations were made using a Metrohm E415 pH-stat along with a pHM85 Precision pH meter from Radiometer equipped with a calomel combination electrode. The titration vessel was thermostated at 25.0 ± 0.1 °C and flushed with purified nitrogen gas previously passed through three successive scrubbers containing alkaline $BaCl_2$, 2 M phosphoric acid, and, finally, a solution identical in composition with that used in the titration vessel. The electrode and instrument were calibrated immediately prior to experiments by using a set of certified standard buffer solutions (Analytical Products Inc.) at pH 4.000 ± 0.002 and 7.000 ± 0.002. In the case of potentiometric titrations of the native forms of α-Ct and F-Ct, the 0.015 M HCl titrant used contained 0.2 M KCl. The exact concentration of HCl titrant was determined by using freshly prepared (CO_2 free) Trizma base as a primary standard in 0.2 M KCl. Several determinations of the equivalence point were made using appropriate Gran plots (Rossotti & Rossotti, 1965).

Either F-Ct or α-Ct in the range of 5–10 mg/mL was dialyzed against HCl solution at pH 3.5, 4 °C, before use in titration experiments. An aliquot of 0.5 mL of protein solution was mixed in the titration vessel with 4.5 mL of freshly prepared (CO_2 free) 0.22 M KCl to give a final KCl concentration of 0.2 M. The pH value of the resultant protein solution was adjusted to about pH 7.0 with a very small amount (10–60 μL) of 0.1 M CO_2 free KOH (Fisher Scientific) and then titrated potentiometrically with 15-s intervals between each addition of titrant. Immediately following the titration of protein, a control experiment was performed. The control solution was prepared by mixing 0.5 mL of the HCl solution used to dialyze the protein with 4.5 mL of 0.22 M KCl solution, and the potentiometric titration was carried out as described above. At each pH in titration of the control solution, a ratio was determined of the volume of the titrant added in titrating the solution from pH 7.00 to the particular pH, to the total volume of the solution at that pH. The resulting ratio vs pH data were fitted to a polynomial equation, and this equation was used in calculation of the degree of protonation of the protein.

The number of moles of H^+ bound/mole of protein was determined at each pH by the difference between (1) the total moles of HCl titrant at each point in the titration of protein and (2) the moles of H^+ needed to reach that pH in the absence of protein. The latter quantity (moles of H^+ in the absence of protein) was calculated by multiplying the above-mentioned polynomial equation with the total volume of protein solution accumulated at each point in the protein titration curve.

Titrations of the unfolded forms of α-Ct and F-Ct were performed in 6.0 M GdnHCl containing 0.2 M KCl, using a titrant of about 0.014 M HCl in 6.0 M GdnHCl containing 0.2 M KCl. As described above, the exact concentration of hydrogen ion in the titrant was determined using Trizma base (primary standard) dissolved in 6.0 M GdnHCl containing 0.2 M KCl. F-Ct as well as α-Ct at about 5–10 mg/mL was dialyzed against HCl solution at pH 3.5, 4 °C, prior to use, and 0.5 mL of the protein solution was mixed with 4.5 mL of freshly prepared 6.67 M GdnHCl solution containing 0.22 M KCl in the titration vessel. Titrations were performed as described above. The control experiments were also performed by mixing 0.5 mL of HCl solution, used to dialyze the protein, with 4.5 mL of 6.67 M GdnHCl solution containing 0.22 M KCl. The amount of hydrogen ion bound to protein as a function of pH was evaluated as described.

Kinetic Measurements. Disappearance of F-Ct at pH 7.0 (0.036 M citrate) in the presence or absence of trifluoroethanol (TFE) was monitored by following the change in absorbance at 265 nm at 25.0 ± 0.1 °C. The absorbance was recorded until no further absorbance change occurred with time. The loss of F-Ct at pH 7.0 in the absence of TFE was also monitored by following the change in ellipticity at 230 nm using an Aviv 62DS circular dichroism spectropolarimeter.

Initial velocities for the hydrolysis of *N*-acetyl-L-tryptophan ethyl ester (ATrEE) in the presence of TFEF were measured spectrophotometrically by means of a Varian 2200 spectrophotometer equipped with a Rapid Kinetics Accessory Stopped Flow system (model SFA-11, Hi-Tech Scientific, Wiltshire, England). A solution containing 0.036 M citrate pH 7.0 and different concentrations of ATrEE (98–368 μM) and TFEF (0–2.34 mM) was mixed (1:1) in the stopped flow system with α-Ct solution (0.5 μM) containing 0.036 M citrate pH 7.0, and the change in absorbance at 300 nm was monitored with time. Following mixing, the absorbance as a function of time was observed to be linear for at least the first 10 s for most measurements, and the initial linear portion of the recorded curve was used to evaluate the initial velocity. The stopped flow system, the cuvette holder, and all solutions in the drive syringes were thermostated at 25 ± 1 °C.

In reactions involving either α-Ct or F-Ct with TFEF plus TFE at pH 7.0, the concentration of unacylated enzyme in the reaction mixture was measured by burst experiments using *p*-nitrophenyl acetate (Caplow & Jencks, 1962; Hartley & Kilby, 1952; Inward & Jencks, 1965).

Nonlinear least-squares fitting was performed by a program provided by Dr. Michael Johnson at the University of Virginia and described elsewhere (Johnson & Frasier, 1985). A Macintosh version of this program (NonLin for Macintosh) was made available by Robelko Software, Murphysboro, IL.

RESULTS

Free Energy Data Associated with Scheme II. Difference spectroscopy and circular dichroism measurements were both used to monitor protein unfolding in the presence of urea. Figure 1 gives the denaturation profiles of α-Ct and F-Ct at pH 3.50 using the molar extinction difference ($\Delta\epsilon$) between native and unfolded forms of a protein at 293 nm, and the inset of Figure 2 shows their unfolding monitored by molar ellipticities at 230 nm (θ_{230}). The solid lines in these figures represent the nonlinear least-squares best fits to the linear extrapolation model. Conversion of these fitted unfolding profiles to a common scale of "fraction unfolded" as in Figure 2 illustrates that the degree of unfolding is independent of the variable monitored and provides limited evidence for two-state unfolding for both proteins (Lumry et al., 1966). Previous work showing the lack of equilibrium unfolding intermediates for α-chymotrypsin and phenylmethanesulfonylchymotrypsin (PMS-Ct) as detected by urea gradient gel electrophoresis (Creighton, 1979; Santoro & Bolen, 1988), provides additional evidence that two-state behavior is a reasonable assumption for the unfolding of α-chymotrypsin and its derivatives at low pH.

The unfolding free energy changes ($\Delta G°_{N-U}$) and the transition midpoints ($C_{1/2}$) of α-Ct and F-Ct evaluated from the nonlinear least-squares fits in Figures 1 and 2 are given in Table I along with their 67% confidence intervals. The $\Delta G°_{N-U}$ values and the need for higher urea concentration to unfold F-Ct demonstrate that the acyl–enzyme is more stable than α-Ct at pH 3.5.

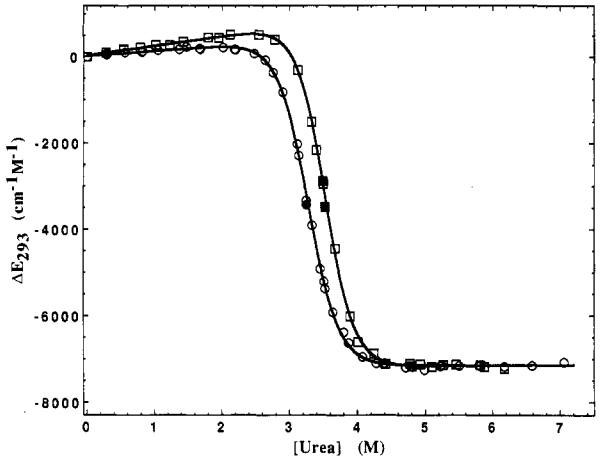


FIGURE 1: Difference extinction coefficients at 293 nm as a function of urea concentration for α-chymotrypsin (○) and furoyl–chymotrypsin (□) in the presence of 0.05 M β-alanine and 0.174 M NaCl at pH 3.50 and 25 °C. Solid lines represent the nonlinar least-squares best fits of the data based on the linear extrapolation model. The refolding points for α-Ct (●) and F-Ct (■) were obtained by first unfolding the proteins in 4.8 M urea with 0.05 M β-alanine in 0.174 M NaCl at pH 3.50 for 5–6 min and then diluting the urea to a final concentration within the transition range as indicated. Refolding of the proteins was allowed to proceed for at least 1 h to achieve the final equilibrium absorbance at 293 nm.

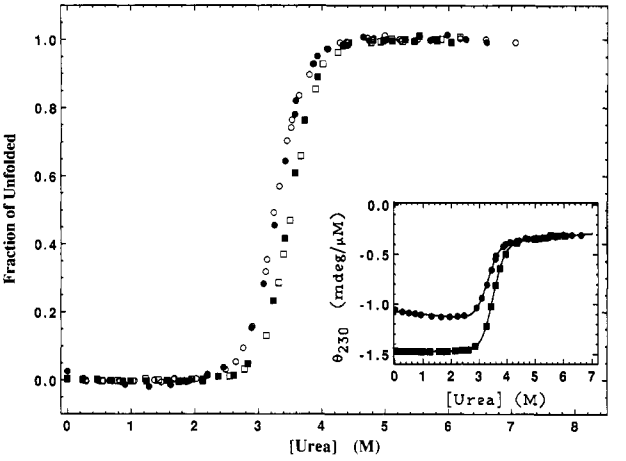


FIGURE 2: Fraction of unfolded protein as a function of urea concentration for α-chymotrypsin (○, ●) and furoyl–chymotrypsin (□, ■) in 0.05 M β-alanine and 0.174 M NaCl at pH 3.50 and 25 °C. The open symbols are data obtained from difference extinction coefficient measurements, while the filled symbols represent data from ellipticity measurements. The inset shows ellipticity as a function of urea concentration for α-Ct (●) and F-Ct (■). Solid lines represent the nonlinear least-squares best fits of the data to the linear extrapolation model (Santoro & Bolen, 1988).

Table I: Unfolding Free Energy Change and Transition Midpoint for α-Chymotrypsin and Furoyl–Chymotrypsin at pH 3.50, 25 °C, Ionic Strength 0.2 M

protein	method	$\Delta G°_{N-U}$ (67% confidence limits) (kcal/mol)	$C_{1/2}$ (M)
α-Ct	$\Delta\epsilon_{293}$	8.69 ± 0.28	3.270
α-Ct	θ_{230}	8.81 ± 0.59	3.280
F-Ct	$\Delta\epsilon_{293}$	9.87 ± 0.35	3.519
F-Ct	θ_{230}	9.90 ± 0.30	3.486

Scheme II requires potentiometric titration of native and unfolded states of α-Ct and F-Ct from pH 3.5 to the neutral pH range. To do this, we need to know how much the native and unfolded states differ in terms of their states of protonation at any given pH. This difference in protonation can be

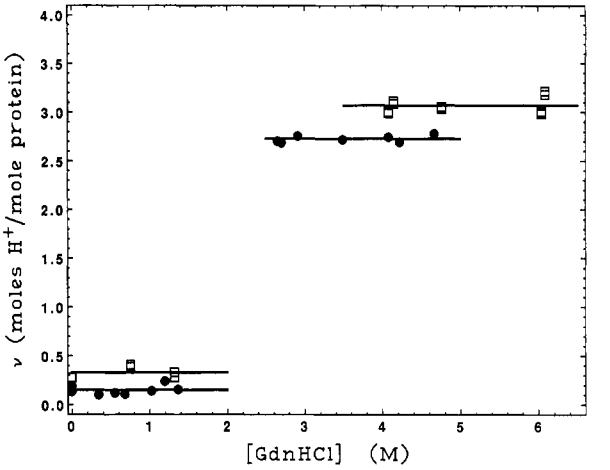


FIGURE 3: Determination of proton uptake by α-Ct (●) and F-Ct (□) on unfolding at pH 3.500 and 25 °C in the presence of 0.2 M KCl. Lines are the linear least-squares fits of the data in pre- and posttransition ranges. Data were obtained by the procedure described under Results.

determined at pH 3.5 by measuring the proton uptake which accompanies unfolding of the protein at this pH. Unbuffered protein solution in 0.2 M KCl at pH 3.500 was mixed with GdnHCl solutions adjusted to a pH slightly higher than 3.5. The resultant mixed solution was back-titrated to pH 3.500 and the amount of titrant recorded. Back-titration of a control experiment involving the mixing of the previously adjusted GdnHCl solution with pH 3.500, 0.2 M KCl in the absence of protein was also performed. The difference in back-titrations for the control and protein-containing solutions is expressed in moles of proton absorbed by protein per mole of protein (ν), and Figure 3 gives the results of such experiments for α-Ct and F-Ct as a function of the final concentration of GdnHCl. The uptake of protons accompanying denaturation of α-Ct and F-Ct at pH 3.500 was found to be 2.58 ± 0.06 and 2.74 ± 0.10 mol of H^+/mol of protein, respectively. It is noted that, in the postunfolding region, ν is independent of GdnHCl concentration for both α-Ct and F-Ct, a result indicating that ionization behavior of the unfolded ensembles is not perturbed by additional GdnHCl.

The standard state free energy changes for titration of native and unfolded protein, $\Delta G°_{I}$ and $\Delta G°_{II}$, in Scheme II can be obtained from integration of pH titration data, involving the relative number of protons (ν) bound to protein versus pH. The exact relationship is given by eq 2, which has been derived by a number of researchers (Chu et al., 1984; Hermans & Acampora, 1967; Pfeil & Privalov, 1976; Schellman & Hawkes, 1980).

$$\Delta G°_{I} \text{ or } \Delta G°_{II} = 2.303RT\int \nu \, \mathrm{dpH}(\nu) \qquad (2)$$

Potentiometric titration curves of unfolded proteins were obtained in the presence of 6 M GdnHCl, and the ν values of the pH titration curves of unfolded proteins were set arbitrarily to zero at pH 7.0. Since hydrogen ions are taken up by the solution on denaturation of α-Ct and F-Ct at pH 3.5, the titration curves of the native form of α-Ct and the native form of F-Ct are frame shifted relative to the titration curves of their unfolded forms by 2.58 and 2.74 protons, respectively, at pH 3.5 (see Figures 4 and 5).

Figure 4 gives the titration curves of native and unfolded forms of α-Ct. In accordance with eq 2, the titration free energy changes from pH 3.5 to 7.0 for the native and unfolded protein species are represented by the integrated areas under

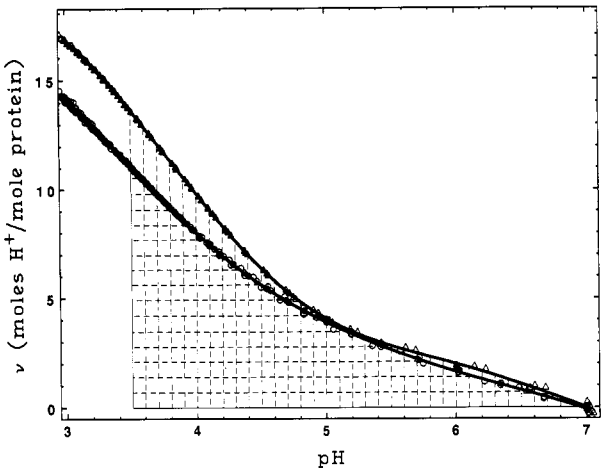


FIGURE 4: Reversible pH titration of the native α-Ct (O) in 0.2 M KCl and unfolded α-Ct (Δ) in 0.2 M KCl and 6 M GdnHCl at 25 °C. The data for both native and unfolded proteins include the titration and retitration of the protein with two different initial concentrations of protein as described under Results. Solid curves are polynomial representations of the data. The hydrogen ion uptake at pH 3.500 of 2.58 ± 0.06 mol of H^+ per mole of α-Ct (see Figure 3) was used to displace the titration curve of the native α-Ct relative to the titration curve of the unfolded α-Ct. The areas under the curves represent the relative free energy change for titration from pH 3.5 to 7.0 of the native (horizontal dashed lines) and unfolded protein (vertical dashed lines) according to eq 2.

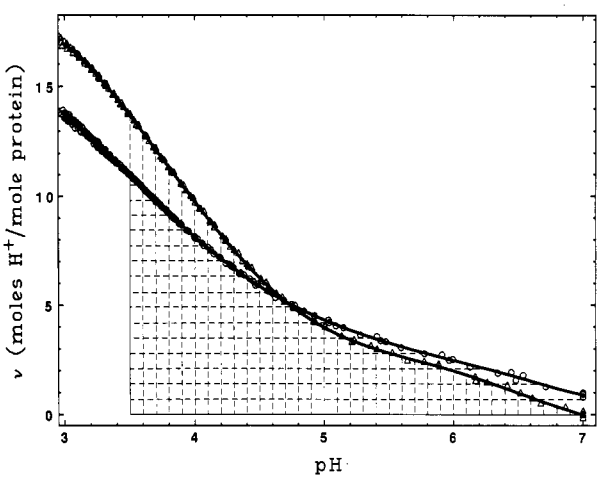


FIGURE 5: Reversible pH titration of the native F-Ct (O) in 0.2 M KCl and unfolded F-Ct (Δ) in 0.2 M KCl and 6 M GdnHCl at 25 °C. The data for both native and unfolded proteins include the titration and retitration of the protein with two different initial concentrations of protein as described under Results. The curves are polynomial representations of the data. The hydrogen ion uptake at pH 3.500 of 2.74 ± 0.10 mol of H^+ per mole of F-Ct (see Figure 3) was used to displace the titration curve of the native F-Ct relative to the titration curve of the unfolded F-Ct. The areas under the curves represent the relative free energy change for titration from pH 3.5 to 7.0 of the native (horizontal dashed lines) and unfolded protein (vertical dashed lines) according to eq 2.

the curves. The standard free energy difference between the native and unfolded forms of α-Ct over this pH range was determined by numerical integration to be 3.33 ± 0.29 kcal/mol. It is noted that, at any given pH in the range of 3–7, the unfolded form of α-Ct is more highly protonated than the native form, indicating that proton uptake accompanies unfolding over the entire pH range studied. Each titration curve in Figure 4 contains four separate sets of titration data with initial protein concentrations ranging from 0.46 to 0.73 mg/mL for the unfolded form of α-Ct and 0.59 to 1.07 mg/mL for the native form of α-Ct. For example, following the first titration of α-Ct (initial concentration of 1.07 mg/mL) from pH 7 to 3, the pH was adjusted back to about pH 7 with 0.1 M KOH, and the protein was subjected to retitration. After correction for the control, the retitration data were found to superimpose on the first titration data, indicating complete reversibility for titration of the native and unfolded forms of α-Ct. Use of approximately 2-fold lower initial concentration of α-Ct along with retitration also falls precisely on the same curve, demonstrating within error that titration is independent of protein concentration over the range investigated. Titrations were also found to be independent of whether 0.2 M NaCl or 0.2 M KCl was used as supporting electrolyte.

Figure 5 gives the titration curves of the native and unfolded forms of F-Ct. Each curve again contains four separate sets of data with protein concentration ranging from 0.58 to 1.03 mg/mL for native F-Ct and 0.62 to 0.99 mg/mL for unfolded F-Ct. Titration followed by retitration for two concentrations each of native and unfolded F-Ct were performed in the manner described above. Within experimental error, the titration curves of the native and unfolded forms of F-Ct are found to be completely reversible and independent of protein concentration over the concentration range used. The standard free energy changes of titration from pH 3.5 to 7.0 are represented by the areas under the curves. The difference in free energy change of titration from pH 3.5 to 7.0 between the native and unfolded forms of F-Ct was determined to be 0.59 ± 0.48 kcal/mol. It is noted that the two titration curves cross over at about pH 4.8, so that, above pH 4.8, the difference in the free energy changes of titration between the native and unfolded forms of F-Ct decreases with increasing pH. The titration of the native form of F-Ct in the presence of 0.2 M NaCl was also performed (data not shown) with no significant titration differences found for NaCl compared with KCl.

It should be noted that the native form of F-Ct will deacylate to form α-Ct and furoate at pH 7.0 with an apparent first-order rate constant of 0.044 ± 0.001 min^{-1}. Reducing the pH from 7.0 results in a decrease of the rate of deacylation to near zero at about pH 6 and below (Inward & Jencks, 1965). Titration experiments require exposing the protein to around pH 7.0 for about 15–30 s. Deacylation of F-Ct resulting from this exposure is estimated to be less than 2% of total EA present.

If unfolding of α-Ct and F-Ct results in full exposure of all titratable groups in the proteins, the titration curves of the unfolded forms of α-Ct and F-Ct should be identical to one another. Direct comparison of the titration curves of the unfolded forms of α-Ct and F-Ct is given in Figure 6, and, within experimental error, the titration profiles in the pH range from 3 to 7 for the unfolded forms of α-Ct and F-Ct are found to be identical.

According to Scheme II, the titration free energy difference ($\Delta G°_{II}-\Delta G°_{I}$) between native and unfolded protein from one pH to another equals the difference in free energy change of unfolding between these two pHs. Therefore, $\Delta G°_{II}-\Delta G°_{I}$ obtained by integration of the area between the titration curves of native and unfolded protein, coupled with unfolding free energy changes at pH 3.5 given in Table I, permits calculation of unfolding free energy change at any pH within the titration range. Figure 7 shows the unfolding free energy changes for both α-Ct and F-Ct as a function of pH from about pH 3 to 7. It is seen in the figure that the unfolding free energy change of F-Ct is larger than that of α-Ct at low pH but becomes smaller than that of α-Ct at pH above 6.

Free Energy Data Associated with Scheme I. The mechanism of α-Ct catalyzed hydrolysis of an ester is given in eq 3 with trifluoroethyl furoate as a substance (Inward & Jencks,

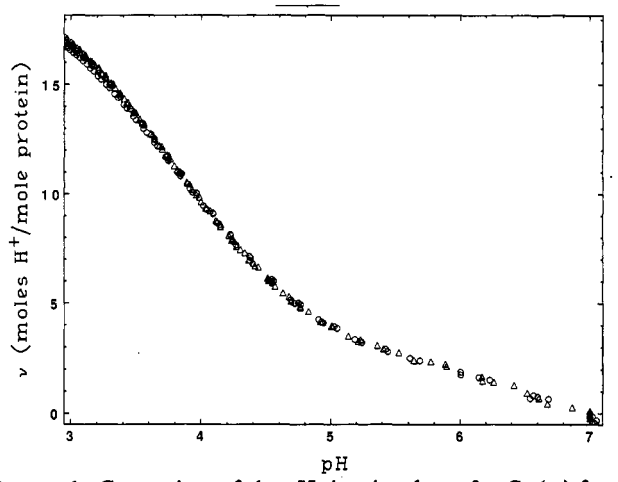


FIGURE 6: Comparison of the pH titration data of α-Ct (○) from Figure 4 and F-Ct (△) from Figure 5 in the presence of 6.0 M GdnHCl and 0.2 M KCl at 25 °C. The points for each protein represent titration and retitration of two different concentrations of F-Ct and α-Ct (see text involving Figures 4 and 5).

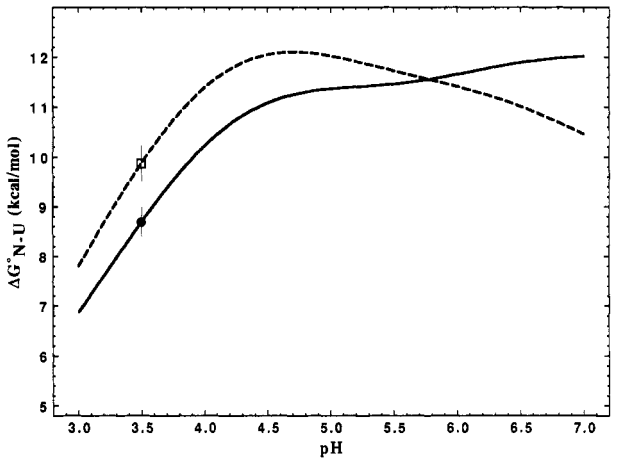


FIGURE 7: Unfolding free energy changes of α-chymotrypsin (solid line) and furoyl–chymotrypsin (dashed line) as functions of pH at ionic strength of 0.2 M at 25 °C. The curves were evaluated from pH titration data of native and unfolded protein along with ΔG°_{N-U} estimated at pH 3.5 for α-Ct (●) and F-Ct (□) as described under Results. The error bars indicate 67% confidence interval ranges of the unfolding free energy changes at pH 3.50 for α-Ct and F-Ct.

1965). Here, α-Ct·TFEF represents the (noncovalent) Michae-

$$\text{TFEF} + \alpha\text{-Ct} \underset{}{\overset{K_1}{\rightleftharpoons}} \alpha\text{-Ct}\cdot\text{TFEF} \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} \text{F-Ct} + \text{TFE} \qquad (3)$$

$$\text{F-Ct} \xrightarrow{k_3,\ H_2O} \alpha\text{-Ct} + \text{Furoate}$$

lis complex between the substrate and the enzyme (binding constant K_1), while k_2 is the rate constant for acylation of α-Ct; k_{-2} and k_3 are the rate constants for alcoholysis and hydrolysis of F-Ct, respectively.

The apparent rate constants for disappearance of furoyl–chymotrypsin in the absence and presence of increasing concentration of trifluoroethanol at 25 °C, pH 7.0, and ionic strength 0.2 M are shown in Figure 8. The disappearance of F-Ct in the presence of TFE was followed spectrophotometrically (265 nm) while F-Ct disappearance in the absence of TFE was monitored by circular dichroism (230 nm) as well as by UV-Vis spectrophotometry (265 nm). For comparison, the inset shows examples of results obtained in the absence and presence of 65 mM TFE. The lines in the inset are the

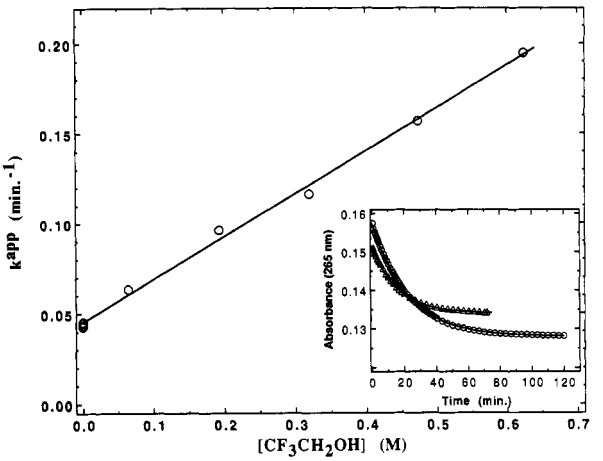


FIGURE 8: Apparent first-order rate constants as a function of trifluoroethanol for hydrolysis or alcoholysis of furoyl–chymotrypsin in 0.036 M sodium citrate at pH 7.0 and 25 °C. The line represents the linear least-squares fit of the data to eq 4 giving values of k_3 = 0.044 ± 0.001 min^{-1} and k_{-2} = 0.238 ± 0.007 M^{-1} min^{-1}. The inset shows the deacylation of F-Ct in the absence of TFE (○) and the presence of 0.065 M TFE (△) monitored by absorbance change at 265 nm. The curves are the nonlinear least-squares best fits of the data using a first-order equation.

first-order fits to the data. In all experiments, the disappearance of F-Ct followed pseudo-first-order kinetics. By both CD and absorbance measurements, a rate constant of 0.044 ± 0.001 min^{-1} was determined in the absence of TFE. In the presence of TFE, the observed rate constants are the sum of the individual rate constants for hydrolysis and alcoholysis as given in eq 4.

$$k^{app} = k_3 + k_{-2}[\text{TFE}] \qquad (4)$$

The linear fit of the plot of apparent rate constants in the presence of TFE versus TFE concentration resulted in an intercept which was identical with the observed rate of hydrolysis in the absence of TFE. The straight line in Figure 8 is the linear least-squares best fit of all data to eq 4, and the slope of this line, giving the second-order rate constant (k_{-2}) for alcoholysis of F-Ct, was determined to be 0.238 ± 0.007 min^{-1} M^{-1}. The rate constant for hydrolysis (k_3) of F-Ct was reported by Inward and Jencks (1965) to be independent of the concentration of TFE, and this was also confirmed under our experimental conditions (data not shown) using their procedure.

The equilibrium constant for the formation of the noncovalent complex between trifluoroethyl furoate and α-chymotrypsin was evaluated by using TFEF as a competitive inhibitor of ATrEE hydrolysis. Initial velocities were determined for ATrEE hydrolysis as a function of the concentration of ATrEE in the absence and presence of 0.39, 0.78, and 1.17 mM TFEF. At constant TFEF concentration, initial velocities as a function of ATrEE were determined as the change in absorbance per minute, and the lines were drawn using the linear least-squares best fitted parameters to the double-reciprocal expression for competitive inhibition. Figure 9 shows the results of individual linear least-squares fits at different fixed TFEF concentrations displayed in a double-reciprocal plot. Within error, the lines converage to a common intercept characteristic of competitive inhibition. An average inhibition constant (K_I) of 1.78 ± 0.09 mM results from the

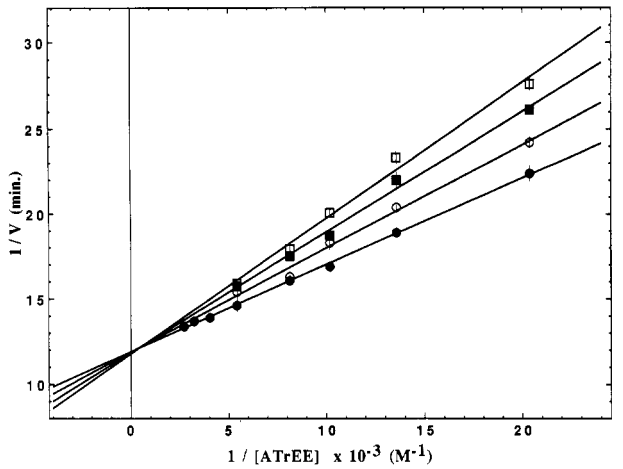


FIGURE 9: Double-reciprocal plot of the apparent velocity of α-Ct catalyzed hydrolysis of ATrEE as a function of ATrEE concentration in the absence (●) and presence of 0.391 mM (○), 0.781 mM (■), and 1.172 mM (□) TFEF at pH 7.0 and 25 °C. The assay solution also contained 0.036 M sodium citrate, 0.5% acetonitrile, and 25 μM α-Ct. Lines are the linear least-squares fits of the data in the fixed concentrations of TFEF. The velocity is reported in terms of absorbance change per minute at 300 nm. The error bars represent the standard deviation of the points from triplicate measurements.

fittings. A simultaneous fit of all four sets of data to eq 5

$$V = \frac{V_m[\text{ATrEE}]}{K_m\left(1 + \frac{[\text{TFEF}]}{K_I}\right) + [\text{ATrEE}]} \quad (5)$$

gives a common value of $K_m = (4.3 \pm 0.4) \times 10^{-5}$ M and K_I = 2.14 (1.77, 2.55) mM along with their respective nonlinear 67% confidence intervals. The K_I translates into a α-Ct·TFEF binding constant (K_1) of 467 (393, 566) M^{-1}. The K_m value for ATrEE of $(7.3 \pm 2.5) \times 10^{-5}$ M reported by Bender et al. (1964) is larger than the K_m of $(4.3 \pm 0.4) \times 10^{-5}$ M determined here. The K_m difference may be due in part to the differences in acetonitrile concentrations in Bender's (0.81%) and our (0.5%) experiments since K_m is highly dependent on acetonitrile concentration (Bender et al., 1964; Zerner et al., 1964).

The equilibrium constant (K_2) for formation of furoyl–chymotrypsin from the Michaelis complex between α-Ct and TFEF was evaluated under steady-state conditions. With solution conditions of 0.036 M citrate at pH 7.0, 25 °C, the reaction was initiated by mixing either α-Ct or F-Ct with TFEF solution in the presence of TFE. The amount of unacylated enzyme at different periods of time was evaluated by determining the initial burst of *p*-nitrophenol released upon mixing aliquots (200 μL) of the reaction mixture with *p*-nitrophenyl acetate solution (2.02 mL) containing 0.036 M citrate and 0.9% acetonitrile at pH 7.5, 25 °C. Figure 10 shows the amount of unacylated enzyme at different periods of reaction time in the presence of different concentrations of TFEF and TFE. It is seen that the concentration of unacylated enzyme reaches a constant value after about 10 min, and the steady-state relationship gives rise to eqs 6

$$d[\text{F-Ct}]/dt = 0 = k_2[\alpha\text{-Ct·TFEF}] - k_{-2}[\text{F-Ct}][\text{TFE}] - k_3[\text{F-Ct}] \quad (6)$$

$$\frac{k_2}{k_{-2}} = K_2 = \frac{[\text{F-Ct}][\text{TFE}] + \frac{k_3}{k_{-2}}[\text{F-Ct}]}{[\alpha\text{-Ct·TFEF}]} \quad (7)$$

and 7. Here, [α-Ct·TFEF] represents the Michaelis complex between α-Ct and TFEF, whose concentration can be

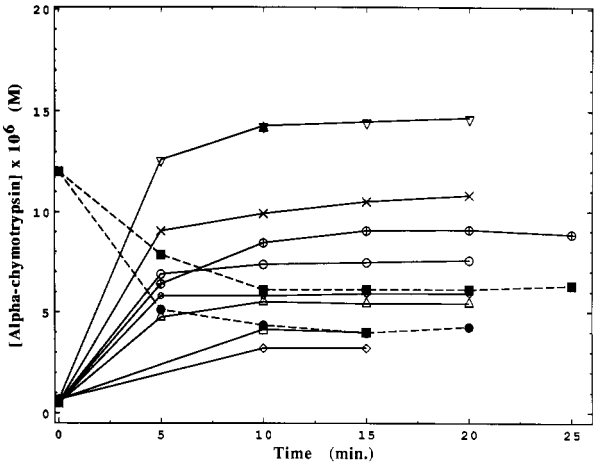


FIGURE 10: Determination of the steady-state concentration of nonacylated α-Ct in 0.036 M sodium citrate at pH 7.0 and 25 °C. At the start of the reaction which was buffered by 0.036 M sodium citrate, pH 7, the reaction mixture contained the following: 0.697 M TFE, 0.56 mM TFEF, and 21.64 μM F-Ct (▽); 0.637 M TFE, 0.565 mM TFEF, and 21.81 μM F-Ct (▲); 0.487 M TFE, 0.569 mM TFEF, and 17.02 μM F-Ct (×); 0.379 M TFE, 0.574 mM TFEF, and 17.15 μM F-Ct (⊕); 0.27 M TFE, 0.579 mM TFEF, and 17.30 μM F-Ct (○); 0.424 M TFE, 1.148 mM TFEF, and 17.15 μM F-Ct (⊗); 0.158 M TFE, 0.584 mM TFEF, and 17.44 μM F-Ct (△); 0.148 M TFE, 1.172 mM TFEF, and 22.63 μM F-Ct (□); 0.091 M TFE, 1.177 mM TFEF, and 22.72 μM F-Ct (◇); 0.424 M TFE, 1.148 mM TFEF, and 12.02 μM α-Ct (●); 0.379 M TFE, 0.574 mM TFEF, and 12.02 μM α-Ct (■).

determined from the equilibrium constant K_1 and concentrations of TFEF and α-Ct. The steady-state concentration of F-Ct can be obtained from knowledge of the free concentrations of α-Ct (see Figure 10), α-Ct·TFEF, and the total amount of enzyme in the reaction mixture. Since large molar excesses of TFEF and TFE were used in the experiment in comparison with the amount of enzyme present, the initial concentrations of TFEF and TFE were taken as the steady-state concentrations. The rate constant k_2 and equilibrium constant K_2 can be determined knowing the concentrations of all species in eqs 6 and 7 along with the previously determined rate constants, k_{-2} and k_3. Table II gives the tabulated values of K_2 determined after a 10-min reaction time using different concentrations of TFEF and TFE. A mean value of 3.09 M is estimated for K_2 with a standard deviation of ±0.85 M. Within the time it takes for the reaction to reach steady state, there is less than a 2% change in TFEF concentration resulting from hydrolysis or from the reaction of TFE with F-Ct or α-Ct with TFEF. It should be noted that the concentration ratio of TFEF to TFE was changed 16-fold, yielding relatively constant values for K_2. The last two experiments in Table II were initiated by mixing α-Ct with TFEF and TFE, while the remainder were initiated by mixing F-Ct with TFEF and TFE. Within experimental error, both procedures result in equivalent K_2 values, demonstrating that the reversible reaction can be driven in both directions.

Free energy changes for the first two reversible steps in eq 1 were calculated from the corresponding equilibrium constants (see Table III). These values are shown in the free energy scheme in Figure 11 along with unfolding free energies of α-Ct and F-Ct at pH 7.0. All free energy changes were determined independently except for the reaction of unfolded α-Ct with TFEF to form unfolded F-Ct plus TFE. This latter free energy change of 5.86 ± 0.75 kcal/mol is obtained by completion of the thermodynamic cycle.

Table II: Evaluation of the Equilibrium Constant for Formation of Furoyl–Chymotrypsin from the Complex of α-Chymotrypsin and Trifluoroethyl Furoate at pH 7.0, 25 °C, Ionic Strength 0.2 M

TFE[a] (M)	TFEF[b] (mM)	α-Ct[c] (μM)	F-Ct[d] (μM)	Ct·TFEF[e] (μM)	K_{eq}[f]
0.158	0.584	5.53	11.91	1.19	3.49
0.270	0.579	7.39	9.91	1.57	2.91
0.379	0.574	8.46	8.69	1.79	2.76
0.487	0.569	9.93	7.09	2.09	2.30
0.424	1.148	5.82	11.33	2.03	3.43
0.091	1.177	3.22	19.50	1.14	4.81
0.148	1.172	4.15	18.47	1.47	4.25
0.697	0.56	14.29	7.35	2.96	2.20
0.637	0.565	14.17	7.64	2.96	2.14
0.424	1.148	4.36	7.66	1.52	3.10
0.379	0.574	6.12	5.90	1.29	2.60
mean					3.09 ± 0.85

[a] Initial concentration of trifluoroethanol. [b] Initial concentration of trifluoroethyl furoate. [c] Steady-state concentration of total unacylated α-chymotrypsin measured by initial burst with *p*-nitrophenyl acetate. [d] Steady-state concentration of furoyl–chymotrypsin, the difference between total enzyme and total unacylated α-Ct. [e] Concentration of the Michaelis complex between α-Ct and TFEF calculated with K_I value of 467 M^{-1}. [f] Equilibrium constant for the reaction α-Ct·TFEF ⇄ F-Ct + TFE. The first nine experiments were initialized by addition of F-Ct into reaction solution, while the last two experiments were initialized by addition of α-Ct into reaction solution.

Table III: Equilibrium Constants and Free Energy Changes at pH 7.0, 25 °C, Ionic Strength 0.2 M[a]

process	K_{eq}	$\Delta G^{\circ\prime}$ (kcal/mol)
E + S ⇄ ES	467 (393, 566)	−3.64 (−3.54, −3.75)
ES ⇄ EA + P_I	3.09 ± 0.85	−0.67 (−0.48, −0.81)
E + S ⇄ EA + P_I	1440 ± 480	−4.31 (−4.07, −4.48)
E + S ⇄ U + S	1.55×10^{-9}	12.01 ± 0.41
EA + P_I ⇄ U–A + P_I	2.13×10^{-8}	10.46 ± 0.59
U + S ⇄ U–A + P_I	19900	−5.86 ± 0.75

[a] Errors reported are derived from one of several methods of analysis including nonlinear least-squares, linear least-squares, or multiple measurements of a quantity. Where practical, the errors are reported as symmetrical (±), but those which are not symmetrical are given in parentheses as the lower and upper nonlinear 67% confidence intervals.

DISCUSSION

In order to determine unfolding free energy changes for α-Ct and F-Ct at pH 7 and avoid problems of autolysis and deacylation, we had to devise an approach that would obviate the need to directly carry out protein unfolding in the neutral pH range. In principle, Scheme II provides such an approach, but to keep the error in ΔG°_{N-U} low, methods had to be developed that would result in good precision and accuracy. Accordingly, we carried out extensive measurements with Scheme II using phenylmethanesulfonylchymotrypsin (PMS-Ct), a serine-195 linked sulfonyl ester which does not hydrolyze upon urea unfolding in the neutral pH range (Bolen & Santoro, 1988; Santoro & Bolen, 1988). More recently, we have improved on the techniques, applying Scheme II to ribonuclease A (Yao and Bolen, unpublished results). With both proteins we determined all four sides of the thermodynamic cycle and demonstrated that the methods used in the independent determination of three sides of the cycle would accurately predict the fourth side. Use of 6 M GdnHCl in determining the titration free energy change for the unfolded protein works equally well in predicting urea or GdnHCl induced unfolding free energy changes at other pH values. These results provide strong support for the methods used in the current work.

Comparisons of the unfolding free energy differences ($\Delta\Delta G^{\circ}_{N-U}$) between E and EA are no different from those

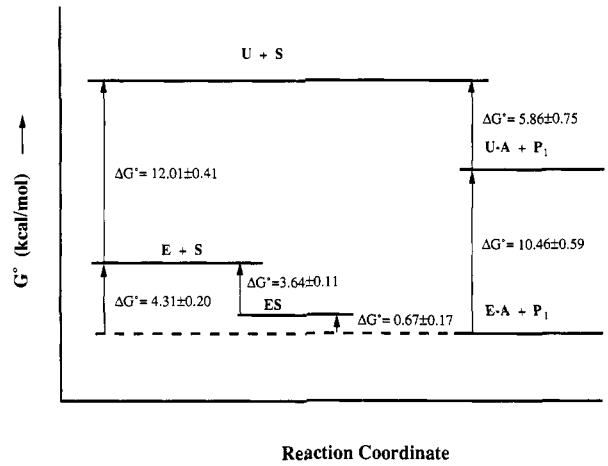


FIGURE 11: Composite free energy diagram for the acylation reaction of α-Ct (E) with TFEF (S) along with the unfolding reactions of α-Ct and furoyl–chymotrypsin (F-Ct) to give unfolded α-Ct (U) and unfolded F-Ct (U–A) at pH 7.0. The species P_I and ES represent trifluoroethanol and the noncovalent Michaelis complex for TFEF binding to α-Ct, respectively. All reactions are at ionic strength 0.2 M, 25 °C, using the standard state of pH 7. The free energy changes along with the corresponding standard deviation for each step are given in the diagram. The free energy change for transesterification TFEF to unfolded α-Ct was calculated by completing the thermodynamic cycle connecting the free energy levels for U + S with U–A + P_I.

commonly made between wild-type and single site mutant proteins. The issue which arises in comparing closely related proteins is whether the $\Delta\Delta G^{\circ}_{N-U}$ changes are due to differences in the native states of the two proteins, differences in their unfolded states, or differences in both states. From the data we have obtained on unfolding and titration, it appears that the native states of α-Ct and F-Ct are clearly different from one another while their unfolded states are indistinguishable. In Figure 1 and also in the inset of Figure 2, it is seen that α-Ct and F-Ct differ in extinction coefficient and ellipticity behavior in the preunfolding or "native" urea concentration range, but in the postunfolding urea concentration range, the spectral characteristics of both proteins are identical to one another. A far more definitive comparison can be drawn from the titration data. Figure 6 shows that unfolded α-Ct and F-Ct are identical in their titration behavior over a range of four orders of magnitude in hydrogen ion concentration. Figures 4 and 5, on the other hand, demonstrate substantial differences between the titration curves of the native forms of α-Ct and F-Ct over the same pH range. These data demonstrate that the differences in unfolding free energy for α-Ct and F-Ct as a function of pH (see Figure 7) arise exclusively from differences in the native states of the two proteins.

A second issue which arises in comparing the unfolding of closely related proteins is whether the extent of unfolding is the same for both proteins. There is evidence from a variety of sources indicating that the degree of unfolding of α-Ct and its serine-195 derivatives is extensive in urea and GdnHCl and that the covalently linked moieties have the same characteristics as corresponding model compounds in these denaturants (Bernhard et al., 1965; Bernhard & Rossi, 1968; Noller & Bernhard, 1965). The ability to quantitatively account for the absorbance of GdnHCl induced unfolded states of α-Ct and PMS-Ct from knowledge of their amino acid composition and molar absorptivities of model amino acids in denaturant indicates that the chromophoric species are extensively exposed to solvent (Edelhoch, 1967; Santoro & Bolen, 1988). Bernhard and co-workers have studied a number

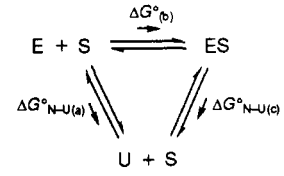
of urea and GdnHCl unfolded arylacryloyl–chymotrypsins related to F-Ct and have shown that the unfolded acylenzyme spectra are in every case the same as that of the corresponding *O*-acyl serine peptide (Bernhard et al., 1965). Additional studies demonstrated that alkaline hydrolysis of unfolded acyl–enzymes is very similar in rate and pH dependence as that of model *O*-acyl serine peptides, again indicating that reactivity involving the acyl moiety is unrestricted in these strong denaturants (Bernhard & Rossi, 1968; Noller & Bernhard, 1965). Perhaps the strongest evidence of extensive unfolding in both α-Ct and F-Ct is from their titration in 6 M GdnHCl. From knowledge of the number and kinds of titratable groups in the pH range of 3–7 and assuming complete unfolding of furoyl– and α-chymotrypsin, we have fitted the data in Figure 6 to obtain the p*K*s of groups being titrated. We find that the fitted p*K*s deviate by no more than 0.1–0.2 p*K* units from the consensus intrinsic p*K* values of titrateable groups reported by Nozaki and Tanford (1967) from their studies of proteins in 6 M GdnHCl. The proton binding polynomial over the pH 3–7 range involves some 17 titratable groups all over the protein, one of which (Asp-194) is adjacent to the *O*-acylserine (195) residue. The p*K*s of these 17 groups reflect that the groups are not constrained in environments that would cause significant p*K* perturbation. The fact that the entire pH dependence of $\Delta\Delta G°_{N-U}$ values for α-Ct and F-Ct is due to the native forms of these proteins and not their unfolded ensembles indicates that regions of incomplete unfolding in one of the proteins and not the other is highly unlikely.

If we begin at pH 3.5, it is seen from Figure 7 that with respect to unfolding furoyl–chymotrypsin is thermodynamically more stable than α-chymotrypsin by about 1.5 kcal/mol. The conventional explanation for this effect would probably be that the acyl moiety interacts favorably with residues at the active site, conferring stability to the protein. As pH is increased from 3.5 to the neutral range, furoyl–chymotrypsin becomes 1.6 kcal/mol less stable than α-chymotrypsin! Thus, the furoyl moiety goes from having a stabilizing influence at low pH to having a destabilizing effect on protein structure relative to α-Ct in the neutral pH range. These data show a relative change in stability between E and EA of about 3 kcal/mol over the pH 3.5–7 range, a change which is a substantial fraction of the total unfolding free energy of a protein.

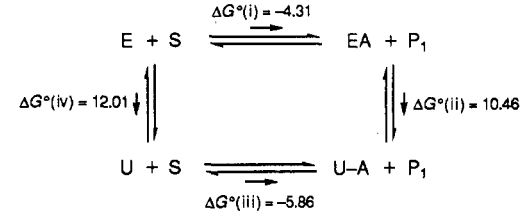
In reference to catalysis it is worth noting that the transformation of the furoyl moiety from a stabilizing to a destabilizing influence on protein structure coincides with F-Ct (and also α-chymotrypsin) being converted from a catalytically inactive species at low pH to near optimal activity at pH 7. The pH–activity profile of F-Ct and the changes in structural integrity of F-Ct as a function of pH both have their origins in the thermodynamic linkage between hydrogen ion and altered p*K*s on the protein brought about by accommodating the acyl moiety in the active site. Thus, there is good reason to believe that the stability changes are directly connected with the catalytic function of the enzyme.

Having evaluated the unfolding free energy changes for α-Ct and F-Ct at pH 7 (reactions ii and iv of Scheme I), only the free energy change associated with the transesterification reaction E + S $\rightleftharpoons$ EA + P_1 remains to be determined. The data described in Figures 8–10 and summarized in Tables II and III were designed to evaluate the equilibrium constant for the reaction α-Ct + TFEF $\rightleftharpoons$ F-Ct + TFE in 0.2 M ionic strength at pH 7, the same conditions used in evaluating unfolding free energy changes for α-Ct and F-Ct. Inward and Jencks (1965) determined the equilibrium constant for

Scheme III



Scheme IV



this reaction at pH 8.0 and 1 M ionic strength, and we applied their techniques in the evaluation of the free energy change under our conditions. The only difference in method is that, in contrast to Inward and Jencks, we have taken into account the finite amount of the Michaelis complex, α-Ct·TFEF, present under steady-state conditions. Accounting for the α-Ct·TFEF concentration results in approximately a 40% increase in the equilibrium constant for the overall reaction, α-Ct + TFEF $\rightleftharpoons$ F-Ct + TFE.

It is evident from the reaction α-Ct + TFEF $\rightleftharpoons$ F-Ct + TFE that noncovalent aspects of the equilibrium will be tied up in the enzyme species. In attempting to look at how noncovalent free energy changes with individual steps during an enzyme-catalyzed reaction, it is important to establish the connection between noncovalent interactions involving enzyme intermediates and the structural integrity of that intermediate. By way of example, consider the case of noncovalent binding of substrate to enzyme. It is seen from Scheme III that the free energy of substrate binding [$\Delta G°_{(b)}$] can be represented as the difference in the unfolding free energy of E compared to that of ES [$\Delta G°_{N-U(a)} - \Delta G°_{N-U(c)}$]. That is, ES is thermodynamically more stable than E + S exactly by the amount associated with the free energy change for substrate binding. This means that the noncovalent free energy changes associated with the interaction of substrate and enzyme are vested in the thermodynamic stability of the enzyme species involved and that the relative thermodynamic stability of a species is an important indicator of how the free energy of functionally important noncovalent interactions change upon conversion of one enzyme species to another.

The overall reaction, α-Ct + TFEF $\rightleftharpoons$ F-Ct + TFE, differs from the case of simple binding in that a covalent linkage is made between enzyme and part of the substrate. This leads to Scheme IV, a reproduction of Scheme I with results of unfolding free energy measurements [$\Delta G°$(ii) and $\Delta G°$(iv)] along with $\Delta G°$(i) obtained in this work, to yield the free energy change in kcal/mol for transesterification, $\Delta G°$(iii). The transesterification reaction given by $\Delta G°$(iii) represents a hypothetical transesterification reaction occurring without the conformational integrity of the protein and, thus, without the noncovalent interactions that give enzymes their special properties.

As stated above, in Michaelis complex formation the reaction is driven entirely by noncovalent interactions, and the favorable free energy change for binding is quantitatively reflected in the increased thermodynamic stabilization of ES relative to E. We have determined that the overall reaction of α-Ct + TFEF $\rightleftharpoons$ F-Ct + TFE (represented as E + S $\rightleftharpoons$ EA + P_1) is also quite favorable, but, in contrast to simple substrate

binding, the thermodynamic stability of acyl–enzyme (EA) has been found to be less than that of E by 1.6 kcal/mol. That is, if noncovalent free energy changes were the only changes taking place, the reaction E + S ⇌ EA + P_1 would be unfavorable. This indicates that the driving force for the overall reaction is forming EA cannot be due to noncovalent processes. Rather, it is the covalent bond-making and bond-breaking process that provides the favorable free energy change which drives the unfavorable noncovalent free energy changes resulting in the observed –4.3 kcal/mol free energy change for the overall reaction.

While we have drawn the conclusion that noncovalent interactions cannot be the driving force for the overall reaction E + S ⇌ EA + P_1, we do not wish to imply that such interactions do not play a role; in fact, noncovalent interactions play a substantial role in the mechanism of EA formation. This becomes evident when the overall reaction E + S ⇌ EA + P_1 is decomposed into its two elementary events, E + S ⇌ ES followed by ES ⇌ EA + P_1. In the first event, involving Michaelis complex formation, TFEF binds to α-Ct with a significant binding free energy of –3.64 kcal/mol (see Table III). The second event, involving covalent (transesterification) and noncovalent contributions, is also shown to be slightly favorable with a measured free energy change of –0.67 kcal/mol (see Table III). As previously discussed, the free energy change for Michaelis complex formation means that ES is thermodynamically more stable than E by 3.6 kcal/mol. Since E is more stable than EA by 1.6 kcal/mol, ES must be more stable than EA by 5.2 kcal/mol. Thus, in the conversion of ES to EA + P_1, which is slightly thermodynamically favorable, a significant reduction in the thermodynamic stability of the enzyme occurs. When the transesterification event involving ES conversion to EA + P_1 takes place, it does so at the expense of more than 5 kcal of noncovalent free energy in the conformational integrity of the ES species, leaving an enzyme species (EA) that is, by comparison, significantly destabilized. Such a reduction in stabilization free energy would have important functional significance if it was used to drive an unfavorable process such as a stress or strain in EA. Catalytic advantage would be gained if the stress or strain would be relieved on reaching the next activated complex. That is, the occurrence of destabilization in the conformational integrity of the EA species relative to ES gives rise to the possibility that energy transduction takes place in the transesterification event, with the endergonic noncovalent free energy part process in forming EA being driven by exergonic covalent bond-breaking and bond-making.

The concept of energy transduction in which a source of favorable free energy is utilized by the enzyme to drive an unfavorable process is common to a number of the major theories of enzyme catalysis which deal with the character of the observed intermediate species in enzyme catalysis. Notable among the theories are the Rack mechanism and the Circe effect in which part of the substrate binding free energy is believed to be used to induce strain or other destabilizing forces that become relieved on reaching the subsequent activated complex (Eyring et al., 1954; Jencks, 1975; Lumry, 1959, 1991). The results of the present experiments provides thermodynamic support for the notion of energy transduction in catalytic events but with the difference in this particular case that covalent bond free energy rather than noncovalent free energy serves as the driving force.

By completing the thermodynamic cycle given above, a free energy change of –5.86 ± 0.75 kcal/mol is calculated for the reaction U + S ⇌ U–A + P_1. The rather large error in this calculated free energy change given in Table III was propagated from the errors of the three contributing branches in the thermodynamic cycle. Since this reaction represents a transesterification reaction in aqueous solution, it is useful to compare the free energy change with that of small molecule model reactions. The closest model reaction we have found is the transesterification of trifluoroethyl acetate with ethanol to give trifluoroethanol plus ethyl acetate. The free energy change for this reaction at pH 7 is –3.3 kcal/mol as estimated from free energy changes for hydrolyses of ethyl acetate and trifluoroethyl acetate (Jencks & Gilchrist, 1964). Within the range of error (±0.75 kcal/mol) we report, the reaction U + S ⇌ U–A + P_1 could be anywhere from 20- to 260-fold more favorable than the small-molecule model transesterification reaction involving trifluoroethyl acetate/ethanol. Consideration of the probable error in the free energy change of the model reaction further increases the uncertainty in the magnitude of the differences between the model reaction and U + S ⇌ U–A + P_1.

The model reaction uses acetyl rather than furoyl esters and ethanol rather than serine hydroxyl. It is not known how the free energy change for transesterification could differ using furoyl instead of acetyl, but some effect is likely. It should also be pointed out that the equilibrium constant for the reaction U + S ⇌ U–A + P_1 in which serine 195 is acylated cannot be measured by direct experimentation since there are many potential acylation sites on unfolded chymotrypsin, including 28 serine side chains.

A Composite Free Energy Diagram. A summary of all of the results of the enzyme mediated hydrolysis and protein unfolding is presented in Figure 11. We have merged the acylation phase of a reaction coordinate free energy diagram for α-chymotrypsin-catalyzed trifluoroethyl furoate hydrolysis with the unfolding free energy changes for α-chymotrypsin and furoyl–chymotrypsin using pH 7 standard state free energy changes. The unfolded state of α-chymotrypsin plus substrate (U + S) provides a useful reference state in relationship to the free energy of the enzyme and small molecule species along the reaction coordinate. The superposition of the free energy diagrams for protein unfolding and enzyme catalysis provides a way of considering the relative free energies of catalytic events in relationship to the thermodynamic stabilities of the enzyme species involved in catalysis.

Conformational energetics in the course of catalysis is a subject of great mystery, largely elusive and generally refractory to investigation. This attempt at obtaining some insight into the subject is, in some ways, crude and limited. Our assumption in this work is that the basic mechanisms of catalysis and basic principles are retained by the enzyme even with the nonspecific substrates to which we are limited. Our interest is in attempting to discover what is possible in terms of noncovalent and covalent free energy changes and how these changes may be involved in the management of energy by the enzyme.

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