development step, and retention of volatiles of interest may provide a solution.

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Ann C. Noble*[1]
Aki A. Murakami[2]
George F. Coope III[2]

[1]Department of Viticulture and Enology
University of California
Davis, California 95616
[2]Miller Brewing Company
Milwaukee, Wisconsin

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Thermodynamic Measurements on the Interaction of Porcine Trypsin with Single- and Two-Chain Trypsin Inhibitors from Corn Seeds

The reactions of the single- and two-chain forms of corn trypsin inhibitor with porcine trypsin in a cacodylate buffer at pH 6.5, 20 °C, both occur with 1:1 stoichiometry, have association equilibrium constants on the order of 10^7 M^{-1}, and indistinguishable enthalpy changes lying near 3.6 kcal/mol. The reactions are therefore driven by positive entropy changes. It is suggested that conversion of this inhibitor from its single- to two-chain form involves more than a simple hydrolysis of a peptide bond.

Complex formation between trypsin and proteinaceous trypsin inhibitors is of considerable interest because of the specificity and strength of these interactions. In the substantial body of investigation on trypsin inhibitors relatively little attention has been given to thermodynamics, even though a thorough grasp of the energetics will be essential to fully understand the nature of the binding reactions. Calorimetric studies have been reported on the interactions of bovine trypsin with the Kunitz and Bowman-Birk inhibitors isolated from soybeans (Turner et al., 1975; Barnhill and Trowbridge, 1975). Here we report the results from a study of the association reactions of porcine trypsin with the single- and two-chain forms of corn trypsin inhibitor, a protein that is quite different structurally from both of the soybean inhibitors (Swartz et al., 1977).

EXPERIMENTAL PROCEDURES

Single- and two-chain forms of trypsin inhibitor were purified from seeds of *opaque-2* corn by salt extraction of ground seeds, affinity chromatography on trypsin-Sepharose, and ion-exchange chromatography on DEAE-cellulose. Inhibitor concentrations were determined using $E_{280nm}^{1\%} = 20$ (Swartz et al., 1977).

Equilibrium constants for the reactions of trypsin inhibitors with porcine trypsin (Sigma; Trypsin Type IX) were estimated as described by Bieth (1974), using *p*-nitrophenyl *p'*-guanidinobenzoate (Chase and Shaw, 1967) to measure concentrations of unbound trypsin. In a spectrophotometer cell, we combined 25 μL of trypsin solution (ca. 5–8 mg/mL in 1 mM HCl) with various amounts of inhibitor solution (ca. 1 mg/mL) that had been dialyzed against 0.001 M $CaCl_2$/0.25 M NaCl/0.05 M sodium cacodylate buffer (pH 6.5). The volume of inhibitor solution added ranged from 25 to 350 μL. Buffer was then added to obtain a precisely known final volume (always ca. 1 mL). The cell was placed in a 20 °C water bath for 10 min. *p*-Nitrophenyl *p'*-guanidinobenzoate (10 μL of a 0.01 M solution in dimethylformamide) was then added quickly, and after the contents of the cell were mixed, 50 μL of 1 M Tris base was added to bring the pH to 8.3. The absorbance at 410 nm was monitored immediately by use of a Beckman Model 25 spectrophotometer. From the initial burst in absorbance we subtracted the small absorbance observed when the enzyme was omitted. In each equilibrium constant determination, the concentration of free trypsin was determined in duplicate or triplicate at each of five to seven inhibitor concentrations. A total of six such experiments was performed with the single-chain inhibitor and one with the two-chain inhibitor.

Plots of inhibitor concentration against $(1 - a)$, where a is the proportion of enzyme that is not bound to inhibitor, indicated that under our conditions the reactions of the inhibitors and trypsin fit case "b" according to Bieth (1974). We therefore obtained the dissociation constant associated with each experiment by plotting $I^o/(1 - a)$ against $1/a$, where I^o is the total inhibitor concentration in each reaction mixture. The slope of each least-squares line gave a value for the appropriate dissociation constant (Bieth, 1974). A value of 12 500 was used as the molecular weight of both the single- and the two-chain forms of the inhibitor (Swartz et al., 1977).

In our calorimetric measurements we used a batch microcalorimeter similar to that described by Kitzinger and Benzinger (1963). In a typical experiment the two bicompartmented microcalorimeter vessels were loaded as follows: the reaction vessel had 5 mL of a buffer solution containing trypsin (ca. 5 mg/mL) in one compartment and 5 mL of the same buffer solution containing trypsin inhibitor (ca. 1 mg/mL) in the other compartment; the blank vessel had 5 mL of buffer solution in each of the two

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compartments. We determined heat changes associated with dilution of trypsin and trypsin inhibitors with analogous experimental set ups but substituted, as appropriate, 5 mL of buffer for one of the protein solutions. Four experiments were performed with trypsin and the single-chain inhibitor in cacodylate buffer, four with these proteins in PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer, and five with trypsin and the two-chain trypsin inhibitor in cacodylate buffer. Cacodylate and PIPES buffers were chosen because their rather different heats of ionization should allow evaluation of proton loss or gain by protein(s) during the reactions. Both buffer solutions were at pH 6.5 at 20 °C and included 0.001 M $CaCl_2$ and 0.25 M NaCl in addition to the buffer (0.05 M). All protein samples were dialyzed for at least 16 h at 0 °C against the appropriate buffer solution. Solutions outside the bags after dialysis were used in blank and sample vessels as indicated above.

To interpret the experimental data, the observed heat changes were first corrected for the appropriate heats of dilution. Then the method of least squares was used to calculate enthalpy changes for reactions of the following type:

$$\text{trypsin} + \text{inhibitor} + n \text{ buffer base} \rightleftarrows \underset{\text{reaction product}}{\text{trypsin–inhibitor} +} \quad \underset{\text{conjugate acid}}{n \text{ buffer}}$$

By knowing the enthalpy changes for such reactions and the heats of ionization of the buffers employed, the number of protons gained or lost by the buffer could be calculated and in turn corrections made for the contributions these exchanges may have made toward the originally calculated enthalpy changes. These subsequent calculations, however, were based on the assumption that the enthalpy changes for the loss or gain of protons from the protein were independent of the nature of the buffer.

We determined the heat changes associated with addition of protons to the buffer systems using an experimental design that was analogous to that outlined above except that prior to loading the reaction vessels the pHs of the buffers were raised to about 7. The other compartment of each reaction vessel was then loaded with a solution of HCl calculated to give a final pH of about 6 after reacting with the buffer. This approach essentially eliminated heat contributions due to protonations that could occur at pHs far removed from 6.5. The observed heat changes were corrected for dilution effects and used to calculate enthalpy changes for the ionization of cacodylic acid and PIPES under the conditions of our studies.

Finally, we chose to study porcine trypsin rather than the more commonly used bovine trypsin because of the much greater stability to autolysis of the porcine enzyme (Vithayathil et al., 1961). We have found that at pH 6.5 in the presence of 0.001 M $CaCl_2$, a 10 mg/mL solution of porcine trypsin loses only 10–15% of its activity in 18 h at room temperature. Thus there should have been little change in the concentration of active trypsin during dialysis at 4 °C and the temperature equilibration period that preceded a calorimetric measurement. Furthermore, the heat produced by autolysis during a measurement should have been negligible.

RESULTS AND DISCUSSION

In the plot suggested by Bieth (1974) the intercept on the ordinate gives the total enzyme concentration if a 1:1 complex is formed. The total enzyme concentration can also be determined directly with the active site titrant in the absence of inhibitor. Thus the ratio of these two values should be unity if a 1:1 complex is formed. The mean ratio and standard deviation from six duplicate determinations involving the single-chain inhibitor were found to be 1.1 ± 0.3 to 1. The ratio found for the single experiment involving the two-chain inhibitor was found to be 0.8 to 1. From these results we conclude that both forms of the corn inhibitor combined with porcine trypsin with a 1:1 stoichiometry.

Table I. Association Constants for Reactions between Trypsin (T) and Corn Trypsin Inhibitor (CTI)

reaction[a]	no. of experiments	K_{assoc} (mean)	SD of the mean
T + single-chain CTI	6	7×10^6 M^{-1}	2×10^6 M^{-1}
T + two-chain CTI	1	7×10^6 M^{-1}	

[a] Reactions carried out in a 0.001 M $CaCl_2$/0.25 M NaCl/0.05 M cacodylate buffer pH 6.5, at 20 °C.

Table II. Enthalpy Changes for the Association of Trypsin (T) and Corn Trypsin Inhibitor (CTI)

reactions and conditions[a]	no. of observations	enthalpy change,[b] kcal/mol of CTI	SD[b]
T + single-chain CTI in cacodylate buffer	4	3.6	1.2
T + single-chain CTI in PIPES buffer	4	3.1	0.5
T + two-chain CTI in cacodylate buffer	5	3.5	0.6

[a] Both solvents were 0.001 M $CaCl_2$/0.25 M NaCl/0.05 M buffer (pH 6.5). The temperature was 20 °C. [b] The enthalpy changes were calculated from slopes of lines having the form $y = bx$. The standared deviations were calculated from the standard deviations of these slopes. These values have been corrected for heats of dilution, but not for possible proton exchange.

As anticipated the corn trypsin inhibitors bound strongly to porcine trypsin. Accordingly, in order for a significant portion of the inhibitor to remain free (not complexed with trypsin), we had to conduct our experiments for estimating the association constants at rather low protein concentrations. This resulted in quite small changes in the absorbances at 410 nm (ca. 0.08 in the absence of inhibitor, corrected for an increase of ca. 0.03 observed in the absence of trypsin). Thus our equilibrium constants should be regarded as estimates that are not significant beyond one figure. Table I lists the association constants we obtained from plots of $I^o/(1 - a)$ vs. $1/a$. Considering these, the error associated with the value for the single-chain inhibitor and the fact that only one determination was made of the constant for the two-chain inhibitor, we conclude that both forms of the inhibitor have association constants of ca. 10^7 M^{-1}.

Table II lists the enthalpy changes obtained from our calorimetric data. From these it can be seen that the association reactions between trypsin and the two forms of corn trypsin inhibitor are endothermic under the conditions we used. Considering the results and errors associated with the single- and two-chain inhibitors in cacodylate buffer we conclude that the small difference between 3.6 and 3.5 kcal/mol is not significant. If the original data used to obtain these two values are pooled,

a value of 3.6 ± 0.7 kcal/mol is obtained. Considering the results involving the single-chain inhibitor in cacodylate and PIPES buffers and that cacodylic acid and PIPES have rather different values for $\Delta H_{ionization}$ (0.3 and −3 kcal/mol, respectively, under our conditions), we further conclude that essentially no protons were taken up by the buffer when trypsin reacted with this form of the inhibitor.

The most recent and thorough calorimetric investigations of the reactions of trypsin inhibitors with trypsin were with the Bowman–Birk inhibitor (Turner et al., 1975) and the Kunitz inhibitor (Barnhill and Trowbridge, 1975) from soybeans. Corn trypsin inhibitor is quite different structurally from both of these proteins. The Bowman–Birk inhibitor has a molecular weight of 7000 and inhibits chymotrypsin as well as trypsin. The corn inhibitor has a molecular weight of 12 500 and does not inhibit chymotrypsin (Swartz et al., 1977). The Kunitz inhibitor, on the other hand, is much larger than the corn trypsin inhibitor, and these two proteins are not immunologically cross reactive (R. S. Corfman and G. R. Reeck, unpublished results, 1977). Furthermore, the corn inhibitor is distinct in having four tryptophan residues (Swartz et al., 1977). A comparison of our results with those reported in the earlier studies is therefore of interest.

Our results agree qualitatively with both those of Turner et al. (1975) and Barnhill and Trowbridge (1975) whose data indicate that at pH 6.5 the association of each soybean inhibitor with trypsin should be favorable thermodynamically with unfavorable enthalpy changes being overcome by favorable entropy changes. Our data lead to the same conclusions for corn trypsin inhibitor. A closer look at certain aspects of the assembled data, however, suggests a difference that is considered worthy of further investigation.

The data for the association of soybean trypsin inhibitor with trypsin which have been provided by Turner et al. do not include the pH we used but those provided by Barnhill and Trowbridge do. Accordingly one can interpolate the latter data to estimate enthalpy changes that would be expected to occur at our pH. When this is done it appears our enthalpy changes are significantly larger than those for the Kunitz inhibitor. Further, this approach suggests that the enthalpy changes for the association reactions of bovine β-trypsin with Kunitz soybean inhibitor and the inhibitor in which the Arg-63-Ile bond has been cleaved should be about 0 and 2 kcal/mol, respectively, at pH 6.5. If this is true the enthalpy change for the conversion of virgin to cleaved Kunitz inhibitors should be about −2 kcal/mol under these conditions. This is in good agreement with calorimetric data on peptide bond hydrolysis taken in Sturtevant's laboratory (Rawitscher et al., 1961) which suggest that ΔH for the hydrolytic reaction involved in converting a single- to a two-chain inhibitor should lie in the range of −1 to −2 kcal/mol. In contrast, our enthalpy data indicate that the conversion of single- to two-chain corn trypsin inhibitor is near zero and suggest that this conversion involves processes other than a simple hydrolysis of a peptide bond. For instance there might be a conformational change and/or changes in solvent interactions.

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R. K. Burkhard*
Shane Adams
Randle S. Corfman
Gerald R. Reeck

Department of Biochemistry
Kansas State University
Manhattan, Kansas 66506

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Thin-Layer Chromatographic Analysis of Nitrofurans in Feed Premixes

A simple method for the assay of furazolidone and furaltadone, substances frequently associated for the prevention of coccidiosis in veterinary medicine, is described. A chromatographic separation technique was carried out before spectrophotometric assay for isolating these nitrofurans from complex pharmaceutical preparations such as feed premixes. The study of the different factors likely to influence recovery and assay of furazolidone and furaltadone from such products allowed an accurate and reproducible quantitative analysis.

Some substances resulting from a reaction of the 5-nitro-2-furaldehyde with different hydrazine groups are frequently used in veterinary medicine for their antimicrobial and anticoccidial activities. These nitro drugs are often blended with other antimicrobial drugs and growth factors in cereal meals; the so obtained premixes are generally in the form of more or less homogeneous powder. Mixed with the normal feed of the animals, generally at low levels (50–200 ppm), these drugs are used in both poultry and swine husbandry for the prevention of enteritis, coccidiosis, and salmonellosis.

Several methods have been reported for the analysis of these compounds. Most of them involved spectrophotometric assays after conversion of the nitrofurans to their

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