The Trypsin-catalyzed Hydrolysis of Nα-Benzoyloxy carbonyl-L-Lysine p-Nitrophenyl Ester in Dimethylsulfoxide at Sub-Zero Temperatures*

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SUMMARY

The effect of sub-zero temperatures and aqueous dimethyl sulfoxide solutions on the trypsin-catalyzed hydrolysis of Nα-benzoyloxy carbonyl-L-lysine p-nitrophenyl ester has been investigated. With increasing dimethyl sulfoxide concentration at 0°, kcat decreases in proportion to the decreased water concentration; however, Kn increases by 2 orders of magnitude. The effect on Kn can be accounted for by a combination of dielectric and competitive inhibition effects. The Arrhenius plot for the decacylation reaction in 05% aqueous dimethyl sulfoxide is linear over the range 0 to -45° and extrapolates to a value of kcat at 25° in excellent agreement with that obtained in the absence of the organic solvent. At -45° and below, turnover occurred very slowly, but acylation was quite rapid. The effect of dimethyl sulfoxide concentration and sub-zero temperatures on the ultraviolet spectral properties of the enzyme showed no evidence of any structural changes. All of the experimental observations were consistent with the conclusion that 65% aqueous dimethyl sulfoxide and sub-zero temperatures have no significant effect on the pathway of trypsin-catalyzed reactions. Preliminary results using Nα-acetyl-L-lysine methyl ester indicated that detectable perturbations in the environment of tyrosine and tryptophan residues in trypsin occur during catalysis.

We are conducting a detailed investigation into the use of sub-zero temperatures as a means of studying the dynamic processes occurring during enzyme catalysis. Although rapid reaction techniques such as stopped flow and temperature jump spectrometry are valuable tools for obtaining kinetic information, they are not well suited for studying the nature of intermediates in the reaction. Furthermore, much of our current knowledge of enzyme mechanisms has been obtained through the use of kinetically nonspecific substrates and substrate analogs in which one cannot be certain that the slower rates are not due to a different reaction pathway. Consequently, a procedure using specific substrates which will allow the detection and study of intermediates on the productive catalytic pathway would be very valuable.

Our low temperature method is based on the following premise. Since a drop of 130° will decrease the rate of a typical step in an enzyme-catalyzed reaction by about 1010 to 10-fold, the rates of individual steps in the overall enzyme-catalyzed reaction will be decreased to such an extent that for some the reaction will be negligible at such low temperatures. If the enthalpies of activation or rate constants (or both) for the several steps in the reaction differ, and are such that at very low temperatures the faster reactions precede the slower ones, it should be possible to accumulate each intermediate successively. Thus, by initiating the reaction at a very low temperature and then raising the temperature by appropriate increments, it should be possible to obtain kinetic, thermodynamic, and most importantly, physicochemical structural information. In order to preclude problems of rate-limiting enzyme-substrate diffusion we are using aqueous-organic solvent systems which are fluid to temperatures in the vicinity of -100°.

For the low temperature technique to be useful it is necessary to show that the enzyme-catalyzed reactions in aqueous-organic solvent systems at sub-zero temperatures involve the same catalytic reaction pathway as occurs under normal conditions. Thus by initially investigating enzymes which have already been studied extensively, there should be ample data available for comparison with those obtained at the low temperatures. Trypsin and chymotrypsin are such enzymes, and the advantage of trypsin is that the intrinsic fluorescence and ultraviolet absorption of the enzyme may be monitored in the presence of specific substrates, since such ultraviolet-transparent substrates of trypsin are available.

Several studies have been reported concerning the effect of organic solvents, usually at concentrations no higher than 25%, on the kinetic parameters of reactions catalyzed by trypsin and chymotrypsin. The general effect observed is an increase in Kn and little or no change in kcat (1-5). Few studies have been performed at high concentrations of organic solvents. Michaelis-Menten kinetics was observed at up to 80% dioxane in the trypsin-catalyzed hydrolysis of Nα-benzoyl-l-arginine ethyl ester (5). Little effect on kcat was observed at 60% dioxane, whereas Kn increased substantially. The same study showed that storing trypsin in 97% dimethyl sulfoxide at 12.5° for 14 hours had little irreversible effect on the activity, and that in 70% aqueous...
dimethylsulfoxide, the initial rates of hydrolysis were relatively unaffected. Rambler (7) observed an increase in relative rate in the presence of up to 30% dimethylsulfoxide in the trypsin-catalyzed hydrolysis of N\textsuperscript{α}-tosyl-L-arginine methyl ester. We have recently shown that 65% aqueous dimethylsulfoxide has no significant adverse effects on the catalytic and structural properties of \(\alpha\)-chymotrypsin (8). Optical rotary studies on trypsin in 100% dimethylsulfoxide indicate that the conformation is essentially the same as in water (9).

Thus, on the basis of the similar structure and catalytic mechanism of trypsin and \(\alpha\)-chymotrypsin (10) and the previously reported effects of organic solvents on trypsin, it appeared likely that 65% aqueous dimethylsulfoxide would be a suitable solvent system.

Very few studies of enzyme-catalyzed reactions at sub-zero temperatures have been reported. Bielski and Freed (11) observed catalytic activity with \(\alpha\)-chymotrypsin in aqueous methanol solutions at temperatures as low as \(-33^\circ\). Denaturation was observed above \(-10^\circ\). Temperatures to \(-65^\circ\) and 70% aqueous \(N, N\)-dimethylformamide have been used by Dousou et al. (12, 13) in studying peroxidase.

Many of the effects of organic solvents on the structure of proteins are due at least in part to the decreased dielectric strength. Sub-zero temperatures would be expected to minimize such effects since the dielectric constant increases with decreasing temperature (14), and since the high enthalpy of activation for protein denaturation will tend to favor the native conformation.

In this initial report we present evidence that 65% aqueous dimethylsulfoxide has little adverse effect on the structure and catalytic properties of trypsin and is suitable for low temperature studies on the mechanism of trypsin-catalyzed reactions.

**EXPERIMENTAL PROCEDURE**

**Materials**—\(N^\alpha\)-Benzyloxycarbonyl-L-lysine \(p\)-nitrophenyl ester was obtained from Cyclo (Lot D-1308) and recrystallized from ethanol-acetonitrile, m.p. 150-151\(^\circ\). \(N^\alpha\)-Acetyl-L-lysine methyl ester was from Cyclo (Lot 10806) and used without further purification. Dimethylsulfoxide, reagent grade from Mallinckrodt, was distilled from calcium hydride under vacuum at 37\(^\circ\) and stored at or below 0\(^\circ\). Trypsin, twice crystallized (Lot TRILDA, Worthington), was used without further purification. Activity was determined using burst or kinetic assays with \(N^\alpha\)-benzyloxycarbonyl-L-lysine \(p\)-nitrophenyl ester (15). Aqueous dimethylsulfoxide buffers (v/v) (acetate), and enzyme and substrate stock solutions were prepared as described previously (8).

**Methods**—Kinetic and ultraviolet absorption measurements were performed on a Cary model 14 or 118C spectrophotometer. Fluorescence spectra were obtained on an Hitachi MPF 2A spectrophotometer modified to use a Varian low temperature probe (8). Unless otherwise specified excitation was at 290 nm. The spectrophotometric experiments at sub-zero temperatures were carried out in a specially constructed double-walled quartz cell, in which temperature control (+0.3\(^\circ\)) was achieved by circulating a temperature-controlled bath. Enzyme and substrate solutions were mixed at -78\(^\circ\) using ethanol-acetonitrile, m.p. 150-151\(^\circ\). Na-Acetyl-L-lysine methyl ester was stored at or below 0\(^\circ\). Trypsin, twice crystallized (Lot TRLBDA, Worthington), was used without further purification. Activity was determined using burst or kinetic assays with \(N^\alpha\)-benzyloxycarbonyl-L-lysine \(p\)-nitrophenyl ester (15).

**RESULTS**

**Effect of Dimethylsulfoxide on Kinetics of Hydrolysis of \(N^\alpha\)-Benzyloxycarbonyl-L-lysine \(p\)-Nitrophenyl Ester**—Trypsin showed no loss in activity toward \(N^\alpha\)-benzyloxycarbonyl-L-lysine \(p\)-nitrophenyl ester when kept at 0\(^\circ\) in 65% aqueous dimethylsulfoxide at pH 4.0 and 0.5 over a 1-hour period. At 25\(^\circ\) and pH > 6.5 precipitation of trypsin occurred in the concentration range used. No precipitation occurred at pH 4.0 and 25\(^\circ\) or at pH 6.5 and 0\(^\circ\). The effect of the concentration of dimethylsulfoxide on the kinetics of the reaction at 0\(^\circ\) is shown in Table I. A large increase in \(K_m\) occurred with increasing concentration of dimethylsulfoxide, whereas the values for \(k_{cat}\) were reasonably close to those expected, based on the decrease in concentration of water.

The effect on \(K_m\) is similar to that observed in the case of \(\alpha\)-chymotrypsin (8) which we have attributed to a combination of dielectric and competitive inhibitor effects (19). The data for \(K_m\) have therefore been plotted (Fig. 1) in accordance with
Equation 1 (3, 4, 19)

\[
\log \left( \frac{K_m^{\text{dim}}}{K_m^{0}} \right) = e^{AX}(1 + [\text{DMSO}]/K_D)
\]

in which \( K_m^{\text{dim}} = K_m \) for 0% dimethylsulfoxide, \( A \) is a constant, \( X \) is the difference in reciprocals of the dielectric strength with and without dimethylsulfoxide, \([\text{DMSO}]\) is dimethylsulfoxide concentration, and \( K_D \) is the dissociation constant for the binding of dimethylsulfoxide to the enzyme. A good fit of the data is obtained, as shown by the solid line in Fig. 1, which is based on values of \( A = 0.78 \) and \( K_D = 1.0 \) M in Equation 1.

Effect of Dimethylsulfoxide on Ultraviolet and Fluorescence Spectra of Trypsin—To investigate whether the high concentrations of dimethylsulfoxide had any adverse effects on the structure of the enzyme, the ultraviolet difference and fluorescence emission spectra were monitored as a function of dimethylsulfoxide concentration. Solvent effects on the exposed residues would be expected to result in linear or smooth monotonic changes with increasing organic solvent concentration, as observed (Figs. 2 and 3), whereas structural effects would be expected to result in distinct deviations in such plots (20). The \( \lambda_{\text{max}} \) for fluorescence emission remained constant at 334 nm.

Effect of Temperature—Fig. 4 shows the changes observed in the fluorescence emission and ultraviolet absorption spectra of trypsin in 65% aqueous dimethylsulfoxide at sub-zero temperatures.

The effect of temperature on the rate of deacylation in the trypsin-catalyzed hydrolysis of \( N^\alpha\)-benzoyloxycarbonyl-L-lysine p-nitrophenyl ester at pH 4.7 is shown in Fig. 5. From the slope of the Arrhenius plot a value of \( E_a = 17.0 \pm 2.5 \) Cal per mole was obtained. The plot extrapolated to a value of 0.15 s\(^{-1} \) at +25°. This is in excellent agreement with the value of 0.2 s\(^{-1} \) calculated on the basis of the rate in the absence of dimethylsulfoxide (21).

The effect of temperature on \( k_{\text{cat}} \) and \( K_m \) at pH 6.4 is also shown in Fig. 6. The Arrhenius plots yield values of 13 ± 3 and 13.0 ± 2.0 Cal per mole for \( K_m \) and \( k_{\text{cat}} \), respectively. The value for \( k_{\text{cat}} \) (12 s\(^{-1} \)) extrapolated to +25° and corrected for decreased water concentration is in reasonable agreement with that obtained in the absence of dimethylsulfoxide (21 s\(^{-1} \)). The extrapolated value of \( K_m \) at 25° is 1.5 × 10\(^3 \) that obtained in the absence of dimethylsulfoxide.

Preliminary Low Temperature Experiments with \( N^\alpha\)-Acetyl-L-lysine Methyl Ester—Trypsin and \( N^\alpha\)-acetyl-L-lysine methyl ester were mixed together at -60° in 65% aqueous dimethylsulfoxide, pH 4.6, and the reaction was monitored at 284 nm. A rapid initial increase in absorbance occurred (Reaction 1),

Fig. 2. The effect of dimethylsulfoxide concentration on the ultraviolet difference spectrum of trypsin. \( A \), difference spectra for 65% (——) and 15% (—) dimethylsulfoxide. \( B \), \( \Delta A_{287} \) (Δ) and \( \Delta A_{340} \) (○) as a function of dimethylsulfoxide concentration. Conditions: pH 6.5, \( \mu = 0.1 \) M, 0°. Trypsin concentration = 2 × 10\(^{-4} \) M.

Fig. 3. The effect of dimethylsulfoxide on the fluorescence emission of trypsin. Conditions: pH 6.5, \( \mu = 0.1 \) M, 0°. Trypsin concentration = 5 × 10\(^{-4} \) M. Excitation at 290 nm, emission at 334 nm.

Fig. 4. The effect of temperature on the fluorescence emission and ultraviolet absorption spectra of trypsin. Conditions: pH 6.5, \( \mu = 0.1 \) M, 65% dimethylsulfoxide.

Fig. 5. Arrhenius plots for \( k_{\text{cat}} \) and \( K_m \) for the trypsin-catalyzed hydrolysis of \( N^\alpha\)-benzoyloxycarbonyl-L-lysine p-nitrophenyl ester. Conditions: 65% dimethylsulfoxide, \( \mu = 0.1 \) M, \( A \), \( k_{\text{cat}} \) at pH 6.4 (Δ); \( B \), \( k_{\text{cat}} \) at pH 4.7 (○); \( C \), \( K_m \) at pH 6.4 (×).
methylsulfoxide a-chymotrypsin was found to undergo fairly rapid denaturation at 25° but to be quite stable at temperatures below 10° (8). Trypsin seems to behave similarly. At 25° we changes in the structural or catalytic properties (or both) of the enzyme. The catalytic parameters in particular should be very effects were caused by such conditions they should appear as Fig. 6. A, derivative spectrum for trypsin + Nα-acetyl-L-lysine methyl ester (---). B, derivative spectrum for trypsin (-- --). Conditions: pH 4.6, 65% dimethyl sulfoxide, μ = 0.1 M, -50°. Trypsin concentration = 7.5 × 10⁻⁴ M. Substrate concentration = 1.5 × 10⁻⁴ M. C, difference spectrum for trypsin + Nα-acetyl-L-lysine methyl ester at -25° (-----). D, difference spectrum after 2 min at -60° (— —). Other conditions same as A and B. which was over within 1 min, followed by a much slower decrease in absorbance (Reaction 2) (kobs = 10⁻³ s⁻¹). Comparison of the spectrum of the enzyme alone under the same conditions with that of the product of Reaction 1 showed a positive maximum at 285 nm, and a negative peak below 280 nm (Fig. 6D). A difference spectrum of the product of Reaction 2 versus trypsin showed a broad positive maximum around 300 nm, a sharp minimum at 290 nm, and a broad minimum at 280 to 284 nm (Fig. 6C). The derivative spectra for trypsin and trypsin + Nα-acetyl-L-lysine methyl ester were obtained at -50° (Fig. 6, A and B). Comparison of the derivative spectrum of the enzyme alone with that in the presence of substrate under otherwise identical conditions provides a very sensitive means of detecting perturbations in the environment of aromatic residues (18). In this case the absorbance spectra appeared identical, but changes caused by the substrate are readily apparent in the derivative spectra. These differences showed maxima at 297 and 275 nm and a minimum at 283 nm. Control experiments in which the substrate was omitted showed that no changes in the ultraviolet spectrum of trypsin in 65% aqueous dimethylsulfoxide occurred over a 1-hour period at -75°, -45°, and -20°.

**DISCUSSION**

The experiments reported here have been undertaken in order to demonstrate that the productive catalytic pathway for trypsin-catalyzed reactions is not adversely affected by 65% aqueous dimethylsulfoxide and sub-zero temperatures. If undesirable effects were caused by such conditions they should appear as changes in the structural or catalytic properties (or both) of the enzyme. The catalytic parameters in particular should be very sensitive to any adverse effects.

**Effect of Dimethylsulfoxide on Catalysis**—In 65% aqueous dimethylsulfoxide α-chymotrypsin was found to undergo fairly rapid denaturation at 25° but to be quite stable at temperatures below 10° (8). Trypsin seems to behave similarly. At 25° we found precipitation to occur at pH > 5 in 65% aqueous dimethylsulfoxide (as observed previously (9)). Presumably the decreased solubility results from a solvent-induced conformational change. At lower pH, or at temperatures below 8° no precipitation was observed, and since the enzyme remained fully active in 65% dimethylsulfoxide for at least 1 hour at 0°, we conclude that the dimethylsulfoxide-induced denaturation has a high enthalpy of activation and the native form is stable at temperatures below 8° in 65% aqueous dimethylsulfoxide.

In the trypsin catalyzed hydrolysis of Nα-benzoylarginine L-lysine p-nitrophenyl ester kcat corresponds closely to k₉, the rate constant for decylation (15, 21). Since water is a participant in the decylation the value of kcat should decrease with increasing concentration of dimethylsulfoxide, as was observed (Table 1). The experimental values for kcat at dimethylsulfoxide concentration 50% and higher were somewhat smaller than calculated on the basis of decreased water concentration, but are not considered to reflect significant effects on the catalytic reaction pathway.

Previous reports (6, 7) have shown that increasing concentrations of dioxane and dimethylsulfoxide cause increases in kcat at up to 50% solvent, in contrast to the results observed in this report. The reason for this difference appears to be related to the pH and temperature of the reaction, and possibly the type of substrate. (The data in Table 1 were obtained at pH 6.4 and 0° and with a lysine derivative as substrate.) In contrast, the previous studies used Nα-benzoyl-L-arginine ethyl ester as substrate at pH 8.0 with dioxane as solvent (6) or Nα-tosyl-L-arginine methyl ester at pH 8.0 with dimethylsulfoxide (7) and were carried out at 25°. Inagami and Sturtevant (6) also investigated the effect of pH on the initial rate of reaction in varying concentrations of dimethylsulfoxide using Nα-benzoyl-L-arginine ethyl ester as substrate. The increases in kcat with increasing dimethylsulfoxide concentration were observed at pH 8.0 to 9.0 but not at pH 7.5 (the lowest value examined).

The observed increase in K₉ could arise from an increase in K₈, a decrease in k₉, or both. A similar increase in K₉ with increasing dimethylsulfoxide concentration was observed with α-chymotrypsin (8) and was shown to be due to a combination of dielectric and competitive inhibition effects by the organic solvent, Equation 1 (19). The data for K₉ in the case of trypsin are also accounted for by such effects, as shown in Fig. 1. The structural and functional similarities in α-chymotrypsin and trypsin (10) suggest that the same factors would be responsible for causing the observed increase in K₉ in both enzymes. Similar dielectric and inhibitory effects of dioxane and 2-propanol on the trypsin-catalyzed hydrolysis of Nα-benzoyl-L-arginine ethyl ester and competitive inhibition by benzamidine have been reported previously (4). The possibility that the increase in K₉ results from a decrease in k₉ cannot be ruled out at this time. We are currently investigating the acylation reaction at sub-zero temperatures and hope to be able to resolve this point. In the corresponding case of α-chymotrypsin, the effect seems to be fully accounted for by the increase in K₈ (19). Kassera and Ladler (22) have reported that 2-propanol causes a decrease in k₉ as well as an increase in K₈ in the trypsin-catalyzed hydrolysis of Nα-benzoylcarbonyl-L-alanine p-nitrophenyl ester.

**Effect of Temperature on Catalysis**—Sub-zero temperatures

1 The accepted reaction pathway for trypsin-catalyzed hydrolysis of acylamino acid esters is

\[
E + S \rightarrow ES \rightarrow k_2 \rightarrow EA \rightarrow k_4 \rightarrow E + P_1 \]

where EA = acyl-enzyme. Under steady state conditions kcat = k₃k₄/(k₅ + k₄) and K₉ = k₅k₄/(k₅ + k₄) where K₉ ≈ k₅/k₄. For Nα-benzoylarginine L-lysine p-nitrophenyl ester k₂ ≫ k₅ (18, 21) and therefore kcat ≈ k₃.
would be expected to be beneficial in overcoming potentially adverse solvent effects on the enzyme's structure for two reasons. The relatively high enthalpies of activation for protein denaturation will tend to stabilize the native form (23), and the increased dielectric constant at lower temperatures (14) will tend to minimize the disruptive influence of decreased dielectric strength on the forces maintaining the native conformation. The linear Arrhenius plots for deacylation under substrate-saturating conditions indicate that no change in the rate-determining step or adverse structural effect occur in the accessible temperature range below 0°C (at temperatures below -45°C the rate of deacylation was too slow to measure conveniently). Additional evidence supporting the premise that sub-zero temperatures and 65% dimethylsulfoxide have no significant effect on at least the deacylation step is the excellent agreement between the values calculated from extrapolating the low temperature data to +25°C and those obtained in the absence of dimethylsulfoxide at +25°C.

Although no detailed analysis of the pH dependence of the reaction was carried out, the values for $k_{cat}$ at pH 4.7 and 6.4 are consistent with a $pK_a$ of 6.8 as reported for the reaction in the absence of solvent (15).

The decrease in $K_m$ with decreasing temperature is consistent with the larger enthalpy of activation for $k_f$ compared with that anticipated for $k_d/K_d$. The difference in energy of activation at the different pH values is not readily explained. It is possible that the enthalpy of activation for deacylation is pH dependent, or the difference may reside in the different procedures used to measure $k_{cat}$. No deviations from Michaelis-Menten kinetics during the course of the complete reaction were observed at sub-zero temperatures and 65% aqueous dimethylsulfoxide.

**Effects of Dimethylsulfoxide and Sub-zero Temperatures on Structure of Trypsin**—The native conformation of the enzyme in aqueous solution is the result of a delicate balance of attractive and repulsive interactions involving mainly electrostatic, hydrophobic, and hydrogen-bonding forces, and including competition between solvent and intramolecular groups. The decreased dielectric strength arising from large concentrations of organic solvents, such as dimethylsulfoxide, would be expected to result in changes in some of these interactions. For example, decreased electrostatic repulsions would be anticipated from the increased counter ion binding. Aqueous dimethylsulfoxide would be expected to have only limited effects on hydrogen bonding since it is a hydrogen bond acceptor, and hydrogen bonding of surface and exposed groups to solvent would probably still occur. Since hydrophobic interactions play a major role in maintaining the tertiary structure of trypsin, their disruption would have a major impact on the structure of the enzyme. The lower dielectric strength of dimethylsulfoxide would tend to decrease the driving force for internal hydrophobic interactions. Such effects are presumably responsible for the lack of activity of papain in 65% aqueous dimethylsulfoxide* and the denaturation of trypsin and α-chymotrypsin at 25°C in 65% aqueous dimethylsulfoxide.

In these low temperature studies on trypsin effects due to decreased dielectric strength of the solvent will be minimized due to the increase in dielectric constant with decreasing temperature (14). We estimate that at -45°C the dielectric constant for 65% aqueous dimethylsulfoxide is 80.

The absence of adverse effects of dimethylsulfoxide on the catalytic properties of trypsin indicates that major structural changes do not occur and the binding and catalytic sites must be essentially intact and unaffected by the solvent. Unfortunately most techniques that could be used to detect small structural changes are also sensitive to changes in solvent composition and hence would not give unequivocal results. Structural changes in proteins are usually also manifested as changes in the spectral properties of the protein. Spectral changes would also be expected due to solvent effects on exposed residues such as tyrosine and tryptophan. However, such solvent effects can be distinguished from solvent-induced structural changes in that they should appear as linear or smooth monotonic changes with increasing solvent concentration, whereas structural effects will be accompanied by distinct breaks in such plots (20). The ultraviolet absorption of dimethylsulfoxide prevents examination of spectral perturbations below 250 nm. Changes in the ultraviolet absorption and fluorescence spectra then reflect changes mainly in the tyrosine and tryptophan residues of the enzyme. Since trypsin has many such residues throughout its structure any substantial change in structure should be apparent from spectral studies.

The utility of ultraviolet difference spectra and fluorescence emission for detection of conformational changes is well established (17, 20, 24, 25). The effects of increasing dimethylsulfoxide concentration, as well as sub-zero temperatures, on such spectra for trypsin (Figs. 2 to 4) are very similar to those observed with α-chymotrypsin (8) and are consistent with solvent effects on the exposed aromatic residues. There is no evidence for any structural effects.

**Reaction with N'-Acetyl-L-lysine Methyl Ester**—A few preliminary experiments with this substrate which is transparent in the region of maximum ultraviolet absorption of trypsin were carried out at low temperatures. The basis for these experiments is as follows. If enzyme-substrate complexes or intermediates prior to the deacylation step in the reaction pathway involve perturbations in the environment of the aromatic residues in the enzyme, then such changes should be potentially detectable in the ultraviolet absorption and fluorescence spectra. Of the 10 tyrosine and 4 tryptophan residues at least one, Trp-199, is in the vicinity of the active site. We are currently carrying out a detailed investigation in which the interactions of trypsin with specific substrates such as N'-acetyl-L-lysine methyl ester are monitored by changes in the ultraviolet spectrum of the enzyme. The present preliminary results are reported only to indicate that such a procedure is feasible at sub-zero temperatures.

In an extensive series of publications, Hess and co-workers have shown that spectral changes in the 200- 290-nm region are associated with binding of inhibitors and substrates to chymotrypsin (26) and have interpreted these changes as corresponding to a substrate- or inhibitor-induced conformational change in the enzyme (27). The difference spectrum of trypsin + N'-tosyl-L-arginine methyl ester under steady state conditions at pH 3.0 and 25°C shows positive maxima at 292, 286, and 280 nm and a negative maximum at 265 nm (28). Evidence for pH dependence of such spectral changes has also been reported (28).

Interpretation of the limited amount of data available in the present study must be considered as mostly speculation. It is clear, however, that the interaction of trypsin with N'-acetyl-L-lysine methyl ester results in perturbations in the environment of tyrosine and tryptophan in the enzyme, since spectral changes centered at 275 and 284 nm have been attributed to perturbations of tyrosine and these at 290 nm to tryptophan (29, 30). The difference spectrum for trypsin (Fig. 6C) is very similar to that

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* In studies with α-chymotrypsin and specific ester substrates we find $\Delta H$ for $k_d/K_d$ to be around 7 Cal per mole.

* A. L. Fink, unpublished results.

* Based on homology with α-chymotrypsin (10).
observed for α-chymotrypsin + N-acetyl-L-alamine amide at pH 8.0 and 24° (26). Based on analogy with corresponding chymotrypsin-catalyzed reactions we would tentatively interpret the initial reaction observed with N-acetyl-L-lysine methyl ester at -60° to involve a substrate-induced conformational change in the enzyme (31), involving perturbation of tryptophan and the subsequent reaction to correspond to acylation and involve perturbations of tryptophan and tyrosine.

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The Trypsin-catalyzed Hydrolysis of $N^\alpha$-Benzyloxycarbonyl-l-Lysine $p$-Nitrophenyl Ester in Dimethylsulfoxide at Sub-Zero Temperatures

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