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# The $\alpha$-Chymotrypsin-Catalyzed Hydrolysis of *N*-Acetyl-L-tryptophan *p*-Nitrophenyl Ester in Dimethyl Sulfoxide at Subzero Temperatures†

Anthony L. Fink

ABSTRACT: The effect of subzero temperatures and aqueous $Me_2SO$ solutions (liquid state) on the $\alpha$-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan *p*-nitrophenyl ester has been investigated. As the $Me_2SO$ concentration increases, the value of $k_{cat}$ decreases proportionately to the decrease in water concentration; however, the value of $K_m$ increases exponentially. The effect on $K_m$ is attributed to the less polar $Me_2SO$ binding more strongly than water to the substrate binding site. The $pK_a$ for $k_{cat}$ (deacylation) is unaffected by the presence of 65% aqueous $Me_2SO$. A Van't Hoff plot of $k_{cat}$ using 65% $Me_2SO$ was linear over the temperature range 0 to $-45°$. Below this temperature turnover occurred extremely slowly; since acylation was still very rapid the acyl-enzyme could be accumulated and was isolated by gel filtration. The intrinsic spectral properties of the enzyme (ultraviolet, circular dichroism, and fluorescence) were examined as a function of $Me_2SO$ concentration in order to detect any $Me_2SO$-induced structural changes. No structural effects were observed, although solvent effects were noted. Similarly the effect of temperature (0 to $-80°$) on the intrinsic fluorescence of $\alpha$-chymotrypsin in 65% $Me_2SO$ did not indicate any structural changes. We conclude that the pathway of the enzyme-catalyzed reaction in 65% aqueous $Me_2SO$ at subzero temperatures is essentially unchanged from that in the absence of $Me_2SO$ and at ambient temperatures.

The rapidity of enzyme-catalyzed reactions of specific substrates has been a major problem in studying the dynamic processes occurring during the catalysis. In an attempt to overcome this problem rapid reaction techniques, *e.g.*, stopped-flow and temperature-jump spectrophotometry, have been used with a considerable degree of success with respect to determining kinetic parameters. However, in order to propose a detailed mechanism one must know the nature of the various intermediates and transition states on the reaction pathway. Some success has been achieved in this direction by the use of nonspecific substrates and substrate analogs. However the fact that intermediates such as acyl-enzymes can be isolated using nonspecific substrates may indicate that they are not on the productive pathway for good (specific) substrates.

We have begun a comprehensive study of the use of subzero temperatures (and fluid solutions) as a means of obtaining information about the nature of intermediates in the catalysis of specific substrates. This approach is predicated on the following basis. The dramatic decrease in reaction rates, and the enhanced difference in rates for different enthalpies of activation which occur with decreases in temperature of the order of 100° could allow the accumulation of intermediates in enzyme-catalyzed reactions. The "trapped" intermediate could then be studied by a variety of chemical and physical techniques to provide information concerning its structure (especially enzyme–substrate interactions), as well as yielding kinetic and thermodynamic data. We are initially applying the technique to some well studied enzymes, such as $\alpha$-chymotrypsin, so that we may compare data obtained at the low temperatures with that obtained by other means.

In order to preclude problems arising from rate-limiting diffusion, fluid solvent systems are required. A few aqueous–organic solvent systems are known which are fluid to temperatures in the range of $-100°$, and which may be suitable for the contemplated experiments. Previous studies have shown that some enzymes are stable in high concentrations of potentially useful aqueous–organic solvents, *e.g.*, trypsin

† From The Division of Natural Sciences, University of California, Santa Cruz, California 95060. *Received December 1, 1972.* This research was supported by Faculty Research Funds granted by the University of California, Santa Cruz, Calif.

in 88% dioxane and 70% $Me_2SO$ (Inagami and Sturtevant, 1960), lysozyme in 60% $Me_2SO$ (Hamaguchi, 1964).

In order for the data obtained from the low-temperature studies to be of value in determining the mechanism of the enzyme-catalyzed reaction under normal (*i.e.*, physiological) conditions, it must first be established that the reactions under both sets of conditions are essentially the same. It is to this end that the work reported herein was undertaken. We have examined one particular system, that of 65% aqueous $Me_2SO$, and its effect on the catalytic properties and structure of $\alpha$-chymotrypsin.

Previous studies of the effects of organic solvents on enzyme-catalyzed reactions have generally shown an increase in $K_m$ and little effect on $k_{cat}$. Various attempts have been made to correlate these observations with dielectric constant and competitive inhibition (Clement and Bender, 1963; Kasserra and Laidler, 1970; Mares-Guia and Figueiredo, 1972). Many previous studies have shown that significant structural changes in a protein can be detected by physical techniques such as ultraviolet (uv) difference spectra, and fluorescent and circular dichroic spectra (Timasheff, 1970; Donovan, 1969; Chen *et al.*, 1969).

There have been very few reported studies of enzyme-catalyzed reactions at subzero temperatures. Freed (Bielski and Freed, 1964) investigated the $\alpha$-chymotrypsin-catalyzed hydrolysis of AcTrpOEt[1] in aqueous methanol in the temperature range +25 to −33°. No denaturation was observed below +10° and activity was detected at temperatures as low as −33° and at methanol concentrations as high as 80%. Since significant transesterification would have occurred (Bender *et al.*, 1964a), and in view of the fluorescent assay used, the observed rates were probably much slower than the true catalytic rate. More recently the potential of the proposed technique has been demonstrated by Douzou (Douzou *et al.*, 1970; Douzou and Leterrier, 1970) using horseradish peroxidase in 70% aqueous dimethylformamide and temperatures to −65°. Both complexes I and II were trapped using low temperatures, and their spectra and the kinetics of their interconversion studied.

AcTrpONph was chosen as a substrate for this study because of its low value of $K_m$, and because of the large difference in rates of acylation and deacylation. The results reported here demonstrate that the reaction pathway of $\alpha$-chymotrypsin-catalyzed reactions is essentially unchanged by 65% $Me_2SO$ and subzero temperatures. This finding coupled with some of our subsequent results (A. L. Fink, in preparation) indicates that the technique of low-temperature trapping of enzyme–substrate intermediates will play a significant role in furthering our understanding of the mechanisms of enzyme action.

## Materials and Methods

### *Materials*

AcTrpONph was obtained from Cyclo (lot H4285) and recrystallized several times from ethyl acetate–hexane (mp 134–135°). $Me_2SO$ (MCB or Mallinkrodt), reagent grade, was distilled under vacuum from calcium hydride at temperatures below 37° and stored at 0°. Doubly distilled water and reagent grade methanol (MCB) were used. Chymotrypsin, three-times recrystallized from Worthington (lot CDI IIB and CDI OBK), was purified by affinity chromatography using Agarose–ε-aminocaproyl-D-tryptophan methyl ester (Miles lot 300/3).

Stock solutions of AcTrpONph were prepared by dissolving it in 65% aqueous $Me_2SO$ and were stored at −20°. Stock solutions of $\alpha$-chymotrypsin were prepared by slowly adding the aqueous enzyme solution to 65% aqueous $Me_2SO$ at 0° with gentle stirring. Such stock solutions were stored at −20° until required. Fresh solutions of aqueous $Me_2SO$ buffer, substrate and enzyme were made up daily. Unless otherwise stated all experiments were carried out in 65% (v/v) aqueous $Me_2SO$, $\mu$ = 0.1 M, and pH 5.5.

### *Methods*

Aqueous $Me_2SO$ solutions were prepared as follows. The appropriate volume per cent of $Me_2SO$ was slowly added to a cooled solution of acetate of phosphate buffer with stirring, in an ice bath. The ionic strength was adjusted to 0.1 M with 1 M KCl solution; and the pH was determined by the reading on a Corning Model 12 pH meter using a No. 476050 electrode (Bates, 1964).

Kinetic measurements were performed on a Cary 14 spectrophotometer using a specially constructed double-walled quartz cell. Temperature control was achieved either by circulating ethanol from an Hetofrig Ultra Cryotherm constant-temperature bath, or by using nitrogen gas, cooled by passing through a coil submersed in liquid nitrogen and then heated to the appropriate temperature by passage over a heating coil. Both systems could maintain the cell contents within ±0.2° over several hours. The hydrolysis of AcTrpONph was followed at 350 nm, the extinction coefficient being determined by adding a known quantity of *p*-nitrophenol to the corresponding solution under appropriate conditions of pH and temperature.

Fluorescent measurements were performed with an Hitachi Perkin-Elmer MPF 2A spectrophotometer. Low temperature experiments were conducted using a 3-mm i.d. Suprasil tube as sample cell. The sample-cell holder of a Varian EP3 electron spin resonance spectrometer was adapted to the fluorescence spectrophotometer and was used to hold the tube. Temperature control was maintained using nitrogen and a Varian temperature controller.

Circular dichroism experiments were carried out using a Jasco J20 spectropolarimeter equipped with a thermostated brass block sample cell holder. A 1-cm path-length cell was used.

Gel filtration experiments were done using Sephadex LH-20 in an all-glass, jacketed column (1.0 × 30 cm). The column was packed in the usual fashion using 65% aqueous $Me_2SO$ as solvent. Prior to beginning an experiment the temperature was dropped to −40° while buffer was pumped through with a peristaltic pump. After thermal equilibrium was reached the substrate and enzyme solutions were mixed at −40° on top of the column and then elution begun. The eluent was monitored at 280 nm using an Isco UA-4 absorbance monitor.

The enzyme was assayed using a burst titration with *trans*-cinnamoylimidazole (Schonbaum *et al.*, 1961). Steady-state parameters ($k_{cat}$ and $K_m$) were determined by the initial velocity procedure using plots of $1/V$ *vs.* $1/(S)$ or $V$ *vs.* $V/(S)$.

## Results

$\alpha$-Chymotrypsin was found to be active in both aqueous $Me_2SO$ and aqueous methanol solutions, but not in aqueous dimethylformamide. Gradual loss of catalytic activity was

[1] Abbreviations used are: AcTrpONph, *N*-acetyl-L-tryptophan *p*-nitrophenyl ester; AcTrp, *N*-acetyl-L-tryptophan; AcTrpOEt, *N*-acetyl-L-tryptophan ethyl ester; Cbz, carbobenzoxy.

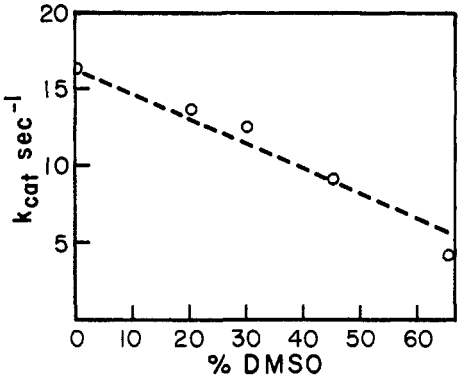


FIGURE 1: The effect of $Me_2SO$ concentration on $k_{cat}$ in the $\alpha$-chymotrypsin-catalyzed hydrolysis of AcTrpONph, pH 5.5, $\mu$ = 0.1 M, 25°. The points are experimental values, the broken line is calculated on the basis of the decreased water concentration.

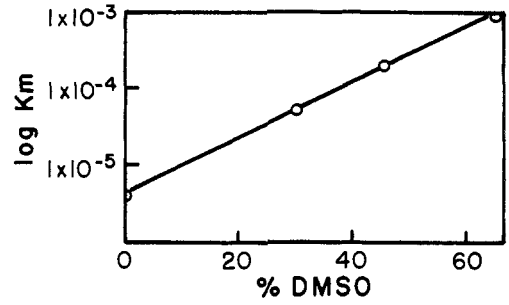


FIGURE 2: The effect of $Me_2SO$ concentration on $K_m$ in the $\alpha$-chymotrypsin-catalyzed hydrolysis of AcTrpANph, pH 5.5, $\mu$ = 0.1 M, 25°.

observed at 25° with both 65% $Me_2SO$ and 70% methanol, but not at temperatures below 10° (at least on the time scale of the reactions investigated). In fact we have stored $\alpha$-chymotrypsin in 65% $Me_2SO$ at −20° for several weeks at concentrations as low as 4 × $10^{-6}$ M without loss of activity. The 65% $Me_2SO$ solutions were fluid to approximately −95°.

*Effect of $Me_2SO$ on $k_{cat}$ and $K_m$.* Figures 1 and 2 show the effect of increasing concentrations of $Me_2SO$ on $k_{cat}$ and $K_m$ for the $\alpha$-chymotrypsin-catalyzed hydrolysis of AcTrpONph. Little effect on $k_{cat}$ is observed if the decrease in water concentration is taken into consideration. However an exponential increase in $K_m$ occurs as the $Me_2SO$ concentration increases. For the comparable experiments with methanol a similar exponential increase in $K_m$ was observed, whereas $k_{cat}$ increased exponentially to 60% methanol and then began to decrease above 75% methanol.

The effect of pH on the $\alpha$-chymotrypsin-catalyzed hydrolysis of AcTrpONph under substrate saturating conditions in 65% $Me_2SO$ was determined over the pH range 4.0 to 8.5. Except for the slightly lower values of $k_{cat}$ ($k_3$)[2] in the $Me_2SO$ solutions, the pH dependence is essentially the same as in the absence of $Me_2SO$. The p$K$ value calculated for $k_3$ in 65% $Me_2SO$ at 25° was 6.7 ± 0.2, as compared to 6.86 at 25° and 0% $Me_2SO$ (Bender *et al.*, 1964b).

*Effects of $Me_2SO$ on the Ultraviolet, Circular Dichroic, and Fluorescent Spectra of $\alpha$-Chymotrypsin.* To determine whether the high concentration of $Me_2SO$ had any significant effect on the native conformation of $\alpha$-chymotrypsin, the intrinsic spectral properties of the enzyme were examined as a function of $Me_2SO$ concentration. Solvent effects on the exposed chromophores should be apparent as monotonic changes with solvent concentration, whereas structural effects, especially cooperative ones, should manifest themselves as distinct deviations in such plots (Chen *et al.*, 1969). Figures 3–5 show the effects of increasing $Me_2SO$ concentration on the uv absorption difference, fluorescent emission and circular dichroic spectra of $\alpha$-chymotrypsin. In each case the effect of increasing $Me_2SO$ concentration results in a linear or smooth monotonic change in the measured spectral property.

*Effect of Temperature.* Figure 6 shows the effect of temperature on the fluorescent emission spectrum of $\alpha$-chymotrypsin in 65% $Me_2SO$. The emission intensity increases linearly with decreasing temperature, whereas the $\lambda_{max}$ shows a gradual blue shift on decreasing the temperature.

The effect of temperature on the turnover rate was determined for AcTrpONph in 65% $Me_2SO$ (pH 5.5) under substrate saturating conditions where the observed zero-order rate = $k_3E_0$. A Van't Hoff plot for $k_3$ (deacylation) is shown in Figure 7. The energy of activation was calculated to be 10.9 ± 1.1 kcal/mol. No deviations from linearity were observed over the range 0 to −46°, below which temperature the rate became too slow to measure accurately. A similar plot was obtained at pH 6.5.

*Formation and Isolation of N-Acetyl-L-tryptophanyl-$\alpha$-Chymotrypsin.* When AcTrpONph and $\alpha$-chymotrypsin were mixed under conditions of [S] ≫ $K_m$ at −40° and pH 5.5, a stoichiometric burst of *p*-nitrophenol was released within 1 min. Since the half-life for deacylation under these conditions is approximately 10 hr the acyl-enzyme was accumulated stoichiometrically and isolated by gel filtration at −40° (Fink, 1973). The elution pattern thus obtained is shown in Figure 8A. Fraction I corresponds to the elution volume for $\alpha$-chymotrypsin and presumably is the acyl-enzyme; fraction II corresponds to the elution volume of AcTrpONph.

Aliquots from fraction I, maintained at temperatures ≤ −40°, were found to be inactive as determined by the lack of a burst of *p*-nitrophenol at −40°. The rates of hydrolysis of AcTrpONph in the presence of aliquots of fraction I at higher temperatures indicated that no significant loss of activity had occurred. Other aliquots of fraction I were rechromatographed either on the same column at 25° or on a column of Sephadex G-25 using acetate buffer as eluent. In each case two fractions were obtained (Figure 8B), the first (III) corresponding to the elution volume of $\alpha$-chymotrypsin and the other (IV) corresponding to that of AcTrp. The uv and fluorescent spectra of fraction IV were obtained and found to be identical with those of an authentic sample of AcTrp. Based on the ratio of absorbance at 280 nm for fractions III and IV, and using values of $\epsilon$ = 5 × $10^4$ and 5 × $10^3$ for $\alpha$-chymotrypsin and AcTrp, respectively, a 1.0:0.9 $\alpha$-chymotrypsin: AcTrp stoichiometry was calculated for fraction I.

[2] The accepted reaction pathway for $\alpha$-chymotrypsin-catalyzed hydrolyses is

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2$$
$$+$$
$$P_1$$

where ES′ = acyl-enzyme. Under steady-state conditions, $k_{cat} = k_2k_3/(k_2 + k_3)$ and $K_m = k_3K_s/(k_2 + k_3)$. For AcTrpONph at 25° $k_2$ = 38 × $10^3$ $sec^{-1}$ and $k_3$ = 30 $sec^{-1}$ (Bender *et al.*, 1964b). Thus $k_2 \gg k_3$ and $k_{cat} = k_3$.

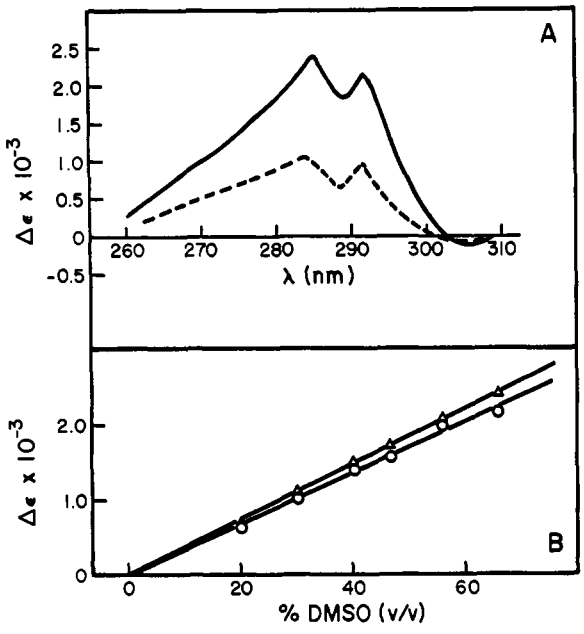


FIGURE 3: The effect of $Me_2SO$ concentration on the uv absorption difference spectrum of α-chymotrypsin, pH 5.5, $\mu$ = 0.1 M, 5°, [E] = $1.0 \times 10^{-5}$ M. (A) Difference spectra for 30% (broken line) and 65% (solid line); (B) $\Delta\epsilon$ as a function of $Me_2SO$ concentration at 292.5 nm (○) and 285 nm (△).

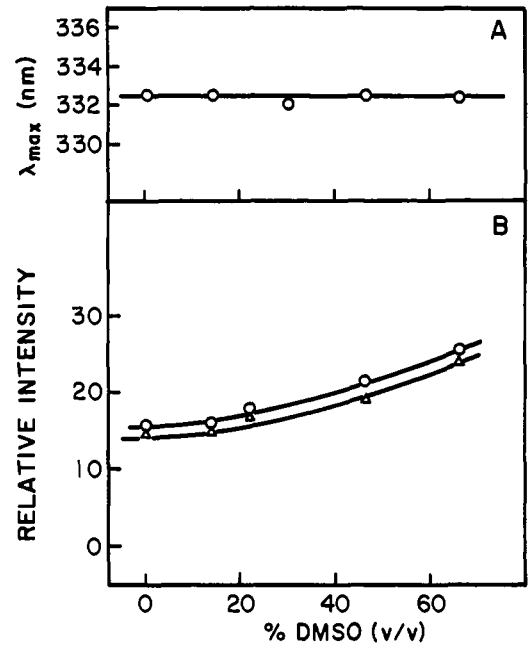


FIGURE 4: The effect of $Me_2SO$ concentration on the intrinsic fluorescence of α-chymotrypsin, pH 5.5, $\mu$ = 0.1 M, 0°, [E] = $4.0 \times 10^{-6}$ M. (A) Effect on $\lambda_{max}$ with excitation at 288 nm; (B) Effect on intensity; excitation at 288 nm (○), excitation at 260 nm (△).

## Discussion

The main purpose of this work was to determine the effects, if any, of 65% aqueous $Me_2SO$ and subzero temperatures on the pathway of α-chymotrypsin-catalyzed reactions. The question has been tackled in two major parts: (1) the effect of the solvent on the catalytic and structural properties of the enzyme, and (2) the effect of subzero temperatures on the catalytic and structural properties of the enzyme.

*Effect of $Me_2SO$ on the Catalytic Properties of α-Chymotrypsin.* Undoubtedly the most sensitive tests for detecting effects on an enzyme are those involving its catalytic activity. It has been shown that minor structural changes can produce drastic effects in enzyme catalysis (*e.g.*, Neet *et al.*, 1968). As shown in Figure 1 the effect on $k_{cat}$ of increasing $Me_2SO$ concentration is essentially only that anticipated from the decrease in water concentration. Thus deacylation is unaffected by $Me_2SO$ since $k_{cat} \approx k_3$ for AcTrpONph. The parallel pH dependence of $k_{cat}$ in the presence and absence of 65% $Me_2SO$ further supports this conclusion and indicates that the $Me_2SO$ does not significantly affect the p$K$ of His-57 in deacylation (*cf.* Kaplan and Laidler, 1967).

Previous studies, both with α-chymotrypsin and with other enzymes, have found that lower concentrations (*e.g.*, up to 20%) of various organic solvents have little effect on $k_{cat}$, but do increase $K_m$ (*e.g.*, Singer, 1962; Clement and Bender, 1963; Applewhite *et al.*, 1958; Inagami and Sturtevant, 1960). For AcTrpONph, $K_m \approx k_3K_s/k_2$, and thus the observed increase in $K_m$ could be due to an increase in $K_s$ or a decrease in $k_2$, or both. An increase in $K_s$ would reflect decreased binding affinity of the substrate and would be expected since the less polar $Me_2SO$ will tend to be more strongly bound in the hydrophobic areas of substrate binding than water. Such an effect can be overcome by using larger substrate concentrations to saturate the enzyme, with no change in the reaction mechanism or pathway. We are currently investigating the effect of $Me_2SO$ on $k_2$, the acylation rate constant for AcTrpONph in 65% $Me_2SO$. Preliminary experiments indicate that no significant effects occur, and certainly no appreciable decrease occurs. Since $k_2$ and $k_3$ are both dependent on the p$K$ of His-57, and since the p$K$ for $k_3$ is unaffected by the presence of $Me_2SO$, it is quite possible that $k_2$ is also unaffected and that the sole effect of $Me_2SO$ on catalysis is competitive binding between $Me_2SO$ and the substrate. There is no reason *a priori* to expect an effect on $k_2$ by $Me_2SO$; however, Laidler (Kasserra and Laidler, 1970) has reported that 2-propanol causes both an increase in $K_s$ and a decrease in $k_2$ in the trypsin-catalyzed hydrolysis of Cbz-L-alanine *p*-nitrophenyl ester.

*Effect of Temperature on Catalysis.* Many of the structural and solvent effects of organic solvents on proteins are due to

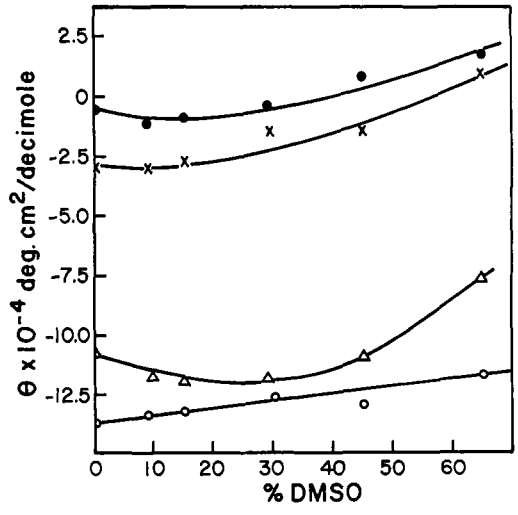


FIGURE 5: The effect of $Me_2SO$ concentration on the circular dichroism spectrum of α-chymotrypsin, pH 5.5 $\mu$ = 0.1 M, 5°, [E] = $2.0 \times 10^{-5}$ M, (○) 252 nm, (△) 272 nm, (×) 290 nm, (●) 298 nm.

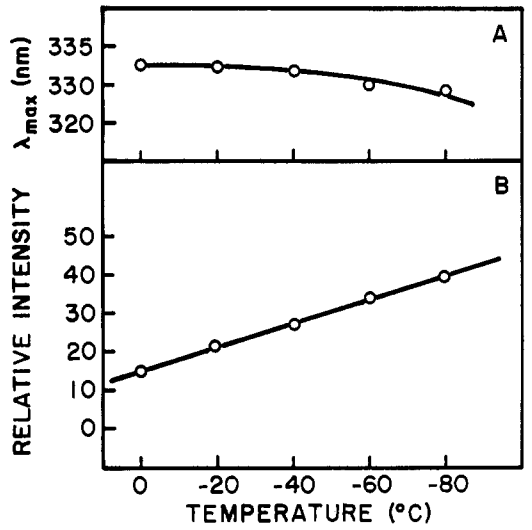


FIGURE 6: The effect of temperature of the fluorescence emission of $\alpha$-chymotrypsin, pH 5.5, 65% $Me_2SO$, $\mu$ = 0.1 M, [E] = $4.0 \times 10^{-6}$ M, excitation at 288 nm: (A) $\lambda_{max}$ and (B) relative intensity.

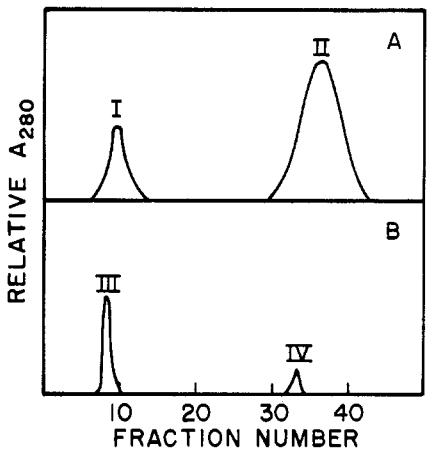


FIGURE 8: (A) Gel filtration of $\alpha$-chymotrypsin + AcTrpONph at $-40°$ on Sephadex LH-20 with 65% $Me_2SO$, pH 5.5. (B) Gel filtration of fraction I in part A at 25°.

the decreased dielectric constant (Kasserra and Laidler, 1970; Mares-Guia and Figueiredo, 1972; Singer, 1962). However, the use of subzero temperatures will tend to negate such effects, since the dielectric constant increases with decreasing temperature (Akerlof, 1932). Also the high enthalpy of activation for protein denaturation will tend to favour the native form at low temperatures (Freed, 1965).

The linear Van't Hoff plot (Figure 7) for $k_{cat}$ indicates no change in the rate-determining step and no adverse structural effects over the accessible temperature range below 0°. The value for the energy of activation, 10.9 ± 1.1 kcal/mol, is in good agreement with that obtained under normal conditions, 12.0 ± 1.0 kcal/mol (Bender *et al.*, 1964c). Since the experiment was done under substrate-saturating conditions, we can assume that $k_{cat} = k_3$. If the plot is extrapolated to +25° we obtain a value for $k_3$ of 2.2 $sec^{-1}$. This is in excellent agreement with the value, 3.0 $sec^{-1}$, reported under normal conditions, *i.e.*, no $Me_2SO$ at the same pH (5.7) (Bender *et al.*, 1964b).

The excellent agreement between the reaction rate and energy of activation at subzero temperatures in 65% $Me_2SO$ with those under normal conditions is very strong evidence that the catalytic reaction is essentially the same in 65% $Me_2SO$ and subzero temperatures as at 25° and in the absence of $Me_2SO$.

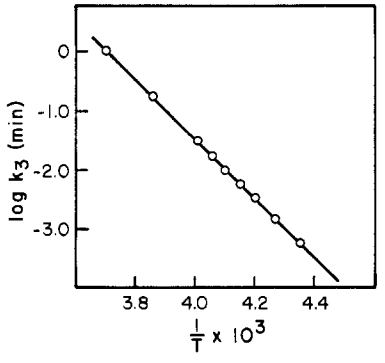


FIGURE 7: The effect of temperature on the $\alpha$-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan *p*-nitrophenyl ester. Conditions; 65% $Me_2SO$, pH 5.5, $\mu$ = 0.1 M, [S] = $4.8 \times 10^{-2}$ M, [E] = $2.2 \times 10^{-5}$ M.

*Effects of $Me_2SO$ on the Structure of $\alpha$-Chymotrypsin.* The major forces involved in maintaining the native conformation of an enzyme are hydrogen bonding, hydrophobic, and electrostatic interactions. Thus effects of organic solvents on the structure of the enzyme are likely to arise from perturbations of these interactions due to the change in medium. According to the Debye–Hückle Theory electrostatic interactions are inversely proportional to the dielectric constant ($D$) of the medium. Based on the relationship between $D$ and temperature (Akerlof, 1932) we estimate that the dielectric constant for 65% aqueous $Me_2SO$ at −40° is the same as that of water at 20°. Thus little effect would be expected on electrostatic interactions in the temperature range of interest. Similarly $Me_2SO$ is unlikely to have any substantial effect on the hydrogen-bonding system involved in maintaining the protein structure. Hydrophobic interactions, which are known to play a major role in maintaining the conformational stability in $\alpha$-chymotrypsin, are the most likely to be perturbed by $Me_2SO$. Several studies have shown that at least some enzymes are apparently unaffected by high concentrations of $Me_2SO$ (Rammler, 1971). For example, an extensive study of the effect of $Me_2SO$ on lysozyme demonstrated no detectable effects up to 65% $Me_2SO$ (Hamaguchi, 1964), and trypsin has been stored in 97% $Me_2SO$ at 12.5° for 14 hr with the loss of only 7% activity (Inagami and Sturtevant, 1960).

The absence of significant effect of $Me_2SO$ on the catalytic properties of $\alpha$-chymotrypsin indicates that major structural changes do not occur, and that minor ones do not occur in the vicinity of the active site. We would expect to observe solvent effects on the exposed residues of the enzyme; for example, six of the eight tryptophan residues are accessible to bulk solvent. A problem arises in detecting minor structural effects which might be induced by $Me_2SO$, since most techniques which would be suitable are also sensitive to the changed solvent composition and thus would not give unequivocal results. Many previous studies have shown that the intrinsic spectral properties of proteins are sensitive to structural changes. However, the inability to observe structural effects superimposed upon solvent effects does not unequivocally rule out their absence. In general solvent effects can be distinguished from structural effects in that they will appear as a linear or monotonic change with increasing organic solvent

concentration, whereas structural changes will be accompanied by distinct breaks in such plots (Chen *et al.*, 1969).

The uv absorption of $Me_2SO$ precludes examination of the spectral properties of the enzyme in the region of interest below 250 nm. Consequently most of the spectral information attainable corresponds to side chain groups, *i.e.*, changes in the tertiary structure but not the secondary structure. Even so, the widely dispersed tryptophan and tyrosine residues in particular should be sensitive to any significant structural changes.

The potential of uv difference spectra (Timasheff, 1970; Herskovits, 1967; Shimaki *et al.*, 1970; Benmouyal and Trowbridge, 1966), fluorescence emission (Timasheff, 1970, and references therein; Chen *et al.*, 1969), and circular dichroism (Timasheff, 1970; Ikeda and Hamaguchi, 1970; Pflumm and Beychok, 1969) to detect conformational changes as well as solvent perturbations of exposed chromophores has been well established. The observed linear dependence of $\Delta\epsilon$ on $Me_2SO$ concentration in the uv difference spectra (Figure 3) thus suggest only solvent effects, presumably due to the exposed tyrosine (4) and tryptophan (6) residues. The intrinsic fluorescence of $\alpha$-chymotrypsin is essentially due only to the tryptophan residues. The results obtained (Figure 4) are consistent with solvent effects and show no evidence of any structural changes. This is further supported by the fact that no changes in the tyrosine $\rightarrow$ tryptophan energy transfer occurred, indicating that the relative positions of the responsible tyrosine and tryptophan residues did not change. Likewise the linear and monotonic changes observed with low temperatures on the fluorescent spectrum of $\alpha$-chymotrypsin in 65% $Me_2SO$ (Figure 6) indicate that the temperature change does not result in any structural effect. The precision of the data obtained by circular dichroism is not as good as that obtained by uv difference or fluorescence, particularly since the changes due to the $Me_2SO$ were rather small and the experimental uncertainty quite large ($\pm 0.6 \times 10^4$ (deg cm$^2$)/dmol). The results, however, are consistent with solvent effects and show no indication of structural effects.

*Conformational Isomers of $\alpha$-Chymotrypsin.* $\alpha$-Chymotrypsin is known to exist in at least two conformations in the pH range in which the reported experiments were conducted, one of which is inactive (*e.g.*, Fersht, 1972; Fersht and Requena, 1971; Kim and Lumry, 1971; Royer *et al.*, 1971). The equilibrium between the active and inactive conformation is both pH and temperature sensitive, such that lower temperatures favor the inactive form (Fersht, 1972). Based on the data of Fersht we estimate the equilibrium constant to be $9.0 \pm 0.6$ at $-45°$ and $85 \pm 5$ at $-75°$. Thus if equilibrium conditions obtained, virtually all the enzyme would be in the inactive form at these low temperatures. However, we estimate the rate of interconversion of active into inactive form to be $5 \times 10^{-4}$ min$^{-1}$ at $-45°$ and $8 \times 10^{-7}$ min$^{-1}$ at $-75°$ (extrapolated from the data of Fersht). Since the temperature of the enzyme is dropped very rapidly from 0° to the low temperature being used,[3] the equilibrium portions existing at 0° are essentially trapped because the presence of excess substrate will stabilize all the active form as such. It is interesting to note that the rate of interconversion of active to inactive conformation at $-45°$ would be almost the same as the rate of deacylation of AcTrpONph (*i.e.*, the rate-determining step in the catalysis). We estimate that at 0° approximately equal amounts of active and inactive form are present.

*Isolation of N-Acetyl-L-tryptophanyl-$\alpha$-Chymotrypsin.* The AcTrpONph-$\alpha$-chymotrypsin system is very well suited to demonstrate the potential of the method of low-temperature trapping of intermediates in enzyme-catalyzed reactions. At $-40°$ in 65% $Me_2SO$ we estimate an acylation:deacylation ratio of approximately 1200,[4] compared with that of 1270 at $+25°$ with no $Me_2SO$ (Zerner *et al.*, 1964). Since the half-life for acylation at $-40°$ is only a fraction of a minute whereas that for deacylation is about 10 hr, the postulated acyl-enzyme can be trapped and isolated using gel filtration. Only covalently bound enzyme–substrate intermediates would be expected to elute at the elution volume of the enzyme (fraction I in Figure 8). That this fraction was indeed AcTrp-$\alpha$-chymotrypsin was demonstrated by raising its temperature enough for deacylation to occur, showing that catalytic activity was then present, and obtaining two fractions on subsequent gel filtration which corresponded to $\alpha$-chymotrypsin and AcTrp. The ratio of enzyme to AcTrp (1.0:0.9) indicates that initially essentially all the enzyme was in the form of the acyl-enzyme. This is to be expected, since substrate saturating conditions were used. The ratio was not 1:1 because it took over an hour for fraction I to elute, and deacylation would have been occurring slowly during this time. This problem has essentially been eliminated in more recent experiments using faster flow rates. The existence of covalent acyl-enzyme intermediates has been conclusively demonstrated with several nonspecific substrates and $\alpha$-chymotrypsin, and indirectly for specific substrates (*e.g.*, Hartley and Kilby, 1954; Bender *et al.*, 1962; Kezdy *et al.*, 1964; Shalitin and Brown, 1966; Miller and Bender, 1968). Fraction I cannot be a tetrahedral intermediate since a stoichiometric burst of *p*-nitrophenol is released under these conditions. The isolation of *N*-acetyl-L-tryptophanyl-$\alpha$-chymotrypsin represents the first direct evidence for the existence of a presumably covalent acyl-enzyme intermediate in the $\alpha$-chymotrypsin-catalyzed hydrolysis of a specific substrate. As long as it is maintained at a sufficiently low temperature, AcTrp-$\alpha$-chymotrypsin is stable and may be stored for a considerable period (*e.g.*, many weeks at Dry-Ice temperature). We are currently carrying out experiments to determine which residue of the enzyme is involved in the covalent linkage.

On the basis of our results we are quite confident that the reaction pathway during catalysis by $\alpha$-chymotrypsin in 65% $Me_2SO$ at subzero temperatures is essentially the same as that under normal conditions (*i.e.*, 0% $Me_2SO$, ambient temperatures). Although the possibility of minor structural changes cannot be eliminated there seem to be no effects on the active center, except for the presence of displaceable $Me_2SO$ in the place of some water molecules. This must mean that at the lower temperatures the bulk solvent structure and properties are very similar to those of water. This supposition is borne out by the effects on the catalytic activity which are observed at higher temperatures (>10°), and which presumably reflect substantial structural changes in the enzyme due to the presence of the $Me_2SO$.

Preliminary investigations of the $\alpha$-chymotrypsin hydrolyses of other specific substrates in this solvent system at low temperatures look very encouraging for the accumulation and detection of intermediates. Also, extrapolation of the acylation and deacylation rate constants for these substrates to 25°

[3] Our normal procedure is to rapidly cool, mix, and inject enzyme and substrate solution together into the sample cell.

[4] The rate of acylation at $-40°$ is too fast to measure accurately without a stopped-flow instrument. The value of $k_2$ was estimated on the basis of the apparent half-life of the acylation reaction.

yields values in good agreement with those obtained in the absence of $Me_2SO$.

Acknowledgments

The author thanks Mr. G. Ringold, Mr. L. Christensen and Mr. D. Fogelson for their technical assistance.

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