Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Norby, J. G., and Jensen, J. (1971), *Biochim. Biophys. Acta 233,* 104.
Post, R. L., Toda, G., and Rogers, F. N. (1975), *J. Biol. Chem. 250,* 691.
Robinson, J. D. (1972), *Biochim. Biophys. Acta 266,* 97.
Schon, R., Dittrich, F., and Repke, K. R. H. (1974), *Acta Biol. Med. Ger. 33,* K9.
Schwartz, A., Matsui, M., and Laughter, A. M. (1968), *Science 160,* 323.
Siegel, G. J., and Albers, R. W. (1967), *J. Biol. Chem. 242,* 4972.
Skou, J. C. (1960), *Biochim. Biophys. Acta 42,* 6.
Skou, J. C. (1974), *Biochim. Biophys. Acta 339,* 246.
Sullivan, W. J., and Perrin, D. D. (1964), *Biochemistry 3,* 18.
Walser, M. (1973), in Handbook of Physiology Section 8: Renal Physiology, American Physiological Society, Chapter 18, p 555.

Papain-Catalyzed Reactions at Subzero Temperatures†

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ABSTRACT: As a first step in the investigation of papain catalysis using subzero temperatures to detect, accumulate, and characterize enzyme–substrate intermediates, we have studied some potential cryosolvents and carried out preliminary intermediate trapping experiments. The effects of subzero temperatures and aqueous dimethyl sulfoxide solutions on the papain-catalyzed hydrolysis of N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester have been investigated in detail. At 0 °C, the value of k_{cat} decreases with increasing dimethyl sulfoxide concentration, decreasing in proportion to the decreased water concentration; however, the value of K_m increases exponentially. The effect on K_m can be accounted for by a combination of both dielectric and competitive inhibition effects. The Arrhenius plot for the deacylation reaction in 7.65 M (60% v/v) dimethyl sulfoxide is linear over the temperature range 0 to −45 °C and extrapolates to a calculated value of k_{cat} at 25 °C in excellent agreement with that obtained in the absence of organic solvent. The pH–rate profile is not substantially perturbed by the presence of 7.65 M dimethyl sulfoxide. At −45 °C and below, turnover occurs extremely slowly, and is essentially negligible, although acylation is still quite rapid. Consequently, the acyl enzyme, N^{α}-carbobenzoxy-L-lysyl-papain, can be readily accumulated and trapped at temperatures below −50 °C. At these low temperatures, under conditions of excess substrate, the amount of *p*-nitrophenol liberated in the acylation reaction is equivalent to the active-site normality of the enzyme, indicating a 1:1 stoichiometry in formation of the acyl enzyme. The effect of dimethyl sulfoxide up to 7.65 M, on the intrinsic ultraviolet, fluorescence, and circular dichroic properties of the enzyme shows no evidence of any solvent-induced structural changes. All experimental observations are consistent with the conclusion that 7.65 M dimethyl sulfoxide and subzero temperatures have no deleterious effects on papain-catalyzed reactions. A related series of experiments indicate that aqueous ethanol cryosolvents up to 13.7 M (80% v/v) are also suitable. Preliminary experiments at subzero temperatures using N^{α}-carbobenzoxy-L-lysine methyl ester suggest the existence of three enzyme–substrate intermediates which can be detected and accumulated.

The rapidity of enzyme-catalyzed reactions and the concomitant short life times of intermediates have been a major problem in studying the dynamic processes which occur during catalysis. However, knowledge of the existence and structure of intermediates on the reaction pathway is necessary before specific details of the mechanism may be successfully resolved. Much of our current understanding of enzyme mechanisms has been obtained through the use of kinetically nonspecific substrates and substrate analogues. However, the fact that intermediates from such pseudo-substrates can be isolated does not necessarily indicate that similar intermediates are on the productive pathway for good substrates. Consequently, a method using specific substrates which permits the detection and characterization of intermediates would be very valuable. We have been developing such a method utilizing subzero temperatures and fluid solvent systems (Fink, 1973a,b, 1976a,b; Fink and Wildi, 1974; Fink et al., 1976). The potential of such an approach was first clearly shown by Douzou's studies on peroxidase (Douzou et al., 1970). The procedure not only allows the accumulation of kinetic and thermodynamic information, which may otherwise be very difficult to obtain, but also permits the trapped intermediates to be studied by a variety of chemical and physical techniques to provide details of their structure.

The methodology is predicated on the following basis. A drop of 100 °C will decrease the rate of a typical step in an enzyme-catalyzed reaction by a factor of 10^5 to 10^8. Thus, 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 free energies and enthalpies of activation are such that at the very low temperatures the faster reactions precede the slower ones, it should be possible to accumulate each intermediate successively. The potential of this cryoenzymological approach for detecting and accumulating intermediates in enzyme-catalyzed reactions has been demonstrated in recent studies with a number of enzymes, e.g., peroxidase (Douzou et al., 1970), chymotrypsin (Fink and Wildi, 1974; Fink, 1976a; Fink

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et al., 1976), elastase (Fink and Ahmed, 1976; Alber et al., 1976), β-glucosidase (Fink and Good, 1974), β-galactosidase (Fink and Angelides, 1975), and bacterial luciferase (Balny and Hastings, 1975).

In order to preclude problems of rate-limiting enzyme-substrate diffusion, it is necessary to employ aqueous-organic solvent systems which are fluid to temperatures in the vicinity of −100 °C or below. For the low-temperature technique to provide meaningful mechanistic data, one must show that the enzyme-catalyzed reaction in aqueous-organic solvent systems at subzero temperatures involves the same catalytic reaction pathway as exists under normal conditions. The initial step in this direction is the demonstration that the catalytic and structural properties of the enzyme are not perturbed in any untoward manner by the cryosolvent.

There is considerable evidence indicating that papain-catalyzed hydrolyses of acyl amino acid derivatives involve an acyl-enzyme intermediate (Lowe and Williams, 1965; Brubacher and Bender, 1966; Glazer and Smith, 1971; Drenth et al., 1975). There have also been reports that papain-catalyzed hydrolyses may involve intermediates in addition to the initial noncovalent Michaelis complex and the acyl enzyme (Lake and Lowe, 1966; Henry and Kirsch, 1967; Holloway and Hardman, 1973). The present study was undertaken to find suitable cryosolvents for carrying out low-temperature intermediate-trapping experiments with papain. Several observations suggest that papain is fairly resistant to organic solvent-induced denaturation (e.g., Barel and Glazer, 1969). To avoid complications from transesterification resulting from nucleophilic competition for the acyl enzyme, a nonalcoholic solvent system was desirable. In this report, we present evidence that 7.65 M aqueous dimethyl sulfoxide has no apparent adverse effects on the structure and catalytic properties of papain and is suitable for subzero temperature studies on the mechanism of papain catalysis. A less detailed investigation also indicates the suitability of aqueous ethanol solvent systems. Preliminary experiments using low temperatures to allow the detection and accumulation of intermediates are also reported.

Experimental Procedures

Materials. N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester was obtained from Cyclo (Lot D-1308) and recrystallized from ethanol–acetonitrile, mp 150–151 °C. N^{α}-carbobenzoxy-L-lysine methyl ester was obtained from Cyclo (Lot N-1710) and used without further purification. Papain, a crystalline suspension, was obtained from Worthington (Lot No. 35M961), activated with mercaptoethanol, and purified by affinity chromatography (Blumberg et al., 1970). The active enzyme was stored at 4 °C. Activity was determined using either a burst assay with N^{α}-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester (Bender et al., 1966) or a kinetic assay with N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester (Bender and Brubacher, 1966). Both gave results in good agreement with each other. Aqueous organic solvent buffers, enzyme, and substrate solutions were prepared as described previously (Fink, 1973a, 1976a). All experiments were done in the presence of 2.5×10^{-5} M EDTA[1]. Dimethyl sulfoxide, reagent grade, from Mallinckrodt was distilled from calcium hydride under vacuum at 37 °C, recrystallized at 2 °C, and stored at or below 4 °C. Cryosolvents were prepared on a v/v basis at 0 °C.

Methods. Kinetic and ultraviolet absorption measurements were performed on a Cary Model 118C spectrophotometer. Fluorescence spectra were obtained on a Perkin Elmer MPF-4 spectrophotometer equipped with a special thermostated sample-cell holder. Excitation was at 290 nm, unless otherwise specified. Circular dichroic spectra were obtained with a Jasco J20 spectropolarimeter equipped with a thermostated brass-block sample-cell holder. A 1-cm pathlength cell was used in all cases. The subzero temperature spectrophotometric (absorption) experiments were carried out in a specially constructed double-walled quartz cell, in which the temperature control (±0.2 °C) was achieved by circulating ethanol from a Hetofrig Ultra Cryotherm or a Neslab Model LT9 constant-temperature bath.

Spectral measurements at subzero temperatures were performed in the following manner. Appropriate concentrations of enzyme and substrate were mixed in aqueous organic buffer solution at 0 °C, taken up into precooled syringes, mounted on a six-jet tangential mixing block, and cooled immediately to the desired temperature. The contents were then injected into the precooled cell in the spectrophotometer using a pneumatic-driven plunger. Mixing was both rapid and homogenous, as determined in tests using dye solutions. To determine k_{cat} and K_m, either initial velocity or a procedure based on the complete reaction curve (Bender et al., 1966) was used. In the experiments with varying concentrations of dimethyl sulfoxide, both procedures were used and in good agreement with each other. In the experiments to measure k_{cat} as a function of temperature, the following procedure was used. Enzyme and substrate solution (saturating conditions, i.e., zero-order kinetics) were mixed at −45 °C and the temperature was raised in 5 °C increments. The slope of the resulting straight line was used to calculate k_{cat}. In addition, for determination of k_{cat} and K_m at low temperatures, the complete reaction curve was used. Ultraviolet difference spectra at 0 °C were obtained by the method of Herskovits (1967). In all spectral experiments, the temperature was monitored throughout the duration of the experiment using a thermocouple and digital voltmeter.

The apparent protonic activity, pH* (the apparent pH *in* aqueous-organic solutions), was determined using a Radiometer glass electrode and calomel reference electrode at either 0 or 25 °C. Values of pH* at subzero temperatures were obtained from the known temperature dependence of pH* (Hui Bon Hoa and Douzou, 1973). Buffer systems used were 0.1 M formate (pH* 3–5), and 0.2 M acetate (pH* 5–7). All aqueous-organic solvents were made up to 0.1 M ionic strength using KCl. The method of Brandts and Lumry (1963) was used to obtain thermodynamic information on protein denaturation in 7.65 M dimethyl sulfoxide, pH* 3.5. All measurements were performed at least in duplicate.

Results

Effect of Dimethyl Sulfoxide on the Catalytic Parameters. N^{α}-Carbobenzoxy-L-lysine *p*-nitrophenyl ester was chosen as the main substrate for this investigation because, in addition to being the most specific (kinetically) known substrate, the deacylation step has been shown to be rate limiting (Bender and Brubacher, 1966). Papain showed no loss of activity toward N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester after 5 h, relative to controls in aqueous solutions, when incubated at 4.0 °C in 7.65 M (60% v/v) dimethyl sulfoxide at pH* 4.0 and 6.1. After 40 h at 4 °C, the dimethyl sulfoxide incubated enzyme had 94% activity of the control. After 140 h of incubation at −17 and −60 °C, the solvent-treated enzyme had, respectively, 100 and 104% of the activity of the control. Gradual loss of activity was observed at 25.0 °C, pH* 6.1 in

[1] Abbreviations used are: uv, ultraviolet; EDTA, (ethylenedinitrilo)-tetraacetic acid.

TABLE I: Effect of Dimethyl Sulfoxide on k_{cat} and K_m for the Papain-Catalyzed Hydrolysis of N^{α}-Carbobenzoxy-L-lysine *p*-Nitrophenyl Ester.

[Me_2SO] (M)	k_{cat}[a] (s^{-1})	K_m (M)
0	5.0	1.0×10^{-5}
1.92	4.8 (4.3)	2.5×10^{-5}
3.84	3.2 (3.5)	5.6×10^{-4}
5.12	2.94 (3.0)	1.1×10^{-3}
6.40	2.54 (2.5)	3.2×10^{-3}
7.68	2.08 (2.0)	1.0×10^{-2}

[a] Values in parentheses are those calculated based on the decreased water concentration. Conditions were: pH* 6.1, 0 °C, μ = 0.1 M, E_0 = 5.5×10^{-7} to 2.2×10^{-6} M, S_0 = 2.0×10^{-3} to 5.0×10^{-2} M, 2.5×10^{-5} M EDTA.

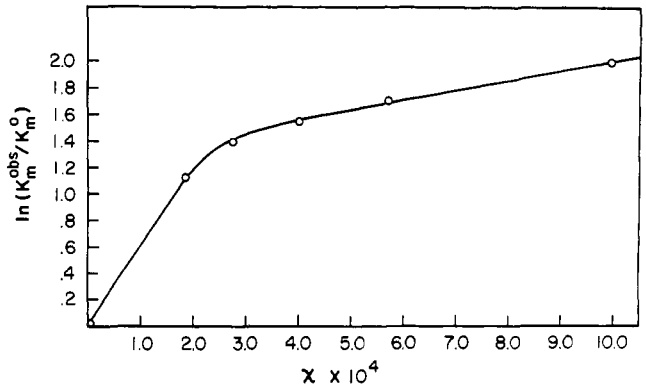

FIGURE 1: The effect of dimethyl sulfoxide on K_m for the papain-catalyzed hydrolysis of N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester, plotted according to eq 1.

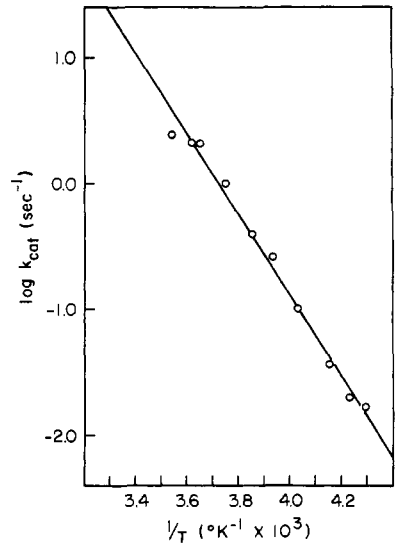

FIGURE 2: The Arrhenius plot of k_{cat} for the papain-catalyzed hydrolysis of N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester. Conditions were 7.65 M dimethyl sulfoxide, pH* 6.1, E_0 = 3.0×10^{-6} M, S_0 = 1.0×10^{-3} M.

7.65 M dimethyl sulfoxide, but not at temperatures below 5 °C, in comparison to the time scale of the reactions being investigated.

The effect of increasing concentrations of dimethyl sulfoxide on k_{cat} and K_m for the papain-catalyzed hydrolysis of N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester is shown in Table I. As would be expected, the value of k_{cat} decreased in proportion to the decreased water concentration. However, an exponential increase in K_m occurred as the solvent concentration increased. This effect on K_m is similar to that previously observed with chymotrypsin (Fink, 1973a) and trypsin (Fink, 1974a), which we have attributed to a combination of dielectric and competitive inhibition effects (Fink, 1974b). The data for K_m, which are inconsistent with competitive inhibition alone, have, therefore, been plotted (Figure 1) in accordance with eq 1 (Clement and Bender, 1963; Mares-Guia and Figueiredo, 1972; Fink, 1974b)

$$K_m^{obsd}/K_m^{\circ} = e^{AX}[1 + ([Me_2SO]/K_D)] \qquad (1)$$

in which K_m° is the value of K_m in the absence of dimethyl sulfoxide, A is a constant, X is the difference in the reciprocals of the dielectric constants with and without dimethyl sulfoxide at 0 °C (Travers and Douzou, 1974), [Me_2SO] is the concentration of dimethyl sulfoxide, and K_D is the dissociation constant for the binding of dimethyl sulfoxide to the active site. A good fit of the data is obtained when $A = 3.42 \times 10^3$ and $K_D = 1.78 \times 10^{-1}$ M in eq 1.

The pH* dependence for the papain-catalyzed hydrolysis of N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester at 0 °C, 7.65 M dimethyl sulfoxide, was not substantially perturbed from that in aqueous solution (Bender and Brubacher, 1966). The values obtained for pK_1 and pK_2 for k_{cat} were 4.2 and ≥8.5, respectively.

Alternate Cryosolvents. The stability of papain in a number of other cryosolvents was investigated. For example, when papain was incubated in 60% (v/v) aqueous *N,N'*-dimethylformamide at 4 °C, a rapid initial decrease in catalytic activity was observed (about 10% remaining at 1 h), followed by a subsequent slow increase to greater than 100% activity of the control. A detailed explanation of this phenomenon will be reported elsewhere.

When papain was incubated at 4 °C in 60% (v/v) aqueous methanol, pH* 6.1, a reduction in catalytic activity to a level 60% that of the control was observed, with a first-order rate constant of 3.9×10^{-5} s^{-1}. In identical experiments using 70% aqueous methanol, a total loss of activity was observed, the loss in activity being first-order with a rate constant of 8.0×10^{-6} s^{-1}.

When papain was incubated in 50, 70, or 80% (v/v) ethanol, pH* 6.1, no loss in activity after 48 h was observed at −17 °C. At 4 °C, more than 80% of the catalytic activity was retained after 48 h at each of these concentrations. The following values for k_{cat} and K_m were obtained at 0 °C, pH* 6.6: for 0%, k_{cat} = 5.0 s^{-1} and $K_m = 1.0 \times 10^{-5}$ M; for 20%, 2.2 s^{-1} and 3.3×10^{-5} M; for 50%, 2.0 s^{-1} and 3.4×10^{-4} M; for 60%, 5.1 s^{-1} and 8.7×10^{-4} M; for 70%, 8.3 s^{-1} and 1.2×10^{-3} M; and for 80%, 12.0 s^{-1} and 6.5×10^{-3} M, respectively.

Effect of Temperature. Figure 2 shows the effect of subzero temperatures on the deacylation rate of the papain-catalyzed hydrolysis of N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester in 7.65 M dimethyl sulfoxide, pH* 6.1. From the slope of the Arrhenius plot, a value of $E_a = 14.6 \pm 0.4$ kcal mol^{-1} was obtained. The value for k_{cat} of 47.4 s^{-1}, obtained by extrapolation to 25 °C and corrected for decreased water concentration, is in excellent agreement with the value of 44.5 s^{-1}, determined in the absence of dimethyl sulfoxide (Bender and Brubacher, 1966). No deviations from linearity were observed in the Arrhenius plot over the temperature range investigated (0 to −45 °C). At temperatures below −45 °C, the rate of

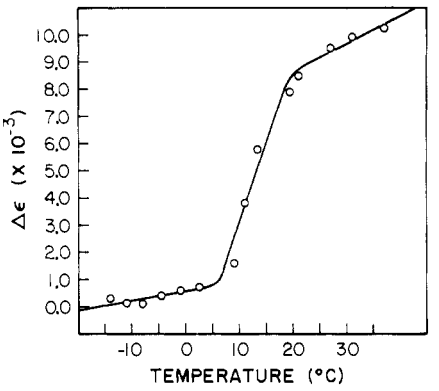

FIGURE 3: The thermal denaturation of papain in 7.65 M dimethyl sulfoxide, pH* 3.5, $E_0 = 1.4 \times 10^{-5}$ M.

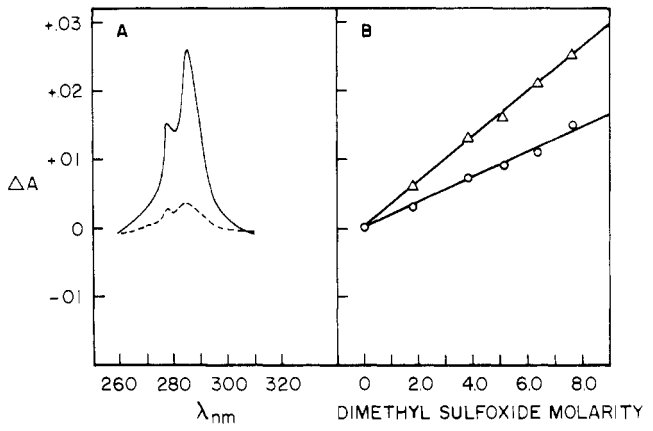

FIGURE 4: The effect of dimethyl sulfoxide concentration on the ultraviolet difference spectrum of papain. (A) Difference spectra for 1.92 and 7.65 M dimethyl sulfoxide. (B) ΔA_{278} (△), and ΔA_{284} (○), as a function of solvent concentration. Conditions: pH* 6.1, 0 °C, $E_0 = 1.0 \times 10^{-5}$ M.

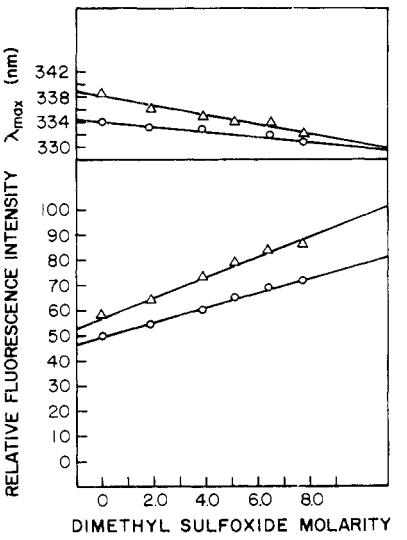

FIGURE 5: The effect of increasing dimethyl sulfoxide concentrations on the fluorescence emission of papain. Conditions: pH* 6.1, 0 °C, $E_0 = 1.8 \times 10^{-6}$ M. Excitation at 260 (○) and 290 (△) nm.

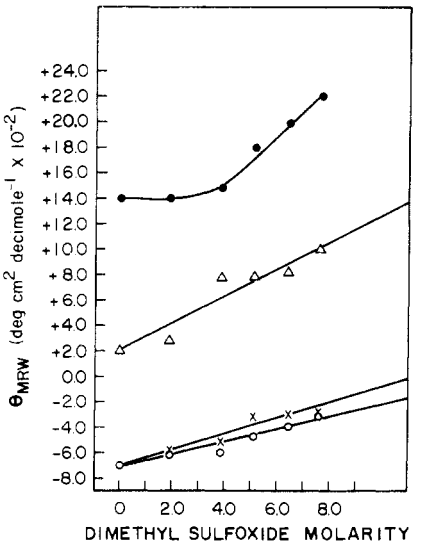

FIGURE 6: The effect of dimethyl sulfoxide concentration on the circular dichroism of papain. Conditions: pH* 6.1, 0 °C, $E_0 = 1.6 \times 10^{-5}$ M; (○) 254, (×) 272, (△) 290, and (●) 298 nm.

deacylation and turnover became too slow for convenient measurement. Values of K_m were constant, within experimental error, over the range 0 to −20 °C.

Acylation Under Nonturnover Conditions. At temperatures below −45 °C, the reaction of papain with N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester appeared as a first-order release of *p*-nitrophenol, corresponding to the formation of the acyl enzyme. A typical acylation, at −70 °C, pH* 7.0, had a first-order rate constant of 7.2×10^{-3} s^{-1} for $E_0 = 1.4 \times 10^{-5}$ M, $S_0 = 1.0 \times 10^{-3}$ M. No subsequent release of *p*-nitrophenol was detected over a time period of hours. The amount of *p*-nitrophenol liberated in the acylation reaction was 1.2×10^{-5} M, in good agreement with the active-site normality of the enzyme used, 1.4×10^{-5} M (determined by burst titration at 25 °C, no dimethyl sulfoxide). Similar 1:1 stoichiometry of the liberated *p*-nitrophenol to enzyme concentration was observed in reactions covering a range of temperatures (−40 to −75 °C) and pH* (4.5 to 7.0), and in 12.0 M aqueous ethanol at −70 °C, pH* 6.6. No deacylation was observed in this latter reaction, for which $k_{obsd} = 5.6 \times 10^{-3}$ s^{-1} and $E_0 = 1.2 \times 10^{-5}$ M and $S_0 = 1.3 \times 10^{-3}$ M.

Effects of Dimethyl Sulfoxide on the Native to Denatured Transition. Experiments were carried out to investigate the effect of dimethyl sulfoxide on the denaturation transition of papain. The unfolding and folding processes were monitored using the intrinsic near-uv spectral properties of the enzyme. The process was made completely reversible. As shown in Figure 3, the midpoint of the transition was 12.0 °C at pH* 3.5, 7.65 M dimethyl sulfoxide. The enthalpy calculated from a Van't Hoff plot over the transition region yields a value of $\Delta H° = 80.0$ kcal mol^{-1}.

Effect of Dimethyl Sulfoxide on the Intrinsic Spectral Properties of Papain. To determine whether the high concentrations of organic solvent had any adverse effects on the structure of the enzyme, the ultraviolet, circular dichroic, and fluorescent spectra were monitored as a function of increasing dimethyl sulfoxide 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 in Figures 4–6, whereas structural effects, especially cooperative ones, would be anticipated to result in distinct deviations in such plots (Chen et al., 1969). Such deviations were, indeed, observed at higher dimethyl sulfoxide concentrations, which also corresponded to loss of catalytic activity. The small blue shift in λ_{max} for fluorescence emission

of papain as the dimethyl sulfoxide concentration increased was also observed when papain was replaced by L-tryptophan in these experiments, and is ascribed to a viscosity effect. The effects of increasing methanol and ethanol concentrations on the intrinsic fluorescence emission of papain were examined at 0 °C, pH* 6.1, $E_0 = 1.8 \times 10^{-6}$ M. In methanol, a sharp break in the relative intensity vs. concentration plot was observed at 10 M (50%) when excitation was at 260 nm and emission monitored at 330 nm. Linear changes in λ_{max} and relative fluorescence emission intensity were observed as a function of increasing ethanol concentration to 13.7 M (80% v/v).

Preliminary Low-Temperature Experiments with N^{α}-Carbobenzoxy-L-lysine Methyl Ester. Both the intrinsic fluorescence emission and near-uv absorption of papain were used to monitor the reaction with N^{α}-carbobenzoxy-L-lysine methyl ester in 7.65 M dimethyl sulfoxide. In addition, the rate of formation and concentration of the acyl enzyme were followed by assaying aliquots at −40 °C with N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester, the "burst" of *p*-nitrophenol liberated being proportional to the amount of free enzyme present. When the reaction was monitored in the uv region, the maximum spectral changes were observed in the vicinity of 276 nm. On mixing, an initial very rapid decrease occurred, followed by a subsequent fast decrease in absorbance ($k_{obsd} = 2.2 \times 10^{-2}$ s^{-1}, −40.8 °C, $E_0 = 1.0 \times 10^{-5}$ M, $S_0 = 1.0 \times 10^{-3}$ M) (reaction 2). A further reaction (3) was observed as a slow increase in A_{276} ($k_{obsd} = 5.7 \times 10^{-5}$ s^{-1}, pH* 6.1, −40 °C). Reaction 2 was found to be pH* independent. Reaction 3 is tentatively ascribed to acylation, based on the similar rates of acylation obtained from corresponding experiments monitored by the *p*-nitrophenol "burst" technique. Changes in the fluorescence emission were also observed during the course of reaction of the methyl ester with papain at subzero temperatures.

Discussion

The experiments reported here were undertaken to demonstrate that the productive catalytic pathway for papain-catalyzed reactions is not adversely affected by 7.65 M dimethyl sulfoxide and subzero temperatures. If undesirable effects were caused by such conditions, they should appear as changes in both the structural and catalytic properties of the enzyme. The catalytic parameters, in particular, should be very sensitive to any adverse effects.

Effect of Dimethyl Sulfoxide on Catalysis. Undoubtedly, the most sensitive tests for detecting effects on an enzyme are those involving its catalytic properties. It is well known that minor structural changes can produce drastic effects on enzyme catalysis. It is also apparent that the stability of proteins in high concentrations of organic solvents is quite temperature dependent. As can be seen from Figure 3, papain is stable in the native form at 5 °C (pH* 3.5) but fully denatured at temperatures above 18 °C.

The large acylation/deacylation rate constant ratio for the papain-catalyzed hydrolysis of N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester means that $k_{cat} \simeq k_3$, the deacylation rate constant in Scheme I (Bender and Brubacher, 1966). Scheme I is the minimum reaction pathway for papain catalysis; ES is the Michaelis complex, EA the acyl enzyme, and K_S the enzyme-substrate dissociation constant. Since water is a participant in the deacylation reaction (i.e., $k_3 = k_3'[H_2O]$) the value of k_{cat} should decrease in proportion to the increasing dimethyl sulfoxide concentrations, as observed (Table I). The lack of perturbation of pH dependence of k_{cat} in the presence of the cryosolvent supports the conclusion that the dimethyl sulfoxide does not affect the deacylation nor the pK of the ion pair in the active site (Drenth et al., 1975).

SCHEME I.

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

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} (e.g., Singer, 1962; Clement and Bender, 1963; Fink, 1973a, 1974a). Various attempts have been made to correlate these observations with dielectric and competitive inhibition effects (Clement and Bender, 1963; Kasserra and Laidler, 1970; Mares-Guia and Figueiredo, 1972; Fink, 1974b). For N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester $K_m = k_3K_S/(k_2 + k_3)$ (for Scheme I) and, thus, the observed increase 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 dimethyl sulfoxide will tend to be more strongly bound in the hydrophobic areas of substrate binding than water. Such an effect can be overcome by using large substrate concentrations to saturate the enzyme, with no change in the reaction mechanism or pathway, and reflects the increasingly less favorable partitioning of the substrate for the active site, relative to the bulk solvent, as the cosolvent concentration increases. Solvation of the substrate binding site, undoubtedly, also plays a significant role. The data for K_m in the case of papain are similar to that observed with α-chymotrypsin and trypsin (Fink, 1974a,b) and can also be accounted for by a combination of dielectric and competitive inhibition effects by the organic solvent, as shown in Figure 1. To further support this conclusion, experiments involving the effect of dimethyl sulfoxide on the binding of competitive inhibitors to papain are planned.

Effects of Dimethyl Sulfoxide and Subzero Temperatures on the Structure of Papain. The native conformation of an enzyme in solution is a delicate balance of attractive and repulsive interactions involving mainly electrostatic, hydrophobic, hydrogen bonding and Van der Waals forces, and including competition between solvent and intramolecular groups. The effects of organic solvents on the structure of the enzyme are likely to arise from perturbations of these interactions. Little effect on the electrostatic and hydrogen-bond interactions would be expected under the experimental conditions (Fink, 1976b). However, since hydrophobic interactions play a major role in maintaining the tertiary structure of papain, their disruption would have a major impact on the structure of the enzyme. It seems likely that the combination of low temperature and high dimethyl sulfoxide concentration will have only minimal effects on *existing* hydrophobic interactions in the native enzyme. A more detailed discussion of the effects of subzero temperatures and organic solvents on protein structure is presented elsewhere (Fink, 1976b). Several precedents exist to indicate that some proteins are apparently unaffected by high concentrations of dimethyl sulfoxide, e.g., lysozyme (Hamaguchi, 1964; Fink and Weber, unpublished results), trypsin (Inagami and Sturtevant, 1960; Vratsanos, 1960; Lukton et al., 1961; Bettleheim and Senatore, 1964; Fink, 1974a), chymotrypsin (Fink, 1973a, 1974b), and β-galactosidase (Fink and Angelides, 1975; Fink and Magnusdottir, in preparation). Additional stabilizing influences include the presence of substrate and the high enthalpy of activation

for protein denaturation (Pohl, 1968), both of which will tend to maintain the native conformation (Fink, 1976b).

The potential of ultraviolet difference spectra, fluorescence emission, and circular dichroism to detect conformational changes, as well as solvent perturbations of exposed chromophores, has been well established. The ultraviolet absorption of dimethyl sulfoxide precludes examination of the spectral properties of the enzyme in the region below 250 nm. Consequently, the ultraviolet absorption and fluorescence spectra reflect changes mainly in the tyrosine and tryptophan residues of the enzyme. Since papain has many such residues throughout its structure, any substantial change in the structure should be apparent from such spectral studies. The effects of increasing dimethyl sulfoxide concentration, as well as subzero temperatures, on the absorption fluorescence and circular dichroic spectra of papain (Figures 4–6) are similar to those observed with chymotrypsin (Fink, 1973a) and trypsin (Fink, 1974a), and are consistent with solvent effects on the exposed aromatic residues (Chignell and Gratzer, 1968). Evidence that these studies would show up structural effects is found in our observations that a sharp break occurs in the relative fluorescence emission vs. methanol concentration plot at 10 M (50%) methanol. The linear relationships observed in Figures 4–6, thus, indicate that major structural changes do not occur in papain in aqueous dimethyl sulfoxide up to 7.65 M concentration. Additional confirmation for this conclusion is found in the fluorescence emission data, which indicate no changes in the tyrosine → tryptophan energy transfer (excitation at 260 nm, emission at 330–340 nm) as a function of solvent concentration.

Alternative Cryosolvents. Methanol and ethanol-based cryosolvents have much lower viscosities than dimethyl sulfoxide solvent systems, which may be advantageous in many situations. The alcoholic solvents have the disadvantage that nucleophilic attack on the acyl enzyme, leading to formation of an ester product, will increase the deacylation rate, hence, necessitating lower temperatures to stop the deacylation reaction. Preliminary investigations indicate that neither methanol nor *N,N'*-dimethylformamide provide suitable cryosolvents for papain. However, ethanol-based systems, up to 80% (v/v), appear suitable, based on the stability of the enzyme, and the effects of ethanol on the catalytic parameters and the intrinsic fluorescence of the enzyme. As in the case with dimethyl sulfoxide, the increase in K_m as a function of increasing ethanol concentration is exponential, yielding a linear log K_m vs. concentration plot. Presumably, the same underlying phenomena, a combination of competitive inhibition and dielectric effects, are responsible. The effect of ethanol on k_{cat} is more complex than simply due to the decreased water concentration and increased nucleophilicity of ethanol. Such anomalous effects have previously been attributed to binding of alcohols to papain (Fink and Bender, 1969).

Subzero Temperature Experiments with N^{α}-Carbobenzoxy-L-lysine p-Nitrophenyl Ester. The linear Arrhenius plot for deacylation under substrate-saturating conditions indicates no change in the rate-determining step or adverse structural effects occurring in the accessible temperature range below 0 °C. Additional evidence supporting the premise that subzero temperatures and 7.65 M dimethyl sulfoxide have no significant effect on at least the deacylation step is the excellent agreement between the values of k_3 obtained at 25 °C in the absence of organic solvent and that calculated from the data of Figure 2.

The advantages of using subzero temperatures to allow the detection and accumulation of an enzyme–substrate intermediate are readily apparent in the experiments carried out below −45 °C. Under these non-steady-state, nonturnover conditions, the acylation reaction appears as a first-order release of *p*-nitrophenol and the acyl-enzyme concentration is stoichiometric with that of the enzyme. The procedure is, thus, admirably suited for producing substantial yields of metastable acyl papains. We are currently using this approach to make crystalline specific acyl papains which will be suitable for x-ray crystallographic studies, analogous to our recent studies on crystalline acyl enzymes of the serine proteases (Fink and Ahmed, 1976).

The results of our preliminary studies of the reaction of papain with N^{α}-carbobenzoxy-L-lysine methyl ester in the −70 to −15 °C range suggest that revision of Scheme I to include an intermediate between ES and EA will be necessary. A more detailed report will be given shortly.

On the basis of the results obtained in this investigation, we are confident that the mechanism of catalysis by papain in 7.65 M dimethyl sulfoxide at subzero temperatures is essentially the same as that in the absence of the solvent and at ambient temperatures.

References

Alber, T., Petsko, G. A., and Tsernoglou, D. (1976), *Nature (London) 263,* 297–300.

Balny, C., and Hastings, J. W. (1975), *Biochemistry 14,* 4719–4722.

Barel, A. O., and Glazer, A. N. (1969), *J. Biol. Chem. 244,* 268–273.

Bender, M. L., Begue-Cantón, M. L., Blakeley, R. L., Brubacher, L. J., Feder, J., Gunter, C. R., Kézdy, F. J., Killheffer, J. V., Jr., Marshall, T. H., Miller, C. G., Roeske, R. W., and Stoops, J. K. (1966), *J. Am. Chem. Soc. 88,* 5890–5898.

Bender, M. L., and Brubacher, L. J. (1966), *J. Am. Chem. Soc. 88,* 5880–5889.

Bettleheim, F. A., and Senatore, P. (1964), *J. Chem. Phys. 61,* 105–111.

Blumberg, S., Schechter, I., and Berger, A. (1970), *Eur. J. Biochem. 15,* 97–102.

Brandts, J., and Lumry, R. (1963), *J. Phys. Chem. 67,* 1484–1494.

Brubacher, L. J., and Bender, M. L. (1966), *J. Am. Chem. Soc. 88,* 5871–5880.

Chen, R. F., Edelhoch, H., and Steiner, R. F. (1969), *in Phys. Princ. Tech. Protein Chem., 1969–1970, Part A,* 171.

Chignell, D. A., and Gratzer, W. (1968), *J. Phys. Chem. 72,* 2934–2941.

Clement, G. E., and Bender, M. L. (1963), *Biochemistry 2,* 836–843.

Douzou, P., Sireix, R., and Travers, F. (1970), *Proc. Natl. Acad. Sci. U.S.A. 66,* 787–792.

Drenth, J., Swen, H. M., Hoogenstraaten, W., and Sluyterman, L. A. AE. (1975), *K. Ned. Akad. Wet., Proc., Ser. C 78,* 104–110.

Fink, A. L. (1973a), *Biochemistry 12,* 1736–1742.

Fink, A. L. (1973b), *Arch. Biochem. Biophys. 155,* 473–474.

Fink, A. L. (1974a), *J. Biol. Chem. 249,* 5027–5032.

Fink, A. L. (1974b), *Biochemistry 13,* 277–280.

Fink, A. L. (1976a), *Biochemistry 15,* 1580–1586.

Fink, A. L. (1976b), *J. Theor. Biol. 61,* 419–445.

Fink, A. L., and Ahmed, A. I. (1976), *Nature (London) 263,* 294–297.

Fink, A. L., and Angelides, K. J. (1975), *Biochem. Biophys.*

Res. Commun. 64, 701-707.
Fink, A. L., and Bender, M. L. (1969), *Biochemistry 8,* 5109-5118.
Fink, A. L., and Good, N. E. (1974), *Biochem. Biophys. Res. Commun. 58,* 126-131.
Fink, A. L., McGarraugh, G., and Farzami, B. (1976), *J. Biol. Chem.* (submitted).
Fink, A. L., and Wildi, E. (1974), *J. Biol. Chem. 249,* 6087-6089.
Glazer, A. N., and Smith, E. L. (1971), *Enzymes 3,* 502-546.
Hamaguchi, K. (1964), *J. Biochem. (Tokyo) 56,* 441-449.
Henry, A. C., and Kirsch, J. F. (1967), *Biochemistry 6,* 3536-3544.
Herskovits, T. T. (1967), *Methods in Enzymol. 11,* 748-775.
Holloway, M. R., and Hardman, M. J. (1973), *Eur. J. Biochem. 32,* 537-546.
Hui Bon Hoa, G., and Douzou, P. (1973), *J. Biol. Chem. 248,* 4649-4654.
Inagami, T., and Sturtevant, J. M. (1960), *Biochim. Biophys. Acta 38,* 64-79.
Kasserra, H. P., and Laidler, K. J. (1970), *Can. J. Chem. 48,* 1793-1802.
Lake, A. W., and Lowe, G. (1966), *Biochem. J. 101,* 402-410.
Lowe, G., and Williams, A. (1965), *Biochem. J. 96,* 199-204.
Lukton, A., Donahue, H., and Bettleheim, F. A. (1961), *Nature (London) 191,* 565-567.
Mares-Guia, M., and Figueiredo, A. F. S. (1972), *Biochemistry 11,* 2091-2098.
Pohl, F. M. (1968), *Eur. J. Biochem. 7,* 146-152.
Singer, S. J. (1962), *Adv. Protein Chem. 17,* 1-68.
Travers, F., and Douzou, P. (1974), *Biochimie 56,* 509-514.
Vratsanos, S. (1960), *Arch. Biochem. Biophys. 90,* 132-138.

Two Functional States of Sarcoplasmic Reticulum ATPase†

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ABSTRACT: The "total" ATPase activity of rabbit sarcoplasmic reticulum (SR) vesicles includes a Ca^{2+}-independent component ("basic") and a Ca^{2+}-dependent component ("extra"). Only the "extra" ATPase is coupled to Ca^{2+} transport. These activities can be measured under conditions in which the observed rates approximate maximal velocities. The "basic" ATPase is predominant in one of the various SR fractions obtained by prolonged density-gradient centrifugation of SR preparations already purified by repeated differential centrifugations and extractions at high ionic strength. This fraction (low density, high cholesterol) has a protein composition nearly identical with that of other SR fractions in which the "extra" ATPase is predominant. In these other fractions the ratio of "extra" to "basic" ATPase activities is temperature dependent, being approximately 9.0 at 40 °C and 0.5 at 4 °C. In all the fractions and at all temperatures studied, similar steady-state levels of phosphorylated SR protein are obtained in the presence of ATP and Ca^{2+}. Furthermore, in all cases the "basic" (Ca^{2+}-independent) ATPase acquires total Ca^{2+} dependence upon addition of the nonionic detergent Triton X-100. This detergent also transforms the complex substrate dependence of the SR ATPase into a simple dependence, displaying a single value for the apparent K_m. The experimental findings indicate that the ATPase of rabbit SR exists in two distinct functional states (E_1 and E_2), only one of which (E_2) is coupled to Ca^{2+} transport. The $E_1 \rightleftharpoons E_2$ equilibrium is temperature-dependent and entropy-driven, indicative of its relation to the physical state of the ATPase protein in its membrane environment. The nonlinearity of Arrhenius plots of Ca^{2+}-dependent ("extra") ATPase activity and Ca^{2+} transport is explained in terms of simultaneous contributions from both the free energy of activation of enzyme catalysis and the free energy of conversion of E_1 to E_2. Thermal equilibrium between the two functional states is drastically altered by factors which affect membrane structure and local viscosity.

Vesicular fragments of sarcoplasmic reticulum (SR)[1] provide an isolated membrane system specifically differentiated for ATP-dependent calcium transport (Ebashi, 1964; Hasselbach, 1964; Weber, 1966). In the absence of Ca^{2+}, rabbit SR vesicles display an ATPase activity ("basic" activity) which markedly increases on addition of Ca^{2+} ("total" activity). The difference between "total" and "basic" activities is known as the "extra" or Ca^{2+}-dependent ATPase. Only this "extra" ATPase is coupled to Ca^{2+} transport (Hasselbach, 1964).

We now find that the ratio of Ca^{2+}-dependent to Ca^{2+}-independent activities varies in SR fractions of different densities but identical protein composition. Furthermore, this ratio is highly temperature dependent. In all cases, Ca^{2+}-independent ATPase acquires Ca^{2+} dependence in the presence of the detergent Triton X-100. These and other experiments described below indicate that the ATPase of rabbit SR resides in thermal equilibrium between two distinct functional states, only one state being coupled to Ca^{2+} transport. Conversion of

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[1] Abbreviations used: SR, sarcoplasmic reticulum; "extra" and "basic" ATPase activity, the Ca^{2+}-dependent and Ca^{2+}-independent components of "total" ATPase activity, respectively; Mops, morpholinopropanesulfonate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetate.