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Effect of Dimethyl Sulfoxide on the Interaction of Proflavine with α-Chymotrypsin†

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With the Technical Assistance of Mr. David E. Griffith

ABSTRACT: The effect of dimethyl sulfoxide on the binding of the competitive inhibitor proflavine to α-chymotrypsin has been investigated as a function of dimethyl sulfoxide concentration. The change in K_p (the dissociation constant for the α-chymotrypsin–proflavine complex) with increasing dimethyl sulfoxide concentration exactly parallels the previously observed increase in K_m (A. L. Fink (1973), *Biochemistry 12*, 1736) indicating that the effect on K_m reflects a change in K_s, *i.e.* substrate binding. The temperature dependence of K_p was found to be negligible. The effect of dimethyl sulfoxide on K_m and K_p could be quantitatively accounted for by a combination of competitive inhibition and dielectric effects by dimethyl sulfoxide on substrate and inhibitor binding.

In connection with our investigations of enzyme-catalyzed reactions at subzero temperatures and in aqueous–organic solvent systems, we have observed that the main difference in the reaction under such conditions compared with normal conditions is an increase in K_m. For example, we have observed a linear relationship between log K_m and dimethyl sulfoxide concentration in the α-chymotrypsin–aqueous dimethyl sulfoxide system (Fink, 1973). This increase in K_m was tenta-

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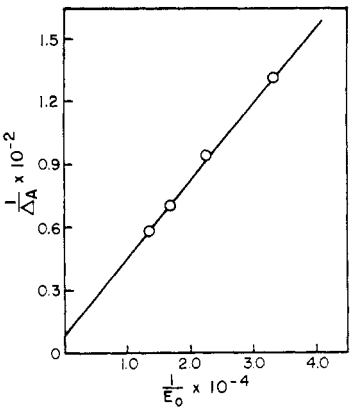

FIGURE 1: Determination of K_p for 30% aqueous dimethyl sulfoxide, pH 6.5, 5°, μ = 0.1 M; [proflavine] = 1×10^{-6} M; $K_p = 5.05 \times 10^{-4}$ M.

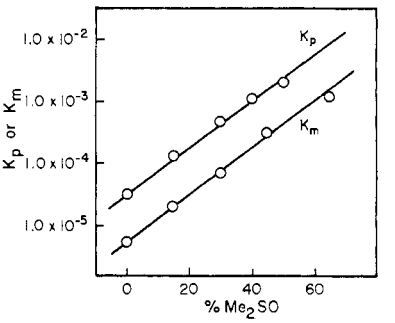

FIGURE 2: The effect of dimethyl sulfoxide on K_m for the reaction with *N*-acetyl-L-tryptophan *p*-nitrophenyl ester, and K_p for proflavine binding, with α-chymotrypsin. The error in K_p is ±15%.

tively ascribed to an increase in K_s (the dissociation constant for substrate binding) which was caused by the less polar dimethyl sulfoxide being more strongly attracted to the substrate binding site than water. Based on the currently accepted equation (1) for α-chymotrypsin-catalyzed hydrolysis of esters

$$E + S \overset{K_s}{\rightleftharpoons} ES \xrightarrow{k_2} EA \xrightarrow{k_3} E + P_2 \qquad (1)$$
$$\qquad\qquad\qquad\qquad\quad + P_1$$

(Hess, 1971) (where EA = acyl-enzyme, k_2 and k_3 represent acylation and deacylation, respectively, and $K_s = k_{-1}/k_1$, *i.e.*, $k_{-1} \gg k_2$) the expression for K_m is given by $K_m = k_3K_s/(k_2 + k_3)$, which reduces to $K_m = k_3K_s/k_2$ since $k_2 \gg k_3$ for most ester substrates. Since the value of k_3 decreases with increasing dimethyl sulfoxide (due to the decreased water concentration) the increase in K_m could arise from either an increase in K_s, a decrease in k_2, or a combination of these. In order to fully ascertain the effect of aqueous–organic solvent systems on the enzyme-catalyzed reaction it is necessary to determine which of the parameters is affected.

For technical reasons (see Discussion) it proved impractical to measure k_2 directly. However, if dimethyl sulfoxide does indeed cause an increase in K_s, *i.e.* decreasing substrate binding, then the effect should also be similar on the binding of competitive inhibitors. We have therefore examined the effect of dimethyl sulfoxide on the binding of the competitive inhibitor proflavine. Previous work has shown that proflavine binds to the active site of α-chymotrypsin in a 1:1 stoichiometry and that it is a competitive inhibitor (Bernhard *et al.*, 1966; Glazer, 1965). The binding of proflavine to α-chymotrypsin and its displacement by substrates has been used advantageously in several investigations (*e.g.*, Himoe *et al.*, 1969; Fersht and Requena, 1971) which utilized the fact that the spectra of the free and bound proflavine are different.

Experimental Section

Materials. α-Chymotrypsin, 3× recrystallized, salt-free, was obtained from Worthington, Lot CDI 11C. Stock solutions, 3×10^{-3} M, were prepared daily in 10^{-3} M HCl and assayed by burst titration procedure (Schonbaum *et al.*, 1961).

Proflavine hemisulfate was obtained from K&K Laboratories and recrystallized in the dark from distilled water. A stock solution, 1×10^{-4} M, in distilled water was kept in the dark. *N-trans*-Cinnamoylimidazole, from Nutritional Biochemicals Corp., was recrystallized from dry cyclohexane (mp 133–134°). Dimethyl sulfoxide (Mallinckrodt) was distilled from calcium hydride under vacuum and stored at −20°. Aqueous dimethyl sulfoxide solutions were prepared daily using the procedure described previously (Fink, 1973).

Methods. A Cary Model 14 spectrophotometer with 0–0.2 Å slide-wire was used for monitoring the binding of proflavine to α-chymotrypsin. Cells of 1.0-cm path length and 1-ml volume were placed in the reference and sample compartments at 5° (unless otherwise noted). To each cell was added 1.0 ml of the appropriate aqueous dimethyl sulfoxide solution (pH 6.5). After a base-line scan, an aliquot (10 μl) of proflavine stock solution was added to each cell and the spectrum again recorded (usually from 550 to 400 nm). A series of five 5-μl aliquots of the stock α-chymotrypsin solution were then added to the cell in the sample compartment (corresponding volumes of 10^{-3} M HCl being added to the reference cell), the spectrum being scanned after each addition. The proflavine concentration (1×10^{-6} M) was kept low to avoid aggregation which occurs at higher concentrations (Glazer, 1965) and to give conditions of $[E]_0 \gg$ [proflavine].

Results

The binding of proflavine to α-chymotrypsin may be represented by eq 2 where EP is the enzyme–proflavine complex and

$$E + P \overset{K_p}{\rightleftharpoons} EP \qquad (2)$$

K_p is the dissociation constant. Since the enzyme has negligible absorbance in the region of maximum proflavine–complex absorbance difference it can be shown that $\Delta A = (\epsilon_{EP} - \epsilon_P)[EP]$, where ΔA is the observed difference in absorbance at a given λ and ϵ_{EP} and ϵ_P are the extinction coefficients for bound and free proflavine, respectively, at that λ. If $[E]_0 \gg [EP]$, as would be the case under our experimental conditions, this equation may be rearranged to give

$$\Delta A = \frac{(\epsilon_{EP} - \epsilon_P)[E]_0[P]_0}{K_P + [E]_0}$$

where $[E]_0$ and $[P]_0$ are the total concentrations of enzyme and proflavine, respectively. Therefore, a plot of $1/\Delta A$ *vs.* $1/[E]_0$ will yield a straight line from which K_p (= slope/intercept) may be determined.

When the experimental data were plotted in this manner, a linear relationship resulted (Figure 1) from which the values of K_p were obtained. These are plotted in Figure 2 as a function of dimethyl sulfoxide concentration. The values of K_m for

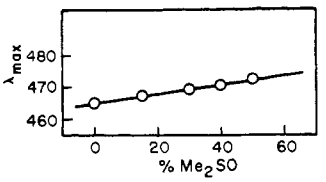

FIGURE 3: The effect of dimethyl sulfoxide on λ_{max} for the absorbance difference between free and bound proflavine.

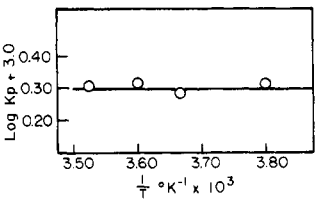

FIGURE 4: The effect of temperature on K_p, pH 6.5, 50% aqueous dimethyl sulfoxide.

N-acetyl-L-tryptophan *p*-nitrophenyl ester are also shown in this figure for comparative purposes.

It was noted that the change in absorbance for a given enzyme concentration decreased with increasing dimethyl sulfoxide concentration and that the λ_{max} for the difference spectra were shifted toward higher wavelengths; see Figure 3. The effect of temperature on K_p was determined in 50% aqueous dimethyl sulfoxide and as shown in Figure 4 was found to be negligible. This experiment was done with 50% rather than 65% dimethyl sulfoxide because a relatively large error was associated with the K_p value obtained in 65% dimethyl sulfoxide due to the small intercept in the $1/\Delta A$ *vs.* $1/[E]_0$ plot.

Discussion

A simple method to determine whether the observed effect of dimethyl sulfoxide on K_m results from an effect on k_2 or K_s would involve the direct measurement of k_2 by following the kinetics of the pre-steady-state reaction. This reaction may be readily followed at temperatures below −40° in the α-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan *p*-nitrophenyl ester in 65% aqueous dimethyl sulfoxide (Fink, 1973). Under conditions of $[S]_0 \gg [E]_0$ the observed first-order rate constant k_{obsd} is given by eq 3, which reduces to eq 4 if

$$k_{obsd} = \frac{(k_2 + k_3)[S]_0 + k_3K_s}{K_s + [S]_0} \quad (3)$$

$$k_{obsd} = \frac{k_2[S]_0}{K_s + [S]_0} \quad (4)$$

$k_3 \simeq 0$. Consequently if $[S]_0 \geq K_s$ one may calculate k_2 and K_s from the observed rate of acylation. If $K_s \gg [S]_0$, then $k_{obsd} = (k_2/K_s)[S]_0$. We have found that at −60° at substrate concentrations to 5×10^{-2} M the observed first-order rate constant for the pre-steady-state acylation of α-chymotrypsin by *N*-acetyl-L-tryptophan *p*-nitrophenyl ester is directly proportional to $[S]_0$. This indicates that the condition $[S]_0 \ll K_s$ holds. Consequently k_2 and K_s cannot separately be determined by this manner. Furthermore, since K_s must be much larger than 5×10^{-2} M, this finding means that K_s must be greatly increased in the presence of 65% dimethyl sulfoxide since K_s at 0% is 2.5×10^{-3} (Zerner *et al.*, 1964). Similarly under conditions of $[E]_0 \gg [S]_0$ it is not experimentally feasible to determine k_2 or K_s separately. Therefore, on the assumption that if dimethyl sulfoxide perturbed the binding of substrates it would have a similar effect on the binding of an inhibitor, we decided to examine the effect on inhibitor binding.

Several previous reports have conclusively shown that proflavine binds almost exclusively at the active site of α-chymotrypsin, and that the spectrum of the bound proflavine is considerably perturbed (Bernhard *et al.*, 1966; Fersht and Requena, 1971). Thus the binding could be monitored spectrophotometrically, and K_p measured directly as a function of dimethyl sulfoxide concentration.

The parallel effects on K_p and K_m, with increasing dimethyl sulfoxide (Figure 2), indicate that the dimethyl sulfoxide decreases the binding of the inhibitor proflavine to the same extent that it affects K_m for *N*-acetyl-L-tryptophan *p*-nitrophenyl ester. The most reasonable explanation to account for this observation is that the K_s component of K_m is affected, and that a given amount of dimethyl sulfoxide perturbs the binding of substrate (K_s) and inhibitor (K_p) to the same degree. The value obtained for K_p at 0% dimethyl sulfoxide, 3.3×10^{-5} M, is in excellent agreement with the literature values, 3.7×10^{-5} M (Bernhard *et al.*, 1966), and 2.2×10^{-5} M (Glazer, 1965).

Since dimethyl sulfoxide is less polar than water, and since at least part of the substrate binding site in α-chymotrypsin is hydrophobic in character (Blow, 1971), it is reasonable to attribute the decreased binding efficiency of substrates and competitive inhibitors in dimethyl sulfoxide to the greater difficulty in displacing dimethyl sulfoxide from the substrate binding site, *i.e.*, a form of competitive inhibition. Furthermore, the reduced polarity and dielectric constant of the aqueous organic solvent compared to water will probably also affect partitioning of substrates or inhibitors between the solvent and the active site, *i.e.* a dielectric effect. An alternative interpretation would be that dimethyl sulfoxide induces a conformational change in the enzyme such that the induced conformation has a reduced affinity for substrates and inhibitors. Attempts to detect such a conformational change have been unsuccessful (Fink, 1973).

If the effect were solely competitive inhibition by dimethyl sulfoxide, plots of K_m^{obsd}/K_m^0 *vs.* $[Me_2SO]$ should be linear, but in fact are not ($K_m^{obsd} = K_m$ for a given concentration of dimethyl sulfoxide, $K_m^0 = K_m$ for 0%). Previous studies involving the effect of organic solvents on chymotrypsin- and trypsin-catalyzed reactions have revealed that a combination of competitive inhibition and dielectric effect can account for the experimental observations (Clement and Bender, 1963; Mares-Guia and Figueiredo, 1972). If the dielectric effect on the binding of dimethyl sulfoxide to α-chymotrypsin is assumed negligible compared to the effect on substrate or inhibitor binding, then eq 5 is obtained (Clement and Bender, 1963) where A is a

$$K_m^{obsd}/K_m^0 = e^{AX}[1 + ([Me_2SO]/K_D)] \quad (5)$$

constant, X is the difference in reciprocals of the dielectric constants of the dimethyl sulfoxide–water mixture and pure water, $[Me_2SO]$ is the dimethyl sulfoxide concentration, and K_D is the dissociation constant for dimethyl sulfoxide bound to α-chymotrypsin. A similar equation holds for K_p (inhibitor binding) except that the value of A might be different. The data for K_m and K_p have been plotted as a function of X, according to eq 5, in Figure 5. Since the values for K_m and K_p almost coincide, the value of A must be very similar for proflavine and *N*-acetyl-L-tryptophan *p*-nitrophenyl ester binding. The solid line in Figure 5 is based on eq 5, using values of $A = 1.74$ and $K_D = 10.0$ M. This seems a reasonable value for the binding

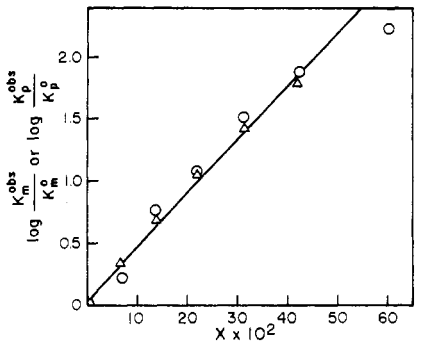

FIGURE 5: The data for K_m (○) and K_p (△) plotted according to eq 5. The solid line is calculated according to eq 5 with $A = 1.74$ and $K_D = 10.0$ M.

of dimethyl sulfoxide. As shown in Figure 4 binding of proflavine to α-chymotrypsin in aqueous dimethyl sulfoxide is essentially temperature independent.

The results of this investigation lend further support to the theory that α-chymotrypsin-catalyzed reactions in 65% aqueous dimethyl sulfoxide and at subzero temperatures follow essentially the same reaction pathway as under "normal" conditions.

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Changes in Sulfhydryl Groups of Honeybee Glyceraldehyde Phosphate Dehydrogenase Associated with Generation of the Intermediate Plateau in Its Saturation Kinetics†

W. G. Gelb,‡ J. F. Brandts,§ and J. H. Nordin*

ABSTRACT: Experiments with honeybee (*Apis mellifera*) and rabbit muscle glyceraldehyde phosphate dehydrogenases were conducted to obtain information at the chemical level regarding anomolous saturation kinetics of the honeybee enzyme. Results demonstrate that the enzyme's sulfhydryl groups are implicated in the process. Measured by 5,5′-dithiobis(nitrobenzoic acid) titration, native honeybee glyceraldehyde phosphate dehydrogenase has one less active sulfhydryl than the native rabbit muscle enzyme and displays changes in overall sulfhydryl reactivity after preincubation with glyceraldehyde 3-phosphate or glyceraldehyde 3-phosphate plus NAD^+. The total 5,5′-dithiobis(nitrobenzoic acid) reactive sulfhydryls of rabbit muscle glyceraldehyde phosphate dehydrogenase are not changed by preincubation with NAD^+ or glyceraldehyde 3-phosphate; honeybee glyceraldehyde phosphate dehydrogenase, under certain conditions of preincubation with these ligands, shows a decrease of two total 5,5′-dithiobis(nitrobenzoic acid) reactive sulfhydryl groups. This difference has been confirmed by an independent experiment in which the two enzymes were carboxymethylated with [^{14}C]bromoacetic acid. The loss of sulfhydryl groups in honeybee glyceraldehyde phosphate dehydrogenase is not a result of its acylation by glyceraldehyde 3-phosphate. After generation of the stable form of the honeybee enzyme with glyceraldehyde 3-phosphate plus NAD^+ it is possible to regenerate the anomolous kinetic curve by treatment of the enzyme with dithiothreitol. It is proposed that in honeybee glyceraldehyde phosphate dehydrogenase, an intrachain disulfide bond forms in conjunction with the conversion of the enzyme from the "metastable" to the stable state.

Intermediate plateau and transition regions in ligand saturation curves have been reported for a number of enzymes. These include glutamate dehydrogenase (LeJohn and Jackson, 1968), phosphoenolpyruvate carboxylase (Corwin and Fanning, 1968), adenosine diphosphoglucose pyrophosphorylase (Gentner and Preiss, 1968), cytidine triphosphate synthetase (Levitski and Koshland, 1969), pyruvate kinase (Somero, 1969), lactate dehydrogenase (Somero and Hochachka, 1969), acetylcholinesterase (Kato *et al.*, 1972), and L-threonine dehydratase (Kagan and Dorozhko, 1973). However, except for glutamate dehydrogenase, no satisfactory explanation for these anomalous kinetics is presently available (Teipel and Koshland, 1969). A similar transition in substrate saturation curves of glyceraldehyde phosphate dehydrogenase (EC 1.2.1.12) obtained from the honeybee *Apis mellifera* and car-

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