$$v^*/v = k_3[\text{MgATP}]/[k_2(1 + x[\text{MgADP}]/K_{\text{Ip}})(1 + k_4/k_5)] \quad (18)$$

where x is the ratio of rate constants for release of glucose in the presence and absence of MgADP (see eq 12). With the rate constants we have adopted and $x = 1$, v^*/v should be about 2.0. The experimental value in the presence of citrate is near 1 at pH 7, becomes almost 4 at pH 8.43, and decreases at low pH. In contrast to the situation in the forward reaction, citrate has a large effect on the reverse isotope exchange ratio, with the ratio being as much as an order of magnitude lower in the absence of citrate. Apparently in the absence of citrate, AlATP present as a contaminant in the ATP is specifically inhibiting the exchange reaction by combining with E–glucose to form an E–glucose–AlATP complex (Viola et al., 1980). If glucose can dissociate from this complex, and AlATP dissociates after glucose-6-P binds, the partitioning of the E–glucose complex toward reverse isotope exchange as opposed to dissociation of glucose is specifically decreased.

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Investigation of Diffusion-Limited Rates of Chymotrypsin Reactions by Viscosity Variation†

Antoon C. Brouwer and Jack F. Kirsch*

ABSTRACT: The possibility that the rates of acylation of chymotrypsin by certain highly reactive substrates approach the diffusion-controlled limits was investigated by measuring the values of k_{cat}/K_m for three substrates as a function of increasing viscosity with sucrose and ficoll as the viscosogenic reagents. The values of k_{cat}/K_m (pH 8.0, 25 °C) representing the acylation rate constants are the following: *N*-(methoxycarbonyl)-L-tryptophan *p*-nitrophenyl ester, 3.5×10^7 M^{-1} s^{-1}; *N*-acetyl-L-tryptophan methyl ester, 8×10^5 M^{-1} s^{-1}; *N*-acetyl-L-tryptophan *p*-nitroanilide, 300 M^{-1} s^{-1}. The rate constants decrease significantly with increasing viscosity for the first compound, decrease slightly for the second, and are insensitive to this perturbation for the third. The *p*-nitroanilide results taken together with the observation that the high concentrations of sucrose or ficoll used produce insignificant changes in k_{cat} for the ester substrates argue against a general nonspecific perturbation in the enzyme structure effected by these reagents. The values of the association rate constants calculated from these results are 9×10^7 and 1×10^7 M^{-1} s^{-1} for the *p*-nitrophenyl and methyl esters, respectively. The values of k_{cat}/K_m divided by the association rate constants show that the rates of acylation by the *p*-nitrophenyl ester occur at ca. 40% and by the methyl ester at ca. 10% of the diffusion limits. Possibilities involving reorientation of a nonproductively bound substrate within the ES complex or desolvation of part of the active site of the enzyme are considered to account for the lower association rate constant for the methyl as compared to the *p*-nitrophenyl ester.

Classically, the rate-determining steps in enzyme-catalyzed reactions have been considered to be those involving the chemical conversion of the ES to the EP complexes. While this situation undoubtedly obtains for many enzymes, there is increasing evidence that for others substrate association and/or product dissociation processes proceed more slowly than the chemical steps (Cleland, 1975); therefore, these reactions are at least partly diffusion limited. Albery & Knowles (1976) have remarked that such enzymes can be considered to have been perfected from an evolutionary point of view, since further improvements in catalytic efficiency would not be manifested in enhanced rates of reaction.

While an enzyme of substantial molecular weight, accommodating a single set of substrates and catalyzing a chemical

† From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received October 9, 1981.* This work was supported by National Science Foundation Grant PCM 7910971.

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reaction which does not have an extremely high inherent free energy of activation, might mold its active site to achieve the desideratum described above, smaller enzymes of broader specificity such as the degradative extracellular proteases, nucleases, and glycosidases would be expected to have more difficulty in maximizing the rates of the chemical steps of the reaction. Indeed, the rate constants for such enzymes acting on their natural substrates are typically several orders of magnitude below the diffusion-controlled values. Broad specificity enzymes, such as chymotrypsin, additionally effect the catalytic conversion of a number of highly reactive synthetic substrates. For example, the rate constants for the formation of the acyl enzyme of chymotrypsin, representing the first irreversible step in the reaction, can exceed 1×10^7 M^{-1} s^{-1}. The near identity of these values with those obtained from direct measurements of association rate constants with related pseudosubstrates or inhibitors led to the suggestion that the reactions of chymotrypsin with the *p*-nitrophenyl esters of *N*-acetyl-L-tryptophan and *N*-(benzyloxy)-L-tyrosine might be encounter limited (Philipp & Bender, 1973). At about the same time, Renard & Fersht (1973) interpreted the anomalously low pK_a value in the pH vs. k_{cat}/K_m profile for the chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan *p*-nitrophenyl ester (AcTrpONP)[1] as indicating that this reaction is ca. 50% diffusion controlled at high pH. Similar conclusions were later reached by Hirohara et al. (1977) from the results of a stopped-flow investigation of the acylation reaction.

Since molecular diffusion coefficients vary inversely with the viscosity of the medium (Caldin, 1964), a direct measure of the extent to which an enzymatic or nonenzymatic reaction is diffusion controlled can in favorable cases be obtained from an investigation of the dependence of the observed rate of reaction upon increasing concentrations of a viscosogenic reagent [e.g., see Cerjan & Barnett (1972), Nakatani & Dunford (1979), and Carapellucci (1975)]. We report here the results of an investigation of the dependence of chymotrypsin reaction rates with the three substrates AcTrp-pNA, AcTrpOMe, and MocTrpONP on the viscosity of the aqueous medium. The last of these substrates, representing a typical *p*-nitrophenyl ester of a specific substrate, is expected to acylate the enzyme with a near-diffusion-controlled rate. The rate of reaction of the less reactive second ester should be much less sensitive to viscosity. AcTrp-pNA, chosen as a slowly reacting, near-isosteric analogue of MocTrpONP, was chosen as a control for any possible general effects of the viscosogenic reagents on enzyme activity.

Materials and Methods

Materials. Twice-crystallized α-chymotrypsin was purchased from Worthington and used as received. The active-site concentration of the enzyme was determined by titration with *N-trans*-cinnamoylimidazole (Schonbaum et al., 1961), and total enzyme was determined by absorption at 280 nm with $\epsilon = 5 \times 10^4$ M^{-1} cm^{-1} (Dixon & Neurath, 1957). Sucrose (AR grade) was purchased from Mallinckrodt, and ficoll type 400, approximate molecular weight 4×10^6, was obtained from Sigma. AcTrpOMe was obtained from Vega Biochemicals, and MocTrpONP was a gift from Dr. V. Zannis (Zannis, 1975). AcTrp-pNA was prepared by the mixed anhydride method (Caplow, 1969). Two recrystallizations of the resulting yellow compound from ethanol–petroleum ether gave yellow crystals with mp 230–232 °C. Anal. Calcd: C, 62.29; H, 4.95; N, 15.29. Found: C, 62.41; H, 5.04; N, 15.26. The yield of *p*-nitroaniline after ca. a 10-h incubation of 66.3 μM AcTrp-pNA with chymotrypsin ($\sim$60 μM) was 66.0 μM, based on $\epsilon = 11\,700$ M^{-1} cm^{-1} at 400 nm at pH 8.0, indicating negligible racemization during synthesis.

Methods. Buffer solutions of 0.05 M potassium phosphate at $I = 0.5$ M (with KCl) were prepared by mixing a solution containing 0.05 M $K_2HPO_4 \cdot 3H_2O$ and 0.35 M KCl with one containing 0.05 M KH_2PO_4 and 0.45 M KCl until the desired pH was reached. In order to prepare sucrose- or ficoll-containing buffers, the desired weight of viscosogenic reagent was dissolved in ca. 60 g of the 0.05 M K_2HPO_4–KCl solution and made to the desired pH and final weight (100 g) by appropriate additions of the two buffer solutions. Subsequent filtering through sintered glass, followed by a Millipore membrane (0.22 μm), served to remove suspended material.

The relative viscosities ($\eta_{rel} = \eta/\eta^0$) of the buffer solutions containing 2 vol % acetonitrile were measured with an Ostwald viscometer at 25 °C with a solution of 0.05 M phosphate buffer, pH 8.0 and $I = 0.5$ M, containing 2 vol % acetonitrile used as a reference. Approximate relative viscosities of the sucrose or ficoll solutions used in this study are the following: 14% sucrose, $\eta_{rel} = 1.5$; 24% sucrose, $\eta_{rel} = 2.2$; 32% sucrose, $\eta_{rel} = 3.2$; 3% ficoll, $\eta_{rel} = 1.4$; 6% ficoll, $\eta_{rel} = 1.75$; 8% ficoll, $\eta_{rel} = 2.2$. The enzymatic hydrolysis reactions were monitored spectrophotometrically with a Cary Model 118C spectrophotometer equipped with a thermostated cuvette holder at 25 °C. pH values were measured with a Radiometer Model pHM 64 meter and a GK2421C glass electrode.

Reactions of chymotrypsin with AcTrpOMe were followed at 298 nm where the change in molar absorbancy is 280. The reaction of the enzyme with MocTrpONP was monitored at 400 nm, where the pH-sensitive molar absorbancy of *p*-nitrophenol is 16 900 at pH 8.0.

The hydrolysis reactions for the ester substrates were initiated by adding 0.01 mL of an ice-cooled enzyme solution (in aqueous buffer) to 2.55 mL of the temperature-equilibrated reaction mixture. The latter was prepared by adding 2.5 mL of buffer solution to substrate dissolved in 0.05 mL of acetonitrile, followed by thorough mixing. The difficulty in dissolving MocTrpONP in concentrated sucrose solutions (>20% w/w) was overcome by first mixing the acetonitrile solution of MocTrpONP with 1.5 mL of a 14% sucrose-containing buffer, and this solution was further mixed with a more concentrated sucrose buffer to give a reaction mixture with the desired viscosity. The time course of the reaction in each buffer solution was followed twice at three different initial substrate concentrations ranging from 2 to 5 times the K_m value for the reaction studied.

The experimental progress curves were fitted to the integrated form of the Michaelis–Menten equation with a computer program written by Dr. C. B. Sawyer (Rosenberg & Kirsch, 1979). Pseudo-first-order kinetics for the hydrolysis of AcTrp-pNA were obtained by saturating the substrate with enzyme. Care was taken to avoid concentrations of chymotrypsin higher than ca. 10^{-4} M to avoid complications due to possible formation of dimers (Shia & Sturtevant, 1969). Typical substrate concentrations were on the order of 2.5 μM, and enzyme concentrations varied from 30 to 60 μM. Reactions were initiated by adding 0.01 mL of the substrate-containing acetonitrile solution to 2.54 mL of a temperature-equilibrated reaction mixture. The latter was made by mixing buffer solutions and an ice-cold enzyme solution in the

[1] Abbreviations: AcTrp-pNA, *N*-acetyl-L-tryptophan *p*-nitroanilide; AcTrpOMe, *N*-acetyl-L-tryptophan methyl ester; MocTrpONP, *N*-(methoxycarbonyl)-L-tryptophan *p*-nitrophenyl ester; AcTrpONP, *N*-acetyl-L-tryptophan *p*-nitrophenyl ester.

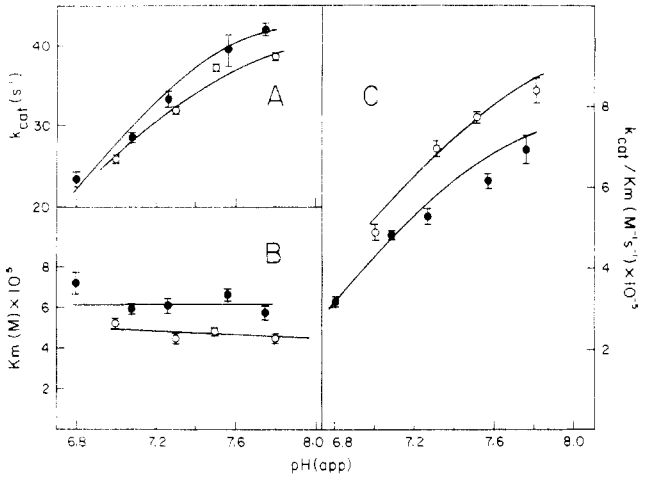

FIGURE 1: Effect of sucrose, (○) 0% and (●) 24% (w/w), on the apparent pH profiles for k_{cat} (A), K_m (B), and k_{cat}/K_m (C) for the α-chymotrypsin-catalyzed hydrolysis of AcTrpOMe in 0.05 M potassium phosphate buffers (I = 0.5 M) and 2 vol % CH_3CN, 25 °C. The points represent the average of at least five runs. The calculated pK_as from the curves are the following: 0% sucrose pK_a (k_{cat}) = 6.81 ± 0.09, pK_a (k_{cat}/K_m) = 6.85 ± 0.13; 24% sucrose pK_a (k_{cat}) = 6.85 ± 0.08, pK_a (k_{cat}/K_m) = 7.01 ± 0.16.

same buffer (100 μM, filtered through a 0.22-μm Millipore membrane) with 0.04 mL of acetonitrile. The reaction was monitored spectrophotometrically at 410 nm, where the molar absorbancy of *p*-nitroaniline is 8800 at pH 8.0. The pseudo-first-order rate constants were obtained by nonlinear regression analysis to the requisite function.

Results

The effect of 24% sucrose on the apparent pH profile for the kinetic parameters describing the chymotrypsin-catalyzed hydrolysis of AcTrpOMe is shown in Figure 1. Both k_{cat} and K_m are somewhat increased in the presence of sucrose, while the ratio of k_{cat}/K_m is reduced by about 10%. The values of k_{cat} and k_{cat}/K_m show the same apparent pH dependence in aqueous and in 24% sucrose-containing buffers. All further comparisons are therefore made at the same apparent pH value of 8.0, where the sensitivity of the rate constants to pH is very small.

The effect of variation in viscosity on the rates of chymotrypsin-catalyzed hydrolysis is substrate dependent. The parameter k_{cat}/K_m is the second-order rate constant including all processes up to the first irreversible step in the enzyme-catalyzed reaction. The value of this rate constant is insensitive to viscosity for AcTrp-pNA (Figure 2), where the slopes of the lines are seen to be identical within experimental error, slightly sensitive for AcTrpOMe, where, e.g., a change in η_{rel} from 1.00 to 2.24 decreases k_{cat}/K_m by 8% (Table I), and quite sensitive for MocTrpONP, where the same increase in viscosity effects a 33% decrease in k_{cat}/K_m (Table II).

Discussion

Viscosity and Diffusion-Controlled Rate Constants. The rate constant for a bimolecular diffusion-limited reaction, i.e., one in which the chemical reaction occurs within the encounter complex with a rate constant greater than that for the dissociation of the reactants ($k_2 \gg k_{-1}$, eq 1)

$$A + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} A{\cdot}B \xrightarrow{k_2} P \qquad (1)$$

is given by

$$k_1 = 4\pi r_0 N_0 (D_A + D_B) \qquad (2)$$

where D_A and D_B are the diffusion coefficients of A and B,

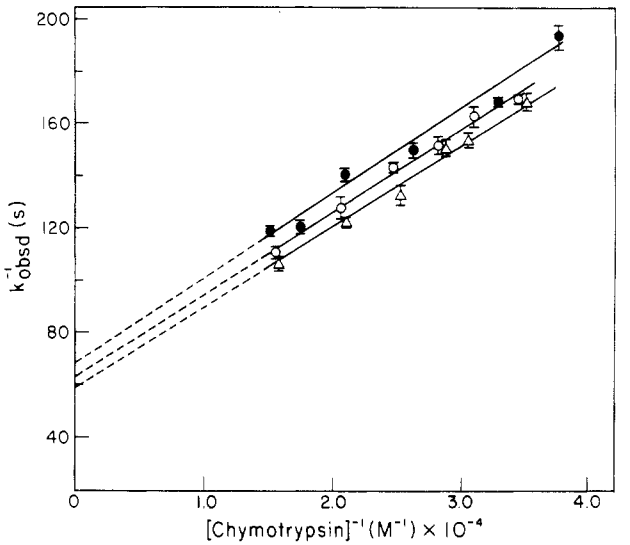

FIGURE 2: Plots of k_{obsd}^{-1} vs. [chymotrypsin]$^{-1}$ for the hydrolysis of AcTrp-pNA in sucrose-containing buffers (0.05 M potassium phosphate, I = 0.5 M, 2 vol % CH_3CN, apparent pH 8.0) at 25 °C. The points represent the weighted average of two runs. The lines are drawn for the calculated values from the equation k_{obsd} = (k_{cat}[chymotrypsin])/(K_m + [chymotrypsin]) for K_m = 50 ± 6 μM and k_{cat} = 0.016 ± 0.001 s^{-1} in 0% sucrose (○), K_m = 48 ± 7 μM and k_{cat} = 0.015 ± 0.001 s^{-1} in 14% sucrose (●), and K_m = 51 ± 8 μM and k_{cat} = 0.017 ± 0.001 s^{-1} in 24% sucrose (△).

Table I: Effect of Viscosity (η_{rel}) on the Kinetic Parameters for the α-Chymotrypsin-Catalyzed Hydrolysis of *N*-Acetyl-L-tryptophan Methyl Ester[a]

η_{rel}	k_{cat} (s^{-1}) (SE)	K_m (M) × 10^5 (SE)	k_{cat}/K_m (M^{-1} s^{-1}) × 10^{-5} (SE)
	(A) In Sucrose-Containing Buffers[b]		
1.00	38.4	4.54	8.45
(0)[d]	(1.6)	(0.22)	(0.25)
1.35	42.09	5.18	8.06
(12)	(0.46)	(0.10)	(0.08)
2.26	43.84	5.61	7.73
(25)	(0.58)	(0.21)	(0.21)
3.26	45.11	6.58	6.55
(33)	(1.2)	(0.48)	(0.30)
	(B) In Ficoll-Containing Buffers[c]		
1.00	39.74	5.00	7.79
(0)[e]	(0.47)	(0.11)	(0.16)
1.43	39.38	4.83	8.07
(3)	(0.59)	(0.17)	(0.18)
1.75	38.66	5.12	7.47
(6)	(0.41)	(0.16)	(0.18)
2.24	39.48	5.49	7.18
(8)	(0.24)	(0.07)	(0.08)

[a] In 0.05 M potassium phosphate buffers, 2 vol % CH_3CN, apparent pH 8.0, I = 0.5 M, 25 °C. Each reported value represents the average of at least five determinations. [b] [Chymotrypsin] = 67 μM, [AcTrpOMe] = 0.17–0.30 mM. [c] [Chymotrypsin] = 87 μM, [AcTrpOMe] = 0.16–0.26 mM. [d] % sucrose (w/w). [e] % ficoll (w/w).

r_0 is the sum of the radii of the idealized spherical molecules, and N_0 is Avogadro's number (Cantor & Schimmel, 1980). In practice, eq 2 gives an upper limit for enzyme-catalyzed reactions because the reaction cross section is not uniform over the enzyme surface (Šolc & Stockmayer, 1973). The diffusion coefficient of a species with radius r_i is inversely proportional to the viscosity, η, according to the Stokes–Einstein equation (Caldin, 1964):

$$D_i = \frac{k_B T}{6\pi\eta r_i} \qquad (3)$$

Table II: Effect of Viscosity (η_{rel}) on the Kinetic Parameters for the α-Chymotrypsin-Catalyzed Hydrolysis of *N*-Moc-L-tryptophan *p*-Nitrophenyl Ester[a]

η_{rel}	k_{cat} (s^{-1}) (SE)	K_m (M) × 10^6 (SE)	k_{cat}/K_m (M^{-1} s^{-1}) × 10^{-6} (SE)
	(A) In Sucrose-Containing Buffers[b]		
1.00	41.73	1.123	36.51
(0)[d]	(0.74)	(0.048)	(0.94)
1.47	44.89	1.493	29.76
(14)	(0.74)	(0.048)	(0.49)
2.16	46.34	1.910	24.09
(24)	(0.54)	(0.044)	(0.32)
3.15	45.85	2.529	18.06
(32)	(0.69)	(0.086)	(0.36)
	(B) In Ficoll-Containing Buffers[c]		
1.00	36.07	1.054	33.98
(0)[e]	(0.44)	(0.025)	(0.67)
1.43	36.46	1.322	27.32
(3)	(0.73)	(0.048)	(0.61)
1.75	39.79	1.465	27.02
(6)	(0.30)	(0.019)	(0.35)
2.24	39.43	1.713	22.89
(8)	(0.44)	(0.030)	(0.39)

[a] In 0.05 M potassium phosphate buffers, 2 vol % CH_3CN, apparent pH 8.0, I = 0.5 M, 25 °C. Each reported value represents the average of at least five determinations. [b] [Chymotrypsin] = 2.02 nM, [MocTrpONP] = 3.5–7.5 μM. [c] [Chymotrypsin] = 1.64 nM, [MocTrpONP] = 4.0–8.0 μM. [d] % sucrose (w/w). [e] % ficoll (w/w).

where k_B is the Boltzmann constant. A combination of eq 2 and 3 gives

$$k_1 = \frac{2RT}{3\eta}\left(2 + \frac{r_A}{r_B} + \frac{r_B}{r_A}\right) \tag{4}$$

so that at a given temperature k_1 is inversely proportional to η.

The observed rate constant for the formation of P (eq 1) is given by

$$k_a = \frac{k_1 k_2}{k_{-1} + k_2} \tag{5}$$

The rate constants k_1 and k_{-1}, representing the diffusion rate constants for association and dissociation, respectively, depend on η according to

$$k_1^0\eta^0 = k_1\eta \qquad k_{-1}^0\eta^0 = k_{-1}\eta \tag{6}$$

where the superscript refers to the reaction in the reference aqueous solution in the absence of added viscosogenic reagent. Substitution into eq 5 gives eq 7 (Nakatani & Dunford, 1979):

$$k_a = k_1^0\frac{\eta^0}{\eta}\Big/\left(1 + \frac{k_{-1}^0\eta^0}{k_2\eta}\right) \tag{7}$$

The reciprocal of eq 7 is eq 8 (Loo & Erman, 1977):

$$\frac{1}{k_a} = \frac{1}{k_1^0}\frac{\eta}{\eta^0} + \frac{k_{-1}^0}{k_1^0 k_2} \tag{8}$$

Therefore, the linear plot of $1/k_a$ vs. η_{rel} ($=\eta/\eta^0$) should give both the rate constant for association as the slope $1/k_1^0$ and the partition ratio of the AB (or ES) complex, k_{-1}^0/k_2, as k_1^0 multipled by the ordinate intercept.

The applicability of the viscosity criterion for a diffusion-controlled reaction to enzyme kinetics is not necessarily straightforward. The large quantities of cosolute required to raise the viscosity significantly can cause changes in the enzyme structure which will make it a worse or possibly an even better catalyst in the more viscous medium, or induce time-dependent denaturation. Such caveats apply equally to any solvent-induced insult, such as substituting D_2O for H_2O in solvent kinetic isotope effect studies (Schowen, 1977) or the addition of organic solvents in cryogenic investigations (Makinen & Fink, 1977). Satisfactory assurances against the occurrence of such generalized perturbations in enzyme structure can be obtained either if the enzyme exhibits a number of kinetically isolable steps which do not involve diffusion processes, and these can be shown to be relatively insensitive to the viscosogenic cosolute, and/or if the range of substrates recognized by the enzyme includes some which do not react with diffusion-controlled rates and for which all kinetic parameters are relatively unperturbed by the cosolute. As an example of the latter criterion, the rate constant for the formation of compound I from the reaction of horseradish peroxidase with *m*-chloroperbenzoic acid is (8.5 ± 0.9) × 10^7 M^{-1} s^{-1}, and it is decreased by increasing concentrations of glycerol, while the rate constant for the formation of compound I from the more slowly reacting hydrogen peroxide is insensitive to the cosolvent (Dunford & Hewson, 1977).

Application to Chymotrypsin. The well-established kinetic mechanism for chymotrypsin-catalyzed reactions is given by eq 9 (Hess, 1971; Fastrez & Fersht, 1973b)

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{+}{AE} \xrightarrow{k_3} E + P_2 \qquad \underset{P_1}{} \tag{9}$$

where AE is a covalent acyl enzyme intermediate, P_1 is generally an alcohol or amine, and P_2 is a carboxylic acid. The deacylation step, k_3, is overall rate determing for most ester substrates (Bender et al., 1964), while the acylation process, k_2, is limiting for amide and peptide substrates (Fastrez & Fersht, 1973b). The first irreversible step in both cases is acylation of the enzyme, because the concentration of P_1 is ordinarily too low to effect a kinetically significant reversal of the ES → AE reaction. Where Michaelis complex formation is at true equilibrium (i.e., $k_{-1} \gg k_2$), eq 5 reduces to

$$k_a = \frac{k_2 k_1}{k_{-1}} = \frac{k_{cat}}{K_m} \tag{10}$$

The second term thus dominates the right-hand side of eq 8, and the values of k_a are insensitive to changes in viscosity. Conversely, covalent capture of the acyl moiety of the substrate by the enzyme with a rate constant greater than that for dissociation ($k_2 \gg k_{-1}$) leads to

$$\frac{k_{cat}}{K_m} = k_a \approx k_1 \tag{11}$$

with the consequence that plots of $1/k_a$ vs. η_{rel} according to eq 8 should give straight lines with near-zero intercepts. Substrates which have partially diffusion-limiting rates ($k_{-1} \approx k_2$) will exhibit intermediate behavior.

Since the values of k_{cat}/K_m for the three substrates investigated here differ by several orders of magnitude, a comparative display of the differential effects of viscosity is best achieved by a normalized plot according to eq 12 (Figure 3):

$$\frac{k_a^0(\text{calcd})}{k_a} = \frac{P}{1+P} + \left(\frac{1}{1+P}\right)\eta_{rel} \tag{12}$$

where $P = k_{-1}^0/k_2$ and k_a^0(calcd) was calculated from the least-squares values of P and k_1^0 from eq 7 with $\eta_{rel} = 1$. These figures are equal within experimental error to the experimentally determined values of k_{cat}/K_m at $\eta_{rel} = 1$ (Tables I and II). The figure of 317 M^{-1} s^{-1} used for AcTrp-pNA is the average experimental value for this substrate. By defi-

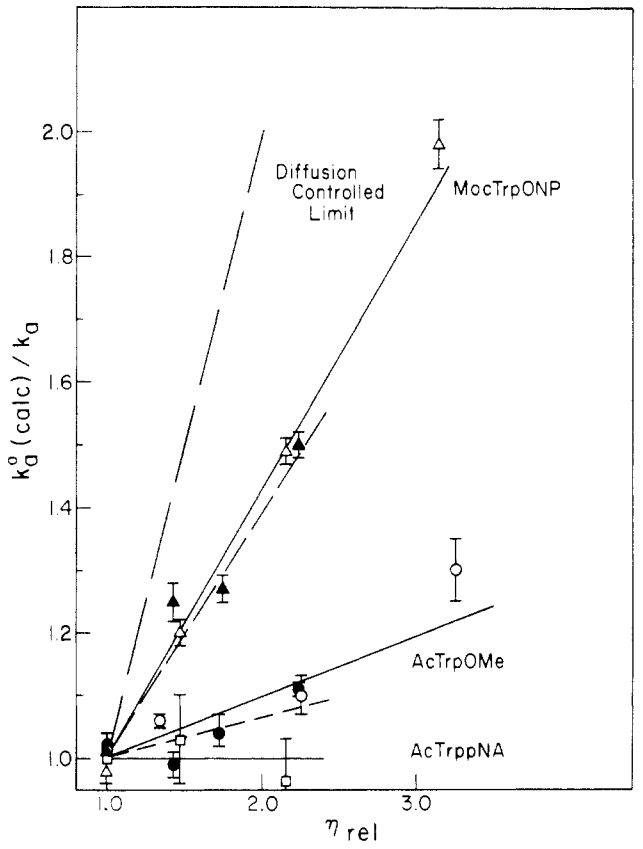

FIGURE 3: Plots of k_a^0(calcd)/k_a vs. η_{rel} for the reaction of α-chymotrypsin with various substrates in sucrose-containing (open symbols) and ficoll-containing (filled symbols) buffers (0.05 M phosphate, I = 0.5 M, 2 vol % CH_3CN, apparent pH 8.0, 25 °C): MocTrpONP (△, ▲); AcTrpOMe (○, ●); AcTrp-PNA (□). The points represent the weighted average of at least five runs. The lines, (—) sucrose and (---) ficoll, are drawn for the calculated values from eq 7 and plotted according to eq 12. The line with the long dashes and slope = 1 is drawn for a completely diffusion-controlled rate of reaction.

nition, all such plots must converge at the coordinate (1,1), and a completely diffusion-limited rate of reaction will have a slope of 1 (long dashes). A qualitative examination of Figure 3 reveals that the reaction rate of MocTrpONP with chymotrypsin approaches the diffusion-controlled limit, while that for AcTrpOMe is partially diffusion controlled and that for AcTrp-pNA is insensitive to viscosity.

Quantitative evaluations of k_1^0 and k_{-1}/k_2 for the two ester substrates were carried out by nonlinear regression analysis of the data according to eq 7, and the values are collected in Table III. Essentially similar results are obtained with sucrose and ficoll. This, together with the facts that for the much less reactive substrate, AcTrp-pNA, k_{cat}/K_m (=300 M^{-1} s^{-1}; Figure 2 legend) is completely insensitive to viscosity and that sucrose or ficoll have only a slight or negligible effect on k_{cat} (Tables I and II), argues strongly that the predominant effect of increasing viscosity is manifest in the diffusion process.

The value of k_{cat}/K_m divided by that of k_1^0 shows that the rate of reaction of MocTrpONP is about 40% and that of AcTrpOMe about 6–10% diffusion controlled. The figure of 9×10^7 M^{-1} s^{-1} for k_1 for the MocTrpONP reaction is similar to those obtained earlier for the very similar substrate AcTrpONP from analyses of the pH vs. rate profiles (6×10^7 M^{-1} s^{-1}, Renard & Fersht, 1973; 1.3×10^8 M^{-1} s^{-1}, Hirohara et al., 1977). The tendency of AcTrpONP to form an oxazolinone in common with other *p*-nitrophenyl esters of *N*-acyl amino acids (de Jersey et al., 1969) precluded its investigation by the progress curve analysis employed in this study.

Magnitudes of the Association Rate Constants. A surprising result is that k_1^0 for AcTrpOMe is 10 times less than that for MocTrpONP (Table III). From a comparison of k_1^0 values with the approximately 10-fold lower values reported by earlier workers for *N*-[(2-furyl)acryloyl]-L-tryptophanamide, Hirohara et al. (1977) had earlier concluded that hydrophobic leaving groups tend to increase the rates of association with chymotrypsin. The exact values of the association rate constants are apparently determined by a multiplicity of factors. Kunugi et al. (1978) have subsequently reported $k_{cat}/K_m = 4.2 \times 10^7$ M^{-1} s^{-1} for the reaction of chymotrypsin with *N*-[(2-furyl)acryloyl]-L-tryptophan methyl ester, a figure representing a minimum value for k_1^0, and the data of Bizzozero et al. (1975) for the reaction of *N*-Ac-Ala-Ala-Tyr methyl ester with chymotrypsin give a value for k_1^0 of close to 1×10^7 M^{-1} s^{-1}. Since all of these compounds have similar diffusion coefficients, and the numbers have been arrived at by alternate experimental techniques, it would seem that the complete process of productive accommodation of the substrate into the active site must be more complicated than simple collision of the substrate molecule with the active-site cavity. At least two possible explanations for the variation in k_1^0 can be envisioned. The first is that residues with smaller, more or less hydrophilic, leaving groups (e.g., OMe or NH_2) bind only to a minor form of the enzyme, perhaps one in which the leaving group binding region is desolvated, whereas hydrophobic leaving groups might bind in a mode which is insensitive to the presence of such a water molecule. The second possibility takes cognizance of the potential for the hydrophobic leaving group to bind nonproductively in the aromatic cavity normally reserved for the amino acid side chain (Hinkle & Kirsch, 1971; Fastrez & Fersht, 1973a) and that reorientation to give the productive complex need not involve dissociation of the substrate from the enzyme. This effectively gives substrate molecules having more than one hydrophobic group a statistical advantage in that a larger fraction of the molecule could be sequestered by the aromatic binding cavity. More generalized discussions of this problem have been given by Burgen et al. (1975) and by Jencks (1980).

Table III: Values of the Association Rate Constants (k_1^0) and Partition Ratios (k_{-1}^0/k_2) for the Reactions of Chymotrypsin with MocTrpONP and AcTrpOMe[a]

substrate	viscosogenic reagent	$10^{-6}k_1^0$ (M^{-1} s^{-1}) (SE)	k_{-1}^0/k_2 (SE)
MocTrpONP	sucrose	89.1 (6.4)	1.49 (0.21)
	ficoll	87.3 (7.7)	1.55 (0.26)
AcTrpOMe	sucrose	8.7 (1.9)	9.2 (2.4)
	ficoll	13.0 (5.8)	15.3 (8.1)

[a] From nonlinear regression analysis of the data in Tables I and II on eq 7.

The values of k_{-1}^0/k_2 of ca. 10 (Table III) indicate that the chemical steps are predominantly rate determining for the reaction of AcTrpOMe. Such a finding is consistent with the 1.8% ethoxy oxygen-18 kinetic isotope effect previously reported for the ethyl ester of *N*-acetyl-L-tryptophan (Sawyer & Kirsch, 1975). This figure, which is close to that observed for model reactions involving general base-catalyzed hydrolysis, is only consistent with a rate-determining step involving some loss of bonding between the acyl carbon atom and the leaving alcohol. Diffusion processes are essentially independent of such an isotopic substitution.

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Steady-State Kinetic Studies on D-Lactate Dehydrogenase from *Megasphera elsdenii*†

Fraser F. Morpeth‡ and Vincent Massey*

ABSTRACT: Initial rate measurements were made of the oxidation of D-lactate and D-α-hydroxybutyrate by oxygen and potassium ferricyanide, catalyzed by D-lactate dehydrogenase from *Megasphera elsdenii*. The detailed kinetic work indicates a "ternary complex" type mechanism, with a complex of keto acid and reduced enzyme reacting with the electron acceptor at pH 8. However, as the pH is lowered, the double-reciprocal plots become nonlinear, with a downward curvature. This seems to be due to negative interactions within the protein rather than to a complexity of the kinetic mechanism. The variation of initial rate parameters at pH 8 with temperature yields nonlinear Arrhenius plots with a greater activation energy above the break point than below. This type of behavior has been previously reported only for fumarase (Massey, 1953). Studies with deuterated D-lactate show only a small isotope effect on ϕ_0 and ϕ_1 (K_M/V_{max} for lactate) but a large effect on ϕ_2 (K_m/V_{max} for ferricyanide).

The first enzyme in lactate metabolism of the anaerobic bacterium *Megasphera elsdenii* is a flavoprotein, D-lactate dehydrogenase (EC 1.1.99.6) (Baldwin & Milligan, 1964; Brockman & Wood, 1975).

This enzyme belongs to a class of oxidation–reduction flavoproteins termed C–N transhydrogenase (Massey & Hemmerich, 1980). It catalyzes the transfer of reducing equivalents from CH substrates, in this case D-lactate, to the flavin N(1)–N(5) center of an electron transferring flavoprotein (Brockman & Wood, 1975; Whitfield & Mayhew, 1974). This electron transferring flavoprotein in turn passes the reducing equivalents on to butyryl-CoA dehydrogenase, another flavoprotein (Engel & Massey, 1971). Many bacteria grown anaerobically possess soluble D-lactate dehydrogenases (Snoswell, 1966; Molinari & Lara, 1960) as does yeast (Cremona, 1964). A mammalian enzyme has also been described (Tubbs & Greville, 1959; Cammack, 1969).

Previous workers (Stachiewicz et al., 1961; Ghiretti-Magaldi et al., 1961) found that the yeast enzyme appears to require zinc as well as FAD for activity. Olson & Massey (1979) showed conclusively that *Megasphera elsdenii* D-lactate dehydrogenase contains one essential zinc per FAD. The role

† From the Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109. *Received July 15, 1981; revised manuscript received November 6, 1981.* This work was supported by U.S. Public Health Service Grant GM 11106.

‡ Present address: School of Molecular Sciences, University of Sussex, Falmer, Brighton BN1 905, England.

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