Riordan, J. F., and Hayashida, H. (1970), *Biochem. Biophys. Res. Commun. 41*, 122-127.
Schmidt, D. E., Jr., and Westheimer, F. H. (1971), *Biochemistry 10*, 1249-1253.
Shen, W. C., and Colman, R. F. (1975), *J. Biol. Chem. 250*, 2973-2978.
Shen, W. C., Mauck, L., and Colman, R. F. (1974), *J. Biol. Chem. 249*, 7942-7949.

Binding Rates, O–S Substitution Effects, and the pH Dependence of Chymotrypsin Reactions†

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ABSTRACT: The pH dependence for acylation of α-chymotrypsin by *N*-acetyltryptophan *p*-nitrophenyl-, *p*-nitrothiophenyl-, ethyl-, and thiolethyl esters has been studied by the stopped-flow technique. Values for the acylation rate constant, k_2, and the binding constant, K_S, were obtained by using measurements of phenolate release, for the *p*-nitrophenyl esters, and proflavin displacement, for the ethyl esters. The oxygen esters tested have slightly higher k_2 values, and substantially higher K_S values relative to the analogous thiol esters. Whereas k_2/K_S for the thiolethyl ester is higher than that for the analogous oxygen ester, the k_2/K_S values for oxy- and thio-*p*-nitrophenyl esters are nearly identical. These data are interpreted to indicate rate-determining formation of a tetrahedral intermediate in acylation of α-chymotrypsin by *p*-nitrophenyl esters, and rate-determining breakdown of such an intermediate in the case of the ethyl esters. It is also concluded that the oxygen to sulfur substitution causes a substantial increase in the proportion of nonproductive binding in these substrates. pH dependent k_2 and K_S values were used to calculate values for k_1 and k_{-1}, the binding and debinding rate constants for the two *p*-nitrophenyl compounds. This is the first such calculation based on experimentally determined acylation rate constants.

The mechanistic description of the action of chymotrypsin requires the observation of individual steps of the enzymatic reactions and a knowledge of all the intermediates on the pathway. It has been well established that the pathway of α-chymotrypsin-catalyzed hydrolysis reactions includes the formation and decomposition of an acyl-enzyme intermediate. This can be described in terms of eq 1 as a minimal requirement (Bender and Kezdy, 1965; Bruice and Benkovic, 1966; Bender, 1971):

$$E + S \overset{K_S}{\rightleftharpoons} E{\cdot}S \xrightarrow{k_2} \underset{P_1}{ES'} \xrightarrow{k_3} E + P_2 \qquad (1)$$

where E·S is the enzyme-substrate complex, ES′ is the acyl-enzyme, and P_1 and P_2 are the alcohol and acid portions of an ester substrate, respectively. Both acylation and deacylation may be clearly categorized as examples of acyl-transfer reactions at a carbonyl carbon atom. Since the occurrence of a tetrahedral intermediate was demonstrated in acyl-transfer reactions of nonenzymatic hydrolysis of esters and amides (Bender, 1960), whether or not a similar intermediate is formed in the enzymatic reaction pathway has been a subject of great interest. Comparisons of the kinetic behavior between oxygen esters and their sulfur counterparts of nonspecific (Frankfater and Kezdy, 1971) and specific substrates (Hirohara et al., 1974) have presented kinetic evidence for the occurrence of a tetrahedral intermediate in the acylation process of α-chymotrypsin-catalyzed reactions. Thus, our data are discussed in terms of a mechanism involving this intermediate and may be summarized by

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}S \rightleftharpoons ET \rightarrow \underset{P_1}{ES'} \xrightarrow{k_3} E + P_2 \qquad (2)$$

where ET is the tetrahedral intermediate.

In the case of the α-chymotrypsin-catalyzed hydrolysis of specific substrates, the rate constant of the acylation step, k_2 (in eq 1), was found to be roughly identical for both thiol esters and their oxygen counterparts (Hirohara et al., 1974). Since, in an SN2 type reaction, an -SR group should be displaced at least 250 times faster than an -OR group (Connors and Bender, 1961; Martin and Hedrick, 1962), and since in the breakdown of similar tetrahedral compounds such as hemithioacetals (Jencks, 1969), acetaldehyde hydrate (Jencks, 1969), and ketene *O,S* acetals (Hershfield and Schmir, 1972) the -SR group has shown a greater leaving ability compared with an -OR group, it was concluded that a one-step transfer does not occur. The results were explained by postulating a metastable tetrahedral intermediate, the formation of which is the rate-determining step in acylation. Proposals for the formation of such an intermediate prior to acylation have been recently made based on a series of kinetic studies on amide substrates (Caplow, 1969; Lucas and Caplow, 1972; Lucas et al., 1973; Fersht and Requena, 1971a; Fastrez and Fersht, 1973; Philipp et al., 1973).

It was suggested in the study of specific ester substrates at two pHs (Hirohara et al., 1974) that: (a) the greater values of k_2/K_S of thiol esters over the corresponding oxygen esters

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(which have been reported previously for alkyl esters (Polgar, 1972)) are mainly due to a lower K_S value of the thiol ester, rather than a larger k_2 value; (b) the values of K_S depend on the nature of the leaving group; (c) the observed k_2 value for *N*-acetyl-L-tryptophan *p*-nitrophenyl ester at pH 8 is one order of magnitude smaller than that estimated from steady-state studies in the past; (d) the K_S values of specific substrates may be dependent upon pH; and (e) the pK governing k_2 may be shifted by substrate binding. These considerations strongly indicated the importance of the direct determination of the pH dependence of k_2 and K_S. Such a complete study for labile specific ester substrates around the neutral pH range as well as that of k_2/K_S has not been reported previously, although some data at acidic pHs (Brandt et al., 1967) and indirect determinations of k_2 over a wide pH range (Rajender et al., 1971) have been published for *N*-acetyl-L-tryptophan ethyl ester. This is partly because the acylation step of such a reaction was believed to be so fast that k_2 could not be determined directly. However, the method used in observation of k_2 for *N*-acetyl-L-tryptophan *p*-nitrophenyl ester at pH 8 (Hirohara et al., 1974) was found to be suitable for determination of k_2 and K_S over a wide pH range. Thus we have undertaken the investigation of the pH dependence of the discrete acylation step of α-chymotrypsin by *N*-acetyl-L-tryptophan *p*-nitrophenyl, *p*-nitrothiophenyl (D-L form), ethyl, and thioethyl esters. These studies not only provide answers for the above-mentioned suggestion brought about from a study at two pHs, but also provide detailed information concerning the effect of rate-determining binding on k_2/K_S and K_S.

Experimental Procedure

Materials and Methods. All materials and methods used in this work were substantially identical with those in the previous article on this subject (Hirohara et al., 1974). Some additional comments are given below.

Enzyme stock solutions were made up in 10^{-3} M HCl, centrifuged for clarity when necessary, and kept (on ice) no more than 1 day. Substrate solutions were made up using either acetonitrile (for *p*-nitrophenyl esters) or 4:1 acetonitrile–dimethylformamide (for ethyl esters). Buffers were degassed before use.

pH dependence studies were done in a manner similar to the single pH studies done previously. However, variations in pH necessitated use of the pH-dependent value of K_F, the dissociation constant for the α-chymotrypsin–proflavin complex. These values are shown in Table A (see paragraph concerning supplementary material at the end of the paper).

The stopped-flow reactions were observed on absorbance vs. time traces.

The reactions were similar to those done previously, except that, at lower pH values, reactions were slower and, hence, easier to observe. The highest k_{obsd} value measurable by the instrument was approximately 2000 s^{-1}. Because of the low value of K_S, k_2 and K_S could be independently determined at relatively low enzyme concentrations. However, in order to satisfy saturation kinetics, it was necessary to maintain the condition that $[E_0] > 8[S_0]$. The failure to satisfy this condition led to erroneously large values of k_2 and K_S. On the other hand, as long as the condition of $[E_0] > 15[S_0]$ held, a small difference of substrate concentration made no difference in k_{obsd} values at the same enzyme concentration (e.g., for the *p*-nitrophenyl ester, $[S_0] = 3.4 \times 10^{-6}$ M and 4.1×10^{-6} M resulted in $k_{obsd} = 1750 \pm 90$ and 1790 ± 50 s^{-1}, respectively, at $[E_0] = 7.17 \times 10^{-5}$ M and pH 8). Therefore, when values of k_{obsd} were close to 2000 s^{-1}, a slightly higher substrate concentration than in the experiments at lower enzyme concentration was often used in order to obtain a clear trace of the transmittance change on the oscilloscope. No deviations from linearity of the Eadie plots due to dimerization of the enzyme were observed in the low pH range.

The kinetic parameters pertaining to the ethyl and thiolethyl esters in the presence of proflavin were evaluated at various substrate concentrations by use of a modified Eadie equation, under the conditions of $[S_0] \gg [E_0] < [F_0]$, as was described previously (Hirohara et al., 1974). Since the value of k_2 is much higher than that of k_3 for the α-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan ester substrates (Hirohara et al., 1974; Zerner et al., 1964), and since oxygen and thiol esters of these specific substrates are hydrolyzed via the same pathway, pH-dependent k_{cat} values for the ethyl ester (Bender et al., 1964) were used as k_3.

Error limits for k_2, K_S, and k_2/K_S, at each pH value reflect the standard deviation calculated from a least-squares analysis of Eadie plot data. Each Eadie plot contained data resulting from at least 30 kinetic determinations under at least ten different sets of conditions. Errors in k_2(lim) and k_2/K_S(lim) as well as pK values of k_2/K_S were calculated from the variation in points in Eadie-type pH dependent plots (as in Figures 2 and 3). Error ranges in K_S(lim) were taken as being similar to the error values for individual pH-dependent K_S values. The error limits do not reflect possible sources of systematic error, error due to pH and ionic strength dependent perturbations, or error due to the use of literature active–inactive enzyme equilibrium constants (Fersht and Requena, 1971b).

Results

The numerical results are summarized in Tables I and II, Tables B–F (see paragraph concerning supplementary material at the end of this paper), and Figures 1–3, together with the experimental conditions. Values of K_S and k_2/K_S are corrected for the active fraction of the enzyme present, using data obtained by Fersht and Requena (1971b), based on original measurements of Oppenheimer et al. (1966). The agreement with previously published data for k_2 and K_S for the ethyl ester at low pH (Brandt et al., 1967) is quite satisfactory. The essential experimental observations are as follows: (a) values of k_2 for the four ester substrates increase according to: thiolethyl < ethyl < *p*-nitrothiophenyl < *p*-nitrophenyl. The k_2 values for the oxygen esters exceed (by a factor of 2 or less) those of their sulfur counterparts at all pH ranges studied except for the alkyl esters at pH 5. (b) Values of K_S decrease with the order ethyl > thiolethyl > *p*-nitrophenyl > *p*-nitrothiophenyl, which clearly indicates some significant effect of the leaving group on binding. The thiol esters always bind better than their oxygen counterparts. The difference is especially remarkable with the alkyl esters. The values of K_S depend on pH for the four specific ester substrates; the values are higher at low pH than around neutral pH. The values of K_S for the *p*-nitrophenyl ester are 10–50-fold lower than those estimated in the past. (c) The second-order rate constant, k_2/K_S, of the *p*-nitrothiophenyl ester is only slightly larger than that of the *p*-nitrophenyl ester, whereas the thiolethyl ester has a much larger k_2/K_S value than the ethyl ester due to the lower K_S term, not to a difference in observed k_2. The observed k_2/K_S values for the oxygen esters are in good agreement with literature values (Bender and Kezdy, 1965; Zerner et al., 1964), or the algebraically equivalent k_{cat}/K_m, considering effects of the organic solvent (Clement and Bender, 1963).

Plots of k_2 and K_S (corrected for active enzyme conformation) against pH for the four substrates are presented in Figure

TABLE I: Limiting Rate and Dissociation Constants for Acylation of α-Chymotrypsin by *N*-Acetyltryptophan Esters at 25.5 ± 0.5 °C and Ionic Strength = 0.1.[a]

Substrate	pK_a of Alcohol	10^3k_2(lim) (s^{-1})	$10^{-4}K_S$[b] (M)	$10^{-4}K_S$[c] (M)	$10^7k_2/K_S$(lim) (M^{-1} s^{-1})
AcTrpOnP	7.04	4.65 ± 0.20	1.30 ± 0.20	0.70 ± 0.10	7.3 ± 0.5
			1.64 ± 0.20	0.90 ± 0.12	5.7 ± 0.5
AcTrpSnP	4.47	2.10 ± 0.10	0.75 ± 0.15	0.25 ± 0.06	9.6 ± 0.6
			0.92 ± 0.18	0.34 ± 0.07	7.2 ± 0.6
AcTrpOEt	16.0	1.12 ± 0.10	15.0 ± 1.0	7.2 ± 0.6	0.150 ± 0.008
			19.0 ± 2.0	8.5 ± 0.8	0.123 ± 0.005
AcTrpSEt	10.50	0.73 ± 0.07	2.6 ± 0.4	0.65 ± 0.05	1.18 ± 0.12
			3.3 ± 0.7	0.85 ± 0.08	0.93 ± 0.10

[a] The first line of the binding and the rate constant for each substrate is the corrected value and the second line is the uncorrected value for the active–inactive enzyme equilibrium (Fersht and Requena, 1971b). [b] Dissociation constant for the protonated form of the enzyme at pH 4.5–5.5. [c] Dissociation constant for the deprotonated form of the enzyme at pH 7.5–8.

TABLE II: Summary of pK Values for the α-Chymotrypsin-Catalyzed Hydrolysis of *N*-Acetyltryptophan Esters.

Substrate	pK_a of Alcohol	pK of k_2	pK_1 of K_S	pK_2 of K_S	pK_1 of k_2/K_S
O-*p*-Nitrophenyl ester	7.04	6.18 ± 0.1	6.65 ± 0.4	6.97 ± 0.1	6.63 ± 0.1
S-*p*-Nitrophenyl ester	4.47	5.88 ± 0.1	6.28 ± 0.5	6.99 ± 0.1	6.76 ± 0.04
O-Ethyl ester	16.0	6.81 ± 0.05	6.40 ± 0.1	6.79 ± 0.1	6.93 ± 0.15
S-Ethyl ester	10.50	6.73 ± 0.08	6.15 ± 0.05	6.86 ± 0.05	7.06 ± 0.15

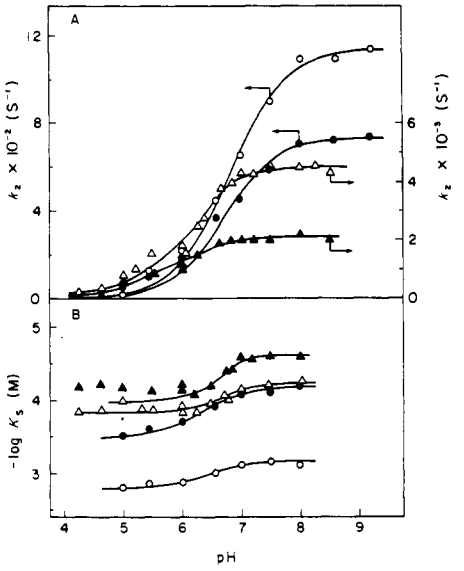


FIGURE 1: The pH dependence of k_2 and log K_S for *N*-acetyl-L-tryptophan *p*-nitrophenyl ester at ionic strength 0.1 (○), and for *N*-acetyl-DL-tryptophan *p*-nitrothiophenyl ester (●), and for the pH dependence of k_2 and log K_S for *N*-acetyl-L-tryptophan ethyl ester (△) and for *N*-acetyl-L-tryptophan thiolethyl ester (▲). Ionic strength 0.1, 25.5 °C. Log K_S is calculated for the active conformation of the enzyme (Fersht and Requena, 1971b).

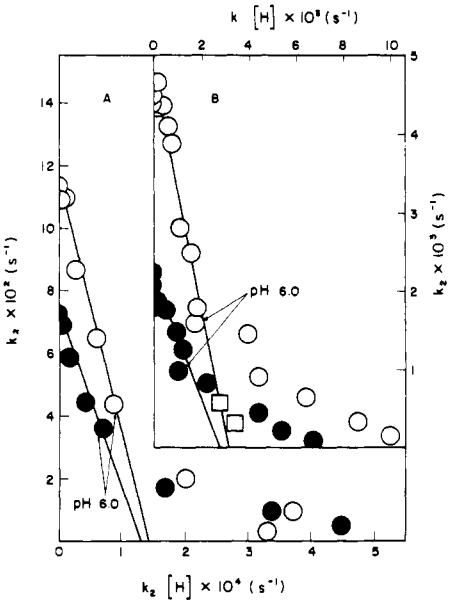


FIGURE 2: Plots of k_2 vs. k_2 [H] for *N*-acetyl-L-tryptophan ethyl (○) and thiolethyl (●) esters in A, and *N*-acetyl-L- (and DL) tryptophan *p*-nitrophenyl (○) and *p*-nitrothiophenyl (●) esters in B; ionic strength 0.1, 25.5 °C [(□) *p*-nitrophenyl ester at ionic strength 1.0].

1. The k_2–pH–rate profile showed a sigmoid curve for all four esters, which indicates that a single ionizable group controls acylation of α-chromotrypsin by specific ester substrates, in contrast to the acylation by nonspecific ester substrates (Hardman et al., 1971).

The data fit the theoretical curve well above pH 6.0 but deviate considerably below pH 6.0. The results obtained at ionic strength 1.0, however, fit the curve (squares in Figure 2B) even at pH 5.0 and 5.3. Values of k_2(lim) and pK_a obtained from Eadie type plots (Eadie, 1942) are shown in the third column of Tables I and II, respectively.

The pH–K_S profile also exhibited a sigmoid curve for all the substrates. Similar pH dependent binding constants were reported for proflavin (Marini and Caplow, 1971) and some amides of *N*-formyl-L-phenylalanine (Lucas et al., 1973; Fersht and Requena, 1971a; Fersht and Renard, 1974). Values of K_S for the conjugate acid and base of the enzyme are summarized in the fourth and fifth columns of Table I, respectively. Values of pK_a governing K_S were calculated according to the

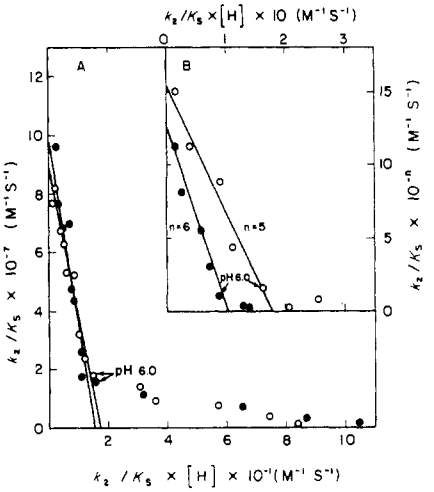


FIGURE 3: Plots of k_2/K_S vs. $(k_2/K_S)[H]$ for *N*-acetyl-L-tryptophan *p*-nitrophenyl ester and *N*-acetyl-DL-tryptophan thiolphenyl ester (A), and *N*-acetyl-L-tryptophan ethyl ester and *N*-acetyl-L-tryptophan thiolethyl ester (B), ionic strength 0.1, 25.5 °C. (□) Ionic strength, 1.0. k_2/K_S is calculated for the active enzyme conformation (Fersht and Requena, 1971b).

method of Dixon (1953) and are given in the fourth and fifth columns of Table II. The pK_a values governing k_2 and the lower pK_a value governing K_S are those of the Michaelis complex and should be identical unless there is a pH-dependent change in the rate determining step during acylation (Fersht and Requena, 1971a). The values of pK_a obtained from k_2 for *p*-nitrophenyl and *p*-nitrothiophenyl esters are substantially lower than the "normal" values. On the other hand, it is interesting to see that the replacement of an oxygen atom of the alcohol by a sulfur atom, which substantially strengthens substrate binding to the enzyme, has little effect on perturbing the pK_a of k_2.

The observed k_2(lim) value for *p*-nitrophenyl ester is one order of magnitude smaller than that previously estimated from steady-state kinetics (Bender and Kezdy, 1964; Zerner et al., 1964). This value is at the usual upper limit of the rate constant for acid–base catalysis (10^3–10^4 s^{-1}), although already slightly faster than the rate constant of a simple proton transfer from water to imidazole (2300 s^{-1}) (Eigen and Hammes, 1963; Tables of Chemical Kinetics, 1950). On the other hand, the k_2(lim) value for the ethyl ester is in fairly good agreement with previous estimates (Bender and Kezdy, 1964; McConn et al., 1971) because K_S had been accurately estimated and k_2 exhibits a pK similar to that of k_2/K_S.

The pH-dependent behavior of k_2/K_S (Figure 3) is similar to that observed for k_2, including the close fit of the data at high ionic strength and low pH. The deviation at low pH and low ionic strength is due to artifactually high k_2 values and low K_S values.[1] These are not caused by dimerization of the enzyme (see below). Values of k_2/K_S(lim) and pK_1 are shown in the sixth columns of Tables I and II. The values of pK_1 for the ethyl esters are in good agreement with literature values for the enzyme (Bender et al., 1964).

The low pH–low ionic strength anomaly in k_2 and k_2/K_S was observed previously on the catalytic rate constant, k_{cat}, of the hydrolysis of *N*-acetyl-L-tryptophan derivatives by α-chymotrypsin at ionic strength 0.05 (Kezdy et al., 1964). At ionic strength 1.0, the k_2 diminished by one-half, whereas the K_S increased from 1.5 to 2 times (see Table F). Consequently, the values of k_2/K_S decreased by a factor of 3–4, and both k_2 and k_2/K_S fit well to pH–rate profile curves with a single ionizable group as is seen in Figures 1–3. This behavior may be rationalized in terms of electrostatic perturbation of the basic group of the active site by the high positive charge on the enzyme at low pH. When the ionic strength is high enough, the effect disappears.

In order to determine if enzyme dimerization affects the rates measured here, the values of k_2 and K_S were also measured by the stopped-flow pH-jump technique. For these experiments, the pH of the enzyme–water solution was adjusted to 7.0 or 5.0 with dilute sodium hydroxide or hydrochloric acid, respectively. Then, the solution was mixed with a substrate solution of the desired buffer in the stopped-flow instrument, resulting in a pH drop or rise. Since the values of rate constants of the interconversions both between active and inactive conformations of the enzyme (Fersht and Requena, 1971b) and between monomer and dimer (unpublished observations of Dr. M. J. Gilleland, in this laboratory) were found to be smaller than the k_2 value for the *p*-nitrophenyl ester at pH 5, the identical results of k_2 and K_S with those obtained by the usual procedure described in the experimental section indicate that there are no effects on k_2 and K_S by a change in the concentration of the active form and/or dimerization. The results for the δ enzyme showed a behavior similar to those for the α enzyme. This may also imply that the deviation of rate constants at low pH is not due to dimerization, as the δ enzyme is believed not to dimerize under the conditions used (Kezdy and Bender, 1965).

The pK values of k_2/K_S for both the *p*-nitrophenyl esters are lower than those of the ethyl esters. This has previously been shown for the oxygen ester to be due to the influence of the approach to rate-determining binding of k_2/K_S, i.e., $k_{-1} \sim k_2$ and $k_2/K_S \sim k_1$ (eq 2) (Philipp and Bender, 1973).

$$K_r = K_S \frac{k_2 + k_{-1}}{k_{-1}} \tag{3}$$

Equation 3 (Renard and Fersht, 1973) applies to this situation. pK_r is the apparent ionization constant of k_2/K_S for rapid substrates and pK_S is the apparent ionization constant of k_2/K_S for analogous slow substrates. The pK of k_2/K_S, in the absence of perturbations, is the pK of the free enzyme (Bender and Kezdy, 1965).

In the two cases studied here, the average pK value of k_2/K_S (Table II) for the ethyl esters was used as pK_S; k_2 and pK_r were the values listed in Tables I and II for the two nitrophenyl esters. The resulting k_{-1} values are listed in Table III. The k_1 values were then calculated from eq 4 (Gutfreund, 1955).

$$K_S = \frac{k_{-1} + k_2}{k_1} \tag{4}$$

To compare values of K_S and k_2/K_S between different substrates where $k_{-1} \sim k_2$, the values of k_{-1}/k_1 (analogous to K_S at $k_{-1} \gg k_2$) and k_2k_1/k_{-1} (analogous to k_2/K_S) were calculated for the nitrophenyl esters. These values are also listed in Table III. It is apparent that unperturbed values of k_2/K_S or (k_2k_{-1}/k_1) for oxy- and thionitrophenyl esters are identical, while the unperturbed K_S or (k_{-1}/k_1) values differ only by a factor of two.

In comparing k_1 values with other such values in the literature, it is apparent that k_1 for AcTrpSnP is the highest such value yet measured. The next highest reported value is 6×10^7 M^{-1} s^{-1} (Hess, 1971). The k_1 value for AcTrpSnP approaches the diffusion-controlled limit (Eigen, 1964).

[1] These values are reported to be sensitive to ionic strength differences (Hague et al., 1971).

TABLE III: Limiting Kinetic Constants for *p*-Nitrophenyl *N*-Acetyltryptophanates.

	Acetyl-L-TrpOnP	AcTrpSnP
k_1(lim)	1.3×10^8 M^{-1} s^{-1}	2.4×10^8 M^{-1} s^{-1}
k_{-1} lim	3.5×10^3 s^{-1}	2.9×10^3 s^{-1}
K_S(uncorr) (lim)[a]	6.5×10^{-5} M	2.1×10^{-5} M
k_{-1}/k_1(lim)[b]	2.8×10^{-5} M	1.2×10^{-5} M
k_2(lim)	4.65×10^3 s^{-1}	2.10×10^3 s^{-1}
k_2k_1/k_{-1}(lim)[c]	1.66×10^8 M^{-1} s^{-1}	1.7×10^8 M^{-1} s^{-1}
k_2/K_S(lim)[a]	7.3×10^7 M^{-1} s^{-1}	9.6×10^7 M^{-1} s^{-1}

[a] Uncorrected for effects due to $k_2 \sim k_{-1}$. [b] Comparable to K_S in cases where $k_2 \ll k_{-1}$. [c] Comparable to k_{cat}/K_m (or k_2/K_S) in cases where $k_2 \ll k_{-1}$.

Discussion

The data presented here show the complete pH dependence of k_2, K_S, and k_2/K_S for four specific *N*-acetyltryptophan ester and thioester substrates of chymotrypsin, as well as pH-independent values of k_1 and k_{-1} for the *p*-nitrophenyl esters and thioesters of *N*-acetyltryptophan. k_1, k_{-1}, and k_2 are all of the kinetic rate constants that can be affected directly by the O-S substitution in eq 2. However, it is useful to consider some of the more complex constants, such as K_S (or its analogue k_{-1}/k_1) or k_2/K_S (or its analogue k_2k_1/k_{-1}). K_S may provide information as to the possibility of nonproductive binding being present, when compared with k_2/K_S, which does not reflect such components (Bender and Kezdy, 1965).

Effects of the Change in Leaving Group on k_2/K_S. Table II shows that the p*K* values for k_2/K_S of the nitrophenyl esters are significantly lower than the analogous values for the ethyl esters. This was the basis, together with the absolute values of k_2 and K_S, for calculation of the values of k_1 and k_{-1} for the nitrophenyl esters (see Results). Since the measured k_2/K_S for the *p*-nitrothiophenyl ester equals k_1 for the analogous oxygen ester, a correction in *p*-nitrophenyl ester k_2/K_S values was made in order to relate them in a meaningful way to each other, and to those of the ethyl esters. Instead of relating k_2/K_S [$\equiv (k_2/((k_2 + k_{-1})/k_1))$], it is more meaningful to relate k_2k_1/k_{-1}, which equals k_2/K_S when $k_{-1} \gg k_2$. Table III gives values of k_2k_1/k_{-1}. This table shows that the values of $(k_2k_1)/k_{-1}$ for oxy- and thio-*p*-nitrophenyl *N*-acetyltryptophanates are identical. Table III shows that O-S substitution in the analogous ethyl esters increases $(k_2k_1)/k_{-1}$ by a factor of eight. These results show much lower O-S substitution effects than those reported in the literature, except literature values for *p*-nitrophenyl acetate (and *p*-nitrophenyl thioacetate) (Table G, see paragraph concerning supplementary material). The only clear difference between the substrates used here and those used previously is that the *N*-acetyltryptophan series is more specific (i.e., possesses higher k_2/K_S values) (Bender and Kezdy, 1965) than the other substrates, when comparisons are made between substrates of identical leaving groups. It is interesting to note that, in previous comparisons of k_2/K_S values between various series of substituted phenyl ester substrates, the most specific series of substrates had the lowest susceptibility of k_2/K_S on any electronic effect (Hammett σ) (Williams and Bender, 1971; Williams, 1970; Philipp, 1971). The O-S substitution can be considered similarly to substituent dependencies, and these data show that, as the leaving group in the ethyl ester series becomes more electron withdrawing, an increase in rate is observed, but that this increase is not observed in the *p*-nitrophenyl esters. However, causes for effects on k_2k_1/k_{-1} will be considered after discussion of k_2 and K_S.

TABLE IV: Binding Constants of *N*-Acetyl-L-tryptophan Derivatives.[a]

Acetyl-L-tryptophan Derivatives	K_S (mM)	pH Range	References
		<7	
Free acid	2	4-5	Johnson and Knowles (1966)
Amide	3	5.75	Bender et al. (1964)
Ethyl ester	2	4-5	Brandt et al. (1967), this work
Thiolethyl ester	0.33	4-5	This work
p-Chloroanilide	5.33	5.60	Caplow (1969)
p-Nitrophenyl ester	0.164	4-5	This work
p-Nitrothiophenyl ester	0.092	4-5	This work
		>7	
Amide	6.3	8	Bender et al. (1966)
Ethyl ester	0.85	8	This work
Thiolethyl ester	0.085	8	This work
p-Chloroanilide	0.70	8.1	Caplow (1969)
p-Nitrophenyl ester	0.090	8	This work
p-Nitrothiophenyl ester	0.034	8	This work

[a] Uncorrected for the active-inactive enzyme equilibrium (Fersht and Requena, 1971b).

The Effect of Leaving Group Changes on K_S. Table I shows that the thiol esters of *N*-acetyltryptophan exhibit lower K_S values (showing better binding) than the comparable oxygen esters. Table III shows that the value of k_{-1}/k_1 for *p*-nitrophenyl *N*-acetyltryptophanate is lower than that of the comparable oxygen ester. Comparisons of the K_S values (or the k_{-1}/k_1 values for the nitrophenyl esters) with literature values of K_S and K_I for *N*-acetyl-L-tryptophan compounds are shown in Table IV. These show that all the compounds used here have considerably lower binding constants than the simple amide and the free acid, with the exception of the ethyl ester at low pH. In addition, the nitrophenyl esters and the thiolethyl ester used here show lower binding constants at all pH values than does the *p*-chloroanilide.

It would appear that any consideration of the magnitude of the K_S values given here must be related to previous considerations concerning K_S values of analogous anilides. *N*-Acetyl-L-tyrosine anilides show strongly pH-dependent K_S values in the neutral and acid pH ranges, as do other hydrophobic substances, such as proflavin (Marini and Caplow, 1971).

Similar but less extreme shifts in K_S values with pH are observable with the substrates used here (Figure 1).

Three interpretations of these pH dependencies have been given: one is that these substrates bind in a nonproductive manner; one that the extent of nonproductive binding is pH dependent; and lastly that the magnitude of K_S at neutral pH, at least with the *p*-chloroanilides, is primarily determined by the hydrophobic nature of the leaving group (Fastrez and Fersht, 1973).

Another interpretation is that, while nonproductive binding is present, the p*K* shifts in K_S (and the related shifts of k_2) result from alterations in the p*K* of the active site by the substrate in its productive binding mode. In this view, the unnatural leaving group, in the productive complex, sterically perturbs the active-site histidine (Philipp et al., 1973).

The third interpretation is that a covalent intermediate on the reaction path to the acyl-enzyme perturbs the K_S value in a pH-dependent way, in the absence of nonproductive binding (Zeeberg et al., 1975).

These differences in interpretation are subject to a number of comments.

First, a covalent reaction intermediate greatly affecting K_S would be expected to show greatest influence either in pH regions of the greatest enzyme activity or in those of best binding. This is not the case, since greatest differences between substrate K_S values and analogous nonsubstrate K_I values are in the pH region below pH 5, where substrate binding becomes relatively weak and enzyme activity very low. In pH regions near neutrality, hydrophobic substrate K_S and analogous inhibitor K_I values differ only slightly, by a factor of less than 3.5 (Bizzozero and Zweifel, 1975; Zeeberg et al., 1975). While substrate–nonsubstrate differences are observable at low pH, it is not necessary to assume the effects by a hypothetical covalent reaction intermediate. Substrate side chain and substrate leaving group interaction with the aspartate–histidine–serine charge-relay system are causes fully consistent with available data. However, imposition of an accumulating reaction intermediate is not consistent with the data since an attempt to observe such an intermediate has failed (Fastrez and Fersht, 1973).

The remaining question, whether pH-dependent productive binding or pH-dependent nonproductive binding is responsible for the pH–K_S effects observed with anilides, is still open. The determinant formulation of Hamilton et al. (1966) has been used in an attempt to resolve this question. There is, however, some question whether the empirical microscopic binding terms derived by Hamilton et al. for unsubstituted amides of *N*-acylamino acids are valid for anilide substrates and inhibitors. For example, it has not been demonstrated that the aniline ring does not interact intramolecularly with the phenolic tyrosine side chain, changing the microscopic binding constants of both. Interestingly, the unsubstituted amides of *N*-acyl amino acids studied by Hamilton et al. all apparently bind in a nonproductive manner. It is difficult to understand why the anilide leaving group, absent in natural substrates, should induce an unusual amount of productive binding. It is true that the anilide benzene ring could fit into chymotrypsin leaving group specificity sites, as suggested by Zeeberg et al. (1975). It appears, however, that this would only provide a new mode of nonproductive binding, since the leaving group site seems to be specific for tyrosine (Morihara et al., 1969; Fersht et al., 1973) and phenylalanine (Bauer et al., 1976) residues, and the phenyl ring in tyrosine and phenylalanine is two carbon atoms further removed from the amide nitrogen atom than is the anilide benzene ring. This new nonproductive binding mode may, in determinant analysis, falsely appear to be productive.

Until the procedure of Hamilton et al. is used in a complete sense, including the calculation of microscopic binding constants for anilides, it is apparent that the method cannot be relied upon to provide an unambiguous answer to the problem of nonproductive binding. Considering the cautions, which Hamilton et al. (1966) discuss in the use of calculations of this type, it might appear that another method, such as crystal structure determinations, might be a more appropriate way of studying this problem (Robertus et al., 1972).

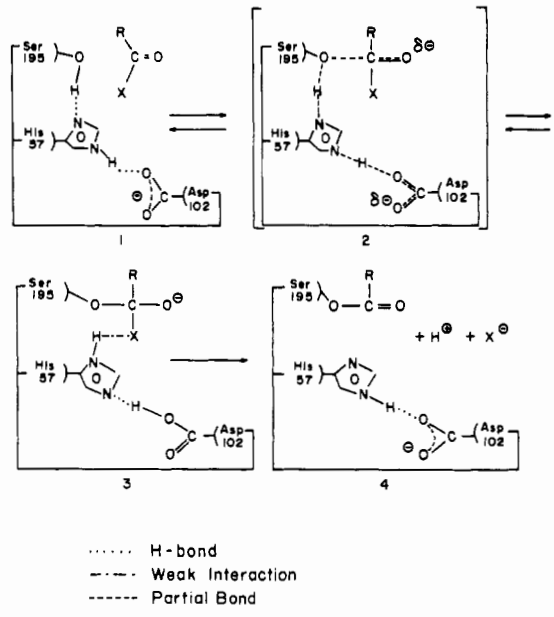
Taking all of the above considerations into account, we conclude that nonproductive binding probably plays a role in the K_S of many *N*-acetyl-L-tyrosine anilides, and therefore, in the K_S values of the compounds used here. Table IV shows that the sulfur and nitrophenyl esters behave, in a qualitative way, similarly to one of the more hydrophobic anilides. However, while it is clear that K_S–pH profiles for the substrates used here are similar to those of hydrophobic anilides, any interpretation of the K_S and pK shifts is made difficult due to interference by pH dependent shifts in active site pK at low pH and ionic strength. Therefore, no such interpretation will be presented here.

The Effect of the O–S Substitution on k_2. It is apparent that any nonproductive binding effects would complicate comparisons of k_2 between oxygen and sulfur esters. The question must arise whether the near identity of oxygen and sulfur ester k_2 values seen in this study can be interpreted in a mechanistic way. In one other such study where k_2 values were determined (involving oxygen and thio forms of *N*-acetylglycine ethyl ester together with chymotrypsin (Ingles and Knowles, 1966)), k_2 increased nearly 6-fold on O–S substitution. However, k_2/K_S values, which are independent of nonproductive binding effects (Bender and Kezdy, 1965), increased nearly 60-fold. With *p*-nitrophenyl acetate, O–S substitution has no effect on either k_2 or k_2/K_S (Frankfater and Kezdy, 1971). However, in the case of the analogous ethyl acetates with subtilisin, a change in k_2/K_S of nearly 50 on O–S substitution is observed (Polgar, 1972). All ethyl esters show large changes in k_2/K_S on O–S substitution, except the most specific such ester, the one used in this study. However, an effect of O–S substitution in nitrophenyl esters has never been observed, either in k_2 or k_2/K_S. Some conclusions can be drawn from these results. The nitrophenyl esters show no real microscopic k_2 changes on O–S substitution since both measured k_2 and k_2/K_S values show no O–S substitution effect. However, nonspecific ethyl esters do show O–S substitution effects in the microscopic k_2 since (1) the measured k_2 does increase on substitution, and (2) the value of k_2/K_S, independent of nonproductive effects, increases in every case by a factor of about 50-fold. Since comparison of K_S values shows that, in the case of the *N*-acetyltryptophan thiolethyl ester, nonproductive binding probably affects (and lowers) the measured k_2 (Bender and Kezdy, 1965), the increase in k_2/K_S (8-fold) must be used as an estimate of the effect of O–S substitution on the microscopic values of k_2. This 8-fold increase is substantially lower than those observed for other, slower substrates.

Previous studies of the substituent effect on k_2/K_S in various series of phenyl esters have shown that, with increased specificity, these substrates become less susceptible to any electronic effect. This is consistent with the pattern observed here (Williams and Bender, 1971; Williams, 1970; Philipp, 1971), when comparing the relatively nonspecific ethyl ester substrates with large O–S substitution effects to the most specific ethyl ester with a lower O–S substitution effect.

Model studies have shown that, in neutral media, breakdown of tetrahedral hemithioacetals and O–S acetals involves preferential elimination of the sulfur-containing leaving group (Jencks, 1969; Hershfield and Schmir, 1972). In the model most similar to the enzymatic case, hydrolysis of an O–S acetal, the sulfur-containing leaving group is eliminated at a rate 110 times that of the oxygen-containing leaving group (Hershfield and Schmir, 1972). This value is not greatly different from the average ratios of k_2/K_S on O–S substitution in less specific ethyl esters (50-fold). This near identity of relative rates strongly indicates that the k_2/K_S values reflect rate-determining breakdown of the tetrahedral intermediate involved during acylation (see Scheme I). Frankfater and Kezdy (1971), using arguments of Fersht and Jencks (1970), have concluded thad k_2 in the chymotrypsin-catalyzed hydrolysis

Scheme I



of *p*-nitrophenyl acetate reflects rate-determining formation of a tetrahedral intermediate on the reaction path. This conclusion must be extended to the very specific *p*-nitrophenyl *N*-acetyltryptophanates. The ethyl *N*-acetyltryptophanates must be substrates in which the rates of tetrahedral intermediate formation must be of the same order of magnitude as the rates of tetrahedral intermediate breakdown.

Thus, it seems that in less specific ethyl esters tetrahedral intermediate breakdown is slower than its formation, and in highly specific ethyl esters the formation and breakdown rates approach each other, while in all nitrophenyl esters tetrahedral intermediate formation rates are lower than their breakdown rates (see Scheme I).

The Magnitude of k_1 and k_{-1}. A previous estimate of k_1 and k_{-1} has been made for the case of *p*-nitrophenyl *N*-acetyl-L-tryptophanate (Renard and Fersht, 1973). The values derived differed from those calculated here since an assumed value of K_S (higher than the actual value observed here) was used. Values of k_1 for *p*-nitrophenyl *N*-acetyltryptophanates (Table III) are the highest yet reported for chymotrypsin substrates. They are comparable to the value of 6.0×10^7 M^{-1} s^{-1} reported for *N*-acetyl-L-phenylalanine amide (Hess, 1971) and are much higher than the value reported for *N*-furylacryolyltryptophanamide, 6.2×10^6 M^{-1} s^{-1} (Hess et al., 1970). The maximal value of 1.49×10^7 M^{-1} s^{-1} for the inhibitor *N*-trifluoroacetyl-D-tryptophan (Smallcombe et al., 1972), the value of 1×10^8 M^{-1} s^{-1} for proflavin (Havsteen, 1967), and the value of 2.1×10^7 M^{-1} s^{-1} for Bieberich's Scarlet (Hague et al., 1971) are all comparable to the values observed here. (See Table H in supplementary material.) These association rate constants vary by a factor of about 40. The values suggest that addition of a hydrophobic leaving group, which induces nonproductive binding, also serves to increase the rate of binding. Values of k_{-1} that have been reported range from 6.6×10^2 s^{-1} (Hague et al., 1971) to 3.9×10^3 s^{-1} (Smallcombe et al., 1972). These values are close to the values reported here, and indeed, many are identical. The k_{-1} values are similar to the rate constant for a water to imidazole proton transfer (Eigen and Hammes, 1963).

Supplementary Material Available

Additional data (Tables A-H) concerning dissociation, kinetic, binding, and limiting rate constants (8 pages). Ordering information is given on any current masthead page.

References

Bauer, C. A., Thompson, R. R. C., and Blout, E. R. (1976), *Biochemistry 15,* 1976.

Bender, M. L. (1960), *Chem. Rev. 60,* 53.

Bender, M. L. (1971), Mechanisms of Homogeneous Catalysis from Protons to Proteins, New York, N.Y., Wiley, Chapter 12.

Bender, M. L., Clement, G. E., Kezdy, F. J., and Heck, H. D'A. (1964), *J. Am. Chem. Soc. 86,* 3680.

Bender, M. L., Gibian, M. J., and Whelan, D. J. (1966), *Proc. Natl. Acad. Sci. U.S.A. 56,* 833.

Bender, M. L., and Kezdy, F. J. (1964), *J. Am. Chem. Soc. 86,* 3704.

Bender, M. L., and Kezdy, F. J. (1965), *Annu. Rev. Biochem. 34,* 49.

Bernhard, S. A., Lee, B. F., and Tashjian, Z. H. (1966), *J. Mol. Biol. 18,* 405.

Bizzozero, S. A., and Zweifel, B. O. (1975), *FEBS Lett. 59,* 105.

Brandt, K. G., Himoe, A., and Hess, G. P. (1967), *J. Biol. Chem. 242,* 3973.

Bruice, T. C., and Benkovic, S. J. (1966), Bioorganic Mechanisms, Vol. 1, New York, N.Y., W. A. Benjamin, Chapter 2.

Caplow, M. (1969), *J. Am. Chem. Soc. 91,* 3639.

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

Connors, K. A., and Bender, M. L. (1961), *J. Org. Chem. 26,* 2498.

Dixon, M. (1953), *Biochem. J. 55,* 161.

Eadie, G. S. (1942), *J. Biol. Chem. 146,* 85.

Eigen, M. (1964), *Angew. Chem., Int. Ed. Engl. 3,* 1.

Eigen, M., and Hammes, G. G. (1963), *Adv. Enzymol. 25,* 1.

Fastrez, J., and Fersht, A. R. (1973), *Biochemistry 12,* 1067.

Fersht, A. R., Blow, D. M., and Fastrez, J. (1973), *Biochemistry 12,* 2035.

Fersht, A. R., and Jencks, W. P. (1970), *J. Am. Chem. Soc. 92,* 5442.

Fersht, A. R., and Renard, M. (1974), *Biochemistry 13,* 1416.

Fersht, A. R., and Requena, Y. (1971a), *J. Am. Chem. Soc. 93,* 7079.

Fersht, A. R., and Requena, Y. (1971b), *J. Mol. Biol. 60,* 279.

Frankfater, A., and Kezdy, F. J. (1971), *J. Am. Chem. Soc. 93,* 4039.

Gutfreund, H. (1955), *Discuss. Faraday Soc. No. 20,* 167.

Hague, D. N., Henshaw, J. S., John, J. A., Pooley, M. J., and Chock, P. B. (1971), *Nature (London) 229,* 190.

Hamilton, C. L., Neimann, C., and Hammond, G. S. (1966), *Proc. Natl. Acad. Sci. U.S.A. 55,* 664.

Hardman, M. J., Valenzuela, P. V., and Bender, M. L. (1971), *J. Biol. Chem. 246,* 5907.

Havsteen, B. H. (1967), *J. Biol. Chem. 242,* 769.

Hershfield, R., and Schmir, G. L. (1972), *J. Am. Chem. Soc. 94,* 1263.

Hess, G. P. (1971), *Enzymes 3rd Ed. 3,* 228.

Hess, G. P., McConn, J., Ku, E., and McConkey, G. (1970), *Philos. Trans. R. Soc. London, Ser. B 257,* 89.

Hirohara, H., Bender, M. L., and Stark, R. S. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1643.

Ingles, D. W., and Knowles, J. R. (1966), *Biochem. J. 99,* 275.
Jencks, W. P. (1969), Catalysis in Chemistry and Enzymology, New York, N.Y., McGraw-Hill, pp 500-501.
Johnson, C. H., and Knowles, J. R. (1966), *Biochem. J. 101,* 56.
Kezdy, F. J., and Bender, M. L. (1962), *Biochemistry 1,* 1097.
Kezdy, F. J., and Bender, M. L. (1965), *Biochemistry 4,* 104.
Kezdy, F. J., Clement, G. E., and Bender, M. L. (1964), *J. Am. Chem. Soc. 86,* 3690.
Lucas, E. C., and Caplow, M. (1972), *J. Am. Chem. Soc. 94,* 960.
Lucas, E. C., Caplow, M., and Bush, K. J. (1973), *J. Am. Chem. Soc. 95,* 2670.
McConn, J., Ku, E., Himoe, A., Brandt, K. G., and Hess, G. P. (1971), *J. Biol. Chem. 246,* 2918.
Marini, J. L., and Caplow, M. (1971), *J. Am. Chem. Soc. 93,* 5560.
Martin, R. B., and Hedrick, R. I. (1962), *J. Am. Chem. Soc. 84,* 106.
Morihara, K., Oka, T., and Tsuzuki, H. (1969), *Biochem. Biophys. Res. Commun. 35,* 210.
Oppenheimer, H. L., Labouesse, B., and Hess, G. P. (1966), *J. Biol. Chem. 241,* 2720.
Philipp, M. (1971), Dissertation, Northwestern University.
Philipp, M., and Bender, M. L. (1973), *Nature (London), New Biol. 241,* 44.
Philipp, M., Pollack, R. M., and Bender, M. L. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 517.
Polgar, L. (1972), *Acta Biochim. Biophys. Acad. Sci. Hung. 7,* 319.
Rajender, S., Lumry, R., and Han, M. (1971), *J. Phys. Chem. 75,* 1375.
Renard, M., and Fersht, A. R. (1973), *Biochemistry 12,* 4713.
Robertus, J. D., Kraut, J., Alden, R. A., and Birktoft, J. J. (1972), *Biochemistry 11,* 4293.
Smallcombe, S. H., Ault, B., and Richards, J. H. (1972), *J. Am. Chem. Soc. 94,* 4585.
Tables of Chemical Kinetics (1950), Homogeneous Reactions, National Bureau of Standards Circular 510.
Weiner, H., and Koshland, D. E., Jr. (1965), *J. Biol. Chem. 240,* 2764.
Williams, A. (1970), *Biochemistry 9,* 3383.
Williams, R., and Bender, M. L. (1971), *Can. J. Biochem. 49,* 210.
Zeeberg, B., Caplow, M., and Caswell, M. (1975), *J. Am. Chem. Soc. 97,* 7346.
Zerner, B., Bond, R. P. M., and Bender, M. L. (1964), *J. Am. Chem. Soc. 86,* 3674.

Comparison of the Biosynthetic and Biodegradative Ornithine Decarboxylases of *Escherichia coli*†

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ABSTRACT: Biosynthetic ornithine decarboxylase was purified 4300-fold from *Escherichia coli* to a purity of approximately 85% as judged by polyacrylamide gel electrophoresis. The enzyme showed hyperbolic kinetics with a K_m of 5.6 mM for ornithine and 1.0 μM for pyridoxal phosphate and it was competitively inhibited by putrescine and spermidine. The biosynthetic decarboxylase was compared with the biodegradative ornithine decarboxylase [Applebaum, D., et al. (1975), *Biochemistry 14,* 3675]. Both enzymes were dimers of 80 000-82 000 molecular weight and exhibited similar kinetic properties. However, they differed significantly in other respects. The pH optimum of the biosynthetic enzyme was 8.1, compared with 6.9 for the biodegradative. Both enzymes were activated by nucleotides, but with different specificity. Antibody to the purified biodegradative ornithine decarboxylase did not cross-react with the biosynthetic enzyme. The evolutionary relationship of these two decarboxylases to the other amino acid decarboxylases of *E. coli* is discussed.

Ornithine decarboxylase is widely distributed in nature and catalyzes the synthesis of putrescine from ornithine (for recent reviews of polyamine biosynthesis, see Tabor and Tabor, 1972; Morris and Fillingame, 1974). In *Escherichia coli,* ornithine decarboxylase is induced to high levels by low pH and the presence of substrate (Applebaum et al., 1975), thus classifying it as one of the biodegradative amino acid decarboxylases (Gale, 1946; Morris and Fillingame, 1974). The biodegradative ornithine decarboxylase of *E. coli* has been purified and characterized (Applebaum et al., 1975). Under noninducing conditions, i.e., at neutral pH and in minimal culture medium, *E. coli* contains low levels of ornithine decarboxylase activity for synthesis of putrescine and spermidine (Morris and Pardee, 1965, 1966). It is not known whether this low activity is due to a distinct ornithine decarboxylase or to simply the uninduced level of the biodegradative enzyme. Both biosynthetic and biodegradative enzymes for the decarboxylation of arginine have been demonstrated in *E. coli* (Blethen et al., 1968; Wu and Morris, 1973a,b). In this paper, we describe the purification of ornithine decarboxylase from uninduced cells and compare its properties with those of the biodegradative enzyme.

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