Acylation of α-Chymotrypsin by Oxygen and Sulfur Esters of Specific Substrates: Kinetic Evidence for a Tetrahedral Intermediate

(enzyme/presteady state/rate constant/binding constant/leaving-group effect)

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ABSTRACT The acylation step of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L (or DL)-tryptophan p-nitrophenyl, p-nitrothiophenyl, ethyl, and thiolethyl esters has been studied by the stopped-flow technique at 25°. The acylation rate constant, k_2, and the enzyme substrate dissociation constant, K_s, were directly determined at pH 4, 5, and 8. Steady-state kinetics were studied at pH 7. The k_2 values are nearly identical for oxygen esters and their sulfur counterparts, whereas the K_s value of the ethyl ester is larger by an order of magnitude than those of the other three. The results strongly suggest that oxygen and thiol esters of these specific substrates are hydrolyzed via the same pathway, and furthermore that acylation consists of more than one step, the formation and breakdown of a tetrahedral intermediate, the former being rate-determining. Effects of leaving-group hydrophobicity on k_2 and K_s are also discussed.

Whether or not a tetrahedral intermediate is formed in the reaction pathway of α-chymotrypsin-catalyzed hydrolysis has been a subject of great interest. Some of the first evidence for such intermediates in nonenzymatic acyl-transfer reactions came from the study of the alkaline hydrolysis of esters labeled specifically in the carbonyl group with ^{18}O (1). The occurrence of a similar intermediate in the α-chymotrypsin-catalyzed hydrolysis of some anilides and hydrazides has been recently proposed from kinetic studies by several research groups (2–9). An important contribution to this problem on (nonspecific) ester substrates has been made by Frankfater and Kézdy (10), who obtained identical rate constants for the α-chymotrypsin-catalyzed hydrolysis of p-nitrophenyl acetate and thiolacetate. Since an –SR group was expected to be a much better leaving group than an –OR group, it was argued that the bond-breaking process could not occur in the rate-determining step. Studies of the breakdown of similar tetrahedral compounds as hemithioacetals and acetaldehyde hydrate (29) and ketene O, S acetals (43) have demonstrated the leaving superiority of –SR over –OR. Because there is no observable accumulation of a tetrahedral intermediate, a rapid decomposition step must follow a slower addition

step. Such a reaction scheme is identical to that postulated for the alkaline hydrolysis of oxygen and thiol esters (10).

The purpose of this investigation is to determine whether such results are applicable to the acylation of α-chymotrypsin by *specific* ester substrates. The acylation rate constant, k_2, and the enzyme-substrate complex dissociation constant, K_s [$=k_{-1}/k_1$, Eq. 4], for N-acetyl-tryptophan esters were directly measured at pH 4.0, 5.0, and 8.0 and 25.5° in this investigation. These constants for specific ester substrates have not previously been determined under conditions close to their physiological environment, although some data for alkyl esters at acidic pH levels has been reported by Hess et al. (11, 12). Therefore, it is expected that the kinetic parameters themselves obtained here will provide further knowledge about the individual steps in the α-chymotrypsin-catalyzed reaction.

MATERIALS AND METHODS

α-Chymotrypsin (three times crystallized) was a Worthington Biochemical Corp. product. Its concentration was determined by active site titration using p-nitrophenyl p'-guanidinobenzoate (13).

N-Acetyl-L-tryptophan p-nitrophenyl ester (AcTrpONp) was obtained from Cyclo Chemical Co., mp 131–132°.

N-Acetyl-L-tryptophan ethyl ester (Cyclo) (AcTrpOEt) was recrystallized from dry ethyl acetate-ether as described previously (14), mp 105–106°.

N-Acetyl-DL-tryptophan p-nitrothiophenyl ester (AcTrpSNp) was prepared from N-acetyl-L-tryptophan (Sigma) and p-nitrothiophenol with dicyclohexylcarbodiimide (DCC) in acetonitrile at room temperature. The urea was filtered and the solvent removed *in vacuo* at room temperature. The residue was repeatedly recrystallized from acetone and anhydrous ether at 4°, mp 195–197°. The product was determined to be 61% racemate (with the L form predominating) from its behavior with α-chymotrypsin.

N-Acetyl-L-tryptophan thiolethyl ester (AcTrpSEt) was also prepared with DCC in acetonitrile according to the method described previously (15). A small amount of acetonitrile was, however, added in order to facilitate the miscibility of N-acetyl-L-tryptophan with ethanethiol. The crude material was purified from ethyl acetate-ether to give white crystals, mp 176.5–177.5°. The product is almost solely the L form as judged by its behavior with α-chymotrypsin.

Proflavin hemisulfate (Aldrich) was recrystallized from water in the dark. All buffers were reagent grade from Mallinckrodt and Sigma. Acetonitrile and N,N'-diethyl-
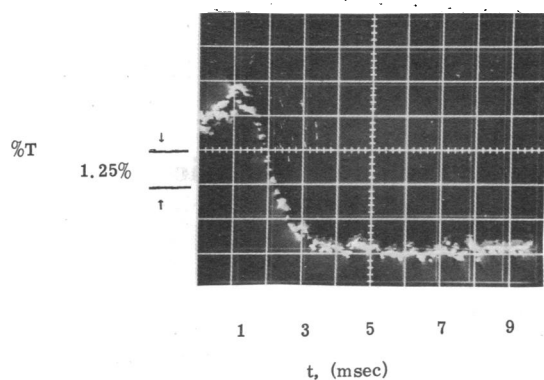
FIG. 1. Oscilloscope trace of acylation of α-chymotrypsin by N-acetyl-L-tryptophan p-nitrophenyl ester shown as percent transmittance at 402 nm versus time. Three consecutive runs are superimposed. Final concentrations: $[E_0] = 4.60 \times 10^{-5}$ M; $[S_0] = 3.85 \times 10^{-6}$ M; 0.1M Tris (pH 8.01); 0.5% (v/v) aceto-nitrile-water; 25.5°.

formamide used for stock solutions of substrates were Eastman Kodak Co. spectro grade products.

Steady-state kinetics were determined at pH 7.0 using a Cary 14 PM recording spectrophotometer with a thermostated cell holder. The hydrolyses of AcTrpONp and AcTrpSNp were followed at 400 nm ($\epsilon = 8,500$ M^{-1} cm^{-1}) and 410 nm ($\epsilon = 13,400$ M^{-1} cm^{-1}), respectively. The hydrolyses of AcTrpOEt and AcTrpSEt were followed at 300 nm ($\Delta\epsilon = 240$ M^{-1} cm^{-1}) described previously (14).

Acylation reactions were measured by a Durrum stopped flow spectrometer (model D-110) with a Durrum Tempera-ture jump accessory, according to methods described pre-viously (16). Volumes of both components were 300 μl per measurement at pH 4.0 and 5.0, and 450 μl at pH 8.0 so as to observe approximately the last 100 μl which is the freshest after mixing. The monitored wavelengths were 340 nm for p-nitrophenol at pH 4.0 and 5.0, 402 nm for p-nitrophenolate ion at pH 8.0, 340 nm for p-nitrothiophenol at pH 4.0, 410 nm for p-nitrothiophenolate ion at pH 5.0 and 8.0, and 465 nm for the enzyme-proflavin complex for both ethyl and thiolethyl esters at pH 5.0 and 8.0. The values of k_2 and K_s were calcu-lated by means of a CDC 6400 computer program written for the least-squares analysis of an Eadie plot (17). The experimental conditions along with the results are summarized in Table 2.

In experiments with the p-nitrothiophenyl ester, the enzyme stock solution was purified with Sephadex G-25 (10).

RESULTS

Steady-state kinetic parameters obtained by conventional methods are shown in Table 1. Values of k_{cat} for all four sub-strates are identical within experimental error.

In the presteady state experiments with AcTrpONp and AcTrpSNp, the pseudo first-order rate constant, k_{obs}, was determined under conditions of $[E_0] \gg [S_0]$. An advantage of these conditions is the elimination of the turnover portion of the enzymatic reaction. Furthermore, the results are not affected by the presence of the D-isomer. An oscillo-scope trace is shown in Fig. 1. Good first-order plots were obtained from such traces. At pH 8.0, only the last part of the reaction is observable because of the "dead" time of the apparatus. The reliability of the data thus obtained was checked by the observation that data at the lower pHs fit well with the first-order plot up to greater than 90% re-

TABLE 1.   *Steady-state kinetic parameters of α-chymotrypsin-catalyzed hydrolysis of N-acetyl-tryptophan esters at pH 6.99 $\pm$ 0.01 and 2.55 $\pm$ 0.2°* *

Substrate	k_{cat} (sec^{-1}) $\times$ 10	K_m (M) $\times$ 10^6	k_{cat}/K_m (M^{-1} sec^{-1}) $\times$ 10^{-6}
AcTrpONp	3.1 $\pm$ 0.3	3.9 $\pm$ 0.4	7.9
AcTrpSNp	2.6 $\pm$ 0.4	4.5 $\pm$ 0.4	5.8
AcTrpOEt	2.9 $\pm$ 0.2	65 $\pm$ 2.5	0.45
AcTrpSEt	2.7 $\pm$ 0.2	6.4 $\pm$ 0.4	4.2

* Phosphate buffer. 6.2% (v/v) acetonitrile-water, I = 0.15 for AcTrpONp and AcTrpSNp. 0.34% (v/v) dimethylform-amide–1.37% (v/v) acetonitrile-water, I = 0.1 for AcTrpOEt and AcTrpSEt.

action. As developed by Kézdy and Bender (16), k_{obs}, under the conditions $[E_0] \gg [S_0]$, is given by

$$k_{obs} = k_2[E_0]/(K_s + [E_0]).   [1]$$

To obtain k_2 and K_s, values of k_{obs} obtained at various enzyme concentrations were plotted according to

$$k_{obs} = k_2 - K_s k_{obs}/[E_0].   [2]$$

Because of the low value of K_s, k_2 and K_s could be indepen-dently determined at relatively low enzyme concentrations, $([E_0] \leq 1.5 \times 10^{-4}$ M). No deviations from linearity of the Eadie plots due to dimerization of the enzyme were observed.

The presteady state kinetic parameters pertaining to the ethyl and the thiolethyl esters were obtained from measure-ments of the displacement of the competitive inhibitor pro-flavin, F, from the enzyme by various substrate concentra-tions using the conditions: $[S_0] \gg [E_0] < [F_0]$. The feasi-bility of this method for the investigation of chymotrypsin's acylation was first suggested by Gutfreund *et al.* (18, 19) and employed by Hess *et al.* (11, 12, 20). Under the above conditions, the form of the Eadie plot is

$$k_{obs} - k_3 = k_2 - K_s(1 + [F_0]/K_F)(k_{obs} - k_3)/[S_0].   [3]$$

Evaluation of k_2 and K_s requires a knowledge of the deacyla-tion rate constant, k_3, and the dissociation constant for enzyme-proflavin complex formation, K_F. The k_3 values for the ethyl ester were determined at various pH levels previously (21). K_F was determined by measuring the difference absorption at 465 nm when $[E_0] > [F_0]$. The results obtained are $K_F = 11.2 \times 10^{-5}$ M and 3.29×10^{-5} M at pH 5.02 and 8.00, respectively, and are in good agreement with published values (11, 22–24). The presteady-state parameters thus obtained are summarized in Table 2 together with k_{cat}/K_m values reported previously.

The important experimental observations are as follows. (a) Values of k_2 are very close for the oxygen esters and their sulfur counterparts, although k_2 values of the oxygen esters are generally slightly larger except for the alkyl esters at pH 5.0. (b) The p-nitrothiophenyl and the p-nitrophenyl esters have roughly identical values of K_s, whereas the K_s value of the thiolethyl ester is much lower than that of the corresponding oxygen ester. The latter is in fairly good agreement with the value estimated from steady state kinetic data (14). In addi-tion, the K_s value seems to decrease with increasing pH for the four specific substrate esters. (c) The second-order rate con-

TABLE 2. *Presteady-state kinetic constants for α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan esters at 25.5 ± 0.4° and I = 0.1* *

Substrate	pH	k_2 (sec^{-1})	K_s† (M) × 10^4	k_2/K_s (M^{-1} sec^{-1}) × 10^{-5}	k_{cat}/K_m (M^{-1} sec^{-1}) × 10^{-5}
AcTrpONp	4.03	67.6 ± 0.6	2.53 ± 0.1	2.7	
	5.02	670 ± 50	1.5 ± 0.1	45	
	8.01	4380 ± 25	0.97 ± 0.2	450	680‡ (25)
AcTrpSNp§	4.03	35.6 ± 0.1	1.51 ± 0.02	2.4	
	5.02	460 ± 30	0.91 ± 0.14	50	
	8.01	225 ± 240	0.32 ± 0.08	700	
AcTrpOEt	5.02	33 ± 4.5	21 ± 3	0.16	0.10¶ (21)
	8.00	1090 ± 130	9.9 ± 1.2	11.0	4.0¶ (21), 5.8–12.4‡ (25)
AcTrpSEt	5.02	47 ± 8.2	4.4 ± 0.7	10.8	
	8.00	695 ± 55	0.85 ± 0.10	82	

* Citrate, acetate, and Tris·HCl buffer for pH 4.0, 5.0, and 8.0, respectively. 0.5% (v/v) acetonitrile–water for AcTrpONp and Ac-TrpSNp. 0.33% (v/v) dimethylformamide–1.36% (v/v) acetonitrile–water for AcTrpOEt and AcTrpSEt.

† K_F used for the calculation of K_s are 11.2 and 3.3 × 10^{-5} M at pH 5.0 and 8.0, respectively, see *text*.

‡ pH 7.9 aqueous solution.

§ DL.

¶ 0.81% (v/v) acetonitrile–water.

stants, k_2/K_s, of the p-nitrothiophenyl and the p-nitrophenyl esters are roughly identical whereas, the thiolethyl ester has a larger k_2/K_s value than the ethyl ester due to the lower K_s term, not to a difference in k_2. (d) The observed k_2/K_s of the oxygen esters are in good agreement with published values of the algebraically equivalent k_{cat}/K_m, considering effects of the organic solvent (26).

DISCUSSION

The foregoing results indicate that α-chymotrypsin catalyzes the hydrolysis of specific thiolesters with a pathway identical to that of the oxygen analogs and includes the same acyl-enzyme as an intermediate. Thus, the reaction scheme for the enzymatic hydrolysis of the thiol substrates would be

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E·S \overset{k_2}{\rightarrow} ES' \overset{k_3}{\rightarrow} E + P_2 \qquad [4]$$
$$+$$
$$P_1$$
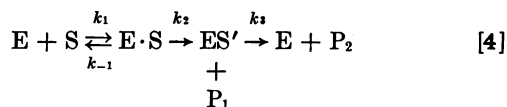
In addition, the results in Table 2 indicate that the mechanism of the acylation part of the reaction is also identical for both the oxygen and sulfur esters.

Since an –SR group is a better leaving group than an –OR group by at least two or three orders of magnitude (ref. 27–29, 43, see *introduction*) the near identity of the k_2 values for the thiol esters and their oxygen counterparts demonstrate that a one-step acyl-transfer reaction does not occur. The simplest way to explain the data is to postulate a *metastable tetrahedral intermediate*, the formation of which is the rate-determining step of the acylation reaction. The first slower bond making step is rate-determining and precedes the second faster bond breaking step. On the assumption that the stationary concentration of the intermediate, TI, is invariably very small, the observed overall acylation rate constants k_2 can be expressed in terms of Eqs. 5 and 6. Since no tetrahedral intermediate is

$$E·S \underset{k_{-a}}{\overset{k_a}{\rightleftarrows}} TI \overset{k_b}{\rightarrow} ES' + P_1 \qquad [5]$$
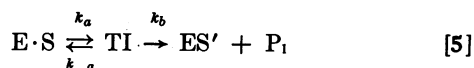
$$k_2 = k_a k_b/(k_{-a} + k_b) \qquad [6]$$

directly observed, $k_b \gg k_a$, and since k_2 is the same for the thiol and oxygen esters, $k_b \gg k_{-a}$. This is in agreement with

the results obtained for the alkaline hydrolysis of oxygen and thiol esters (27, 29–31), and also for the α-chymotrypsin-catalyzed hydrolysis of p-nitrophenyl acetate and thiolacetate (10).

Combining the experimental results that $k_2 \simeq k_a \simeq (1 - 5) × 10^3$ sec^{-1} at pH 8.0 and that $k_b \gg k_a$, with the expectation that –SR ions are at least 2 to 3 orders of magnitude better leaving groups than –OR ions, the values of k_b for the thiol esters should be 10^6–10^{10} sec^{-1}. Such large rate constants were indeed reported recently for the breakdown of tetrahedral intermediates in the general base-catalyzed hydrolysis of thiolesters (32). It is estimated that the rate constants of the breakdown of the intermediate are 1.7 × 10^7 and 5.8 × 10^{10} sec^{-1} for ethyl trifluorothiolacetate and methyl S-trifluoroacetylmercaptoacetate, respectively. The value for the latter compound approaches that of a diffusion-controlled process (33).

In the determination of k_2 by the proflavin-displacement method, the absorption at 465 nm decreases at first very rapidly due to the formation of a noncovalent enzyme–substrate complex. This is followed by a further decrease at 465 nm, which was measured by the stopped-flow technique, due to the formation of a covalent tetrahedral intermediate. It is assumed that the decomposition step of the tetrahedral intermediate to acyl-enzyme does not involve further proflavin displacement. In this case the observed rate constants by this method correspond to the formation of the tetrahedral intermediate and not to their breakdown. In terms of the Hammond postulate, some ambiguity may exist as to the magnitude of k_b of the ethyl ester as the pK_a of its leaving group (about 16) is probably larger than that of the serine hydroxyl nucleophile. Nevertheless, it should be emphasized that the conclusion regarding the tetrahedral intermediate in the previous paragraphs is applicable for all four esters since quasi-identical k_2 values were obtained for the ethyl and thiolethyl esters, also.

It is seen from the K_s values of the ethyl ester and its sulfur counterpart that the nature of the atom being displaced during acylation has a fairly large effect on binding to the enzyme, contrary to previous speculation. Since nonproductive binding should be a very minor factor, if any, in reactions

TABLE 3. *Comparison of kinetic parameters of*
α-chymotrypsin-catalyzed hydrolysis of several oxygen
and sulfur esters at pH 7.8–8.0 and 25°

Substrate		k_{cat} (sec^{-1})	K_m (M) $\times 10^3$	k_{cat}/K_m (M^{-1} sec^{-1})
AcGlyOEt*	(15)	0.039	389	0.101
AcGlySEt	(15)	0.23	41	5.61
ZGlyOEt	(31)			3.65
ZGlySEt	(31)	0.274	0.66	420
AcPheOEt	(31)			54,000
AcPheSEt	(31)			1,000,000
		k_2	K_s	k_2/K_s
AcPheOEt	(35)	265	7.14	37,200
NPA†	(10)	4.55	1.47 (21)	3,940 (21)
		4.8 (16)		4,060 (16)
NPTA‡	(10)	4.52	1.20	3,770

* According to Bender *et al.* (14), acylation is the rate-determining step ($k_3 = 2.29$ sec^{-1}). Therefore, the reported k_{cat} and K_m values can be regarded to be close to k_2 and K_s values, respectively.

† NPA, *p*-nitrophenyl acetate.

‡ NPTA, *p*-nitrophenyl thiolacetate.

of *N*-acetyl-tryptophan esters, these data indicate that the hydrophobicity of the leaving group provides additional binding strength but that when the leaving group is strongly hydrophobic to begin with, additional hydrophobicity makes only an additional small effect on binding. For *p*-nitrophenyl acetate and thiolacetate, identical values of K_s were observed (10, see Table 3), which were also similar to the value in the case of AcTrpOEt. It is likely that the binding modes of nonspecific substrates is entirely "nonproductive," which, if so, would lower k_2 values drastically (8). Presumably, *p*-nitrophenyl acetate has good nonproductive binding to begin with so the additional hydrophobicity due to a sulfur atom showed only a small effect on binding. From this viewpoint, the experimental finding that the thiolethyl ester has a larger k_2 value at pH 5.0 than the ethyl ester, may be understandable; the relatively large K_s value of the latter at this pH may imply that the substrate is not bound tight enough to give the optimum orientation. The additional binding due to the sulfur atom, therefore, allows a more favorable orientation for attack by the nucleophile, overcoming any slightly unfavorable reactivity of thiol substrates for the formation of the tetrahedral intermediate. The similar situation can be seen for acetylglycine ethylester (AcGlyOEt) and acetylglycine thiolethyl ester (AcGlySEt) in Table 3. The same argument might be applicable to benzyloxycarbonylglycine ethyl ester (ZGlyOEt) and benzyloxycarbonylglycine thiolethyl ester (ZGlySEt) and also to *N*-acetyl-L-phenylalanine ethyl ester PheOEt) and *N*-acetyl-DL-phenylalanine thiolethyl ester (AcPheSEt), to some extent (also see below). The observation that usually the oxygen ester has slightly greater k_2 may be due to the fact either that the Michaelis complex of the sulfur ester is so tightly bound that a slightly larger activation free energy is required in order to "loosen" the binding in a transition state of the formation of the tetrahedral intermediate, or that within the Michaelis complex the carbonyl group of the oxygen esters are slightly more favorably oriented to attack by the nucleophile than the carbonyl group of the sulfur esters, or a combination of both.

Since the value of K_s depends, more or less, on the nature of the leaving group, and since the difference in k_2/K_s terms are mainly due to differences in K_s, it is obvious that k_2/K_s (= K_{cat}/K_m) should not be used for studies of the acylation process without careful consideration. It can be easily said that the much larger k_{cat}/K_m values of ZGlySEt and AcPheSEt than those of their oxygen counterparts (in Table 3) are mainly due to the stronger binding of the thiolethyl esters, although the larger value for k_2 of thiolethyl esters over their oxygen analogs is also possible, as was mentioned above.

The experimentally obtained k_2 values for AcTrpONp and AcTrpSNp at pH 8.0 are an order of magnitude smaller than that estimated for the oxygen ester from steady state studies in the past (14) and are at the usual upper limit of the rate constant for acid-base catalysis (10^3–10^4 sec^{-1}) (36), although faster than the rate constant of a simple proton transfer from water to imidazole (2300 sec^{-1}) (36). *p*-Nitrophenyl esters are usually two orders of magnitude more reactive toward alkaline hydrolysis than ethyl esters (37). The relative reactivity ratio of the specific ester substrates in enzymic hydrolysis is, however, only 4 to about 5 at pH 8.0. This may reflect that the k_2 value at this pH has approached the maximum rate constant for general base catalysis.

Some k_1 values for α-chymotrypsin-substrate complex formation are 6 $\times$ 10^7 M^{-1} sec^{-1} for N-*trans* (2-furyl) acryloyl-L-tryptophanamide at 15° (38), 10^8 M^{-1} sec^{-1} for proflavin at 12° (39), 2.1 $\times$ 10^7 M^{-1} sec^{-1} for biebrich scarlet at 20° (40), and 1.5 $\times$ 10^7 M^{-1} sec^{-1} for trifluoroacetyl-D-tryptophan at 32° (41). Considering these values and the conditions that $k_1 \geq k_2/K_s$ (42), and that k_1 is lower than diffusion controlled [about 10^9 sec^{-1} (36)], the probable k_1 value for AcTrpONp and AcTrpSNp will be (6–10) $\times$ 10^7 M^{-1} sec^{-1}. Taking a K_s value of (0.5–1) 10^{-4} for both substrates, k_{-1} = (3–10) $\times$ 10^3 sec^{-1}. Therefore, the observed k_2 values for these substrates are at the most comparable to the k_{-1} values.

Finally, an interesting problem arises from the experimental data. Using the value of k_2 at pH 8.0 as the k_2 (lim) value, one can calculate an apparent pK_a for the enzyme-substrate complex according to:

$$k_2 = k_2 \text{(lim)}/(1 + \text{[H]}/K_a). \qquad [7]$$

The estimated pK; values are 5.8, 5.6, 6.5, and 6.2 for AcTropONp, AcTrpSNp, AcTrpOEt, and AcTrpSEt, respectively. The value for the former two are abnormally low, even taking experimental error into account. Further study for this direction, including complete pH profiles of all four substrates, is now in progress, and details will be published elsewhere.

1. Bender, M. L. (1951) *J. Amer. Chem. Soc.* **73**, 1626–1629.
2. Inagami, T., York, S. S. & Patchornick, A. (1969) *J. Biochem. (Tokyo)* **65**, 809–819.
3. Caplow, M. (1969) *J. Amer. Chem. Soc.*, **91**, 3639–3645.
4. Lucas, E. C. & Caplow, M. (1972) *J. Amer. Chem. Soc.* **94**, 960–963.
5. Lucas, E. C., Caplow, M. & Bush, K. J. (1973) *J. Amer. Chem. Soc.* **95**, 2670–2673.
6. Fersht, A. R. & Requena, Y. (1971) *J. Amer. Chem. Soc.* **93**, 7079–7087.
7. Fersht, A. R. (1972) *J. Amer. Chem. Soc.* **94**, 293–295.
8. Fastrez, J. & Fersht, A. R. (1973) *Biochemistry* **12**, 1067–1074.
9. Philipp, M., Pollack, R. M. & Bender, M. L. (1973) *Proc. Nat. Acad Sci. USA* **70**, 517–520.

10. Frankfater, A. & Kézdy, F. J. (1971) *J. Amer. Chem. Soc.* **93**, 4039–4043.
11. Brandt, K. G., Himoe, A. & Hess, G. P. (1967) *J. Biol. Chem.* **242**, 3973–3982.
12. McConn, J., Ku, E., Himoe, A., Brandt, K. G. & Hess, G. P. (1971) *J. Biol. Chem.* **246**, 2918–2925.
13. Chase, T. & Shaw, E. (1967) *Biochem. Biophys. Res. Commun.* **29**, 508–513.
14. Zerner, B., Bond, R. P. M. & Bender, M. L. (1964) *J. Amer. Chem. Soc.*, **86**, 3674–3679.
15. Ingles, D. W. & Knowles, J. R. (1966) *Biochem. J.* **99**, 275–282.
16. Kézdy, F. J. & Bender, M. L. (1962) *Biochemistry* **1**, 1097–1106.
17. Eadie, G. S. (1942) *J. Biol. Chem.* **146**, 85–88.
18. Bernhard, S. A. & Gutfreund, H. (1965) *Proc. Nat. Acad. Sci USA* **53**, 1238–1243.
19. Barman, T. E. & Gutfreund, H. (1966) *Biochem. J.* **101**, 411–416.
20. Himoe, A., Brandt, K. G., DeSa, R. J. & Hess, G. P. (1969) *J. Biol. Chem.* **244**, 3483–3493.
21. Bender, M. L., Clement, G. E., Kézdy, F. J. & Heck, H. D'A. (1964) *J. Amer. Chem. Soc.* **86**, 3680–3690.
22. Bernhard, S. A., Lee, B. F. & Tashjian, Z. H. (1966) *J. Mol. Biol.* **18**, 405–420.
23. Marini, J. L. & Caplow, M. (1971) *J. Amer. Chem. Soc.* **93**, 5560–5566.
24. Fersht, A. R. & Requena, Y. (1971) *J. Mol. Biol.* **60**, 279–290.
25. Bender, M. L. & Kézdy, F. J. (1965) *Annu. Rev. Biochem.* **34**, 49–76.
26. Clement, G. E. & Bender, M. L. (1963) *Biochemistry* **4**, 836–843.
27. Connors, K. A. & Bender, M. L. (1961) *J. Org. Chem.* **26**, 2498–2504.
28. Martin, R. B. & Hedrick, R. I. (1962) *J. Amer. Chem. Soc.* **84**, 106–110.
29. Jencks, W. P. (1969) in *Catalysis in Chemistry and Enzymology* (McGraw-Hill Inc., New York), pp. 500–501.
30. Morse, B. K. & Tarbell, D. S. (1952) *J. Amer. Chem. Soc.* **74**, 416–419.
31. Polgar, L. (1972) *Acta Biochim. Biophys. Acad. Sci. Hung.* **2**, 319–334.
32. Zygmunt, R. J. & Barnett, R. E. (1972) *J. Amer. Chem. Soc.* **94**, 1996–2000.
33. Eigen, M. (1964) *Angew. Chem., Int. Ed. Engl.* **3**, 1–19.
34. Zerner, B. & Bender, M. L. (1964) *J. Amer. Chem. Soc.* **86**, 3669–3674.
35. Berezin, I. V., Kazanskaya, N. F. & Klyosov, A. A. (1971) *FEBS Lett.* **15**, 121–124.
36. Eigen, M. & Hammes, G. G. (1963) *Advan. Enzymol.* **25**, 1–38.
37. e.g., *Tables of Chemical Kinetics. Homogeneous Reactions. National Bureau of Standards Circular 510.*
38. Hess, G. P. (1971) in *The Enzymes*, ed. Boyer, P. D. (Academic Press, New York), 3rd ed., pp 213–248.
39. Havsteen, B. H. (1967) *J. Biol. Chem.*, **242**, 769–771.
40. Hague, D. N., Henshaw, J. S., John, V. A., Pooley, M. J. & Chock, P. B. *Nature New Biol.* **229**, 190–191.
41. Smallcombe, S. H., Ault, B. & Richards, J. H. (1972) *J. Amer. Chem. Soc.* **94**, 4585–4590.
42. Philipp, M. & Bender, M. L. (1973) *Nature New Biol.* **241**, 44.
43. Hershfield, R. & Shmir, G. L. (1972) *J. Amer. Chem. Soc.* **94**, 1263–1270.