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The Presteady-State Kinetics of the Papain-Catalyzed Hydrolysis of Isomeric Nitrophenyl Esters of Carbobenzoxyglycine*

Colin D. Hubbard and Jack F. Kirsch

ABSTRACT: The rate constants for the acylation of papain by the *o*-, *m*-, and *p*-nitrophenyl esters of carbobenzoxyglycine have been determined directly under conditions of [E] $\gg$ [S] by stopped-flow spectrophotometry and the results have been found to agree with the predictions derived from steady-state measurements at [S] $\gg$ [E].

The apparent second-order rate constants did not decrease with increasing enzyme concentrations, implying that the dissociation constant, K_s, is greater than 10^{-4} M for the reactions of the *p*-nitrophenyl ester. The addition of the nucleophile ethanol to the reaction mixture enhanced the over-all rate of reaction by increasing the rate of deacylation but had no effect on the velocity of acylation determined either directly or from the steady-state parameters.

Reactions of nitrophenyl esters with proteolytic enzymes have been particularly instrumental in helping to clarify their mechanism of action. Hartley and Kilby (1952, 1954) made the initial observation that the chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate proceeds in two steps, the first measured by an initial rapid release of *p*-nitrophenol in approximate stoichiometry with the enzyme followed by a slower reaction which is zero order in substrate concentration. The kinetics of both phases were carefully investigated in later work (Kézdy and Bender, 1962; Faller and Sturtevant, 1966, and references therein). Similar experiments have been carried out with trypsin (Stewart and Ouellet, 1959). These initial studies were later extended to steady-state measurements on *p*-nitrophenyl esters of so-called specific substrates, *i.e.*, of *N*-acylamino acids (Bender and Kézdy, 1965, and references therein). The experiments with these compounds, together with other kinds of evidence, have led to the now widely accepted kinetic scheme for the reaction of chymotrypsin and trypsin with esters (eq 1) (Gutfreund and Sturtevant, 1956a,b; Bender and Kézdy, 1965).

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES_1 \xrightarrow{k_2} \underset{+\ P_1}{ES_2} \xrightarrow{k_3} E + P_2 \qquad (1)$$

where ES_1 is the noncovalent enzyme–substrate complex, ES_2 an acyl-enzyme with the acyl moiety of the substrate covalently bound to the β-OH of the active site serine residue, P_1 the alcohol released from the substrate, and P_2 the carboxylic acid.

While fewer experiments have been done with the plant sulfhydryl proteases, papain, bromelin, and ficin, the picture that is emerging, at least for papain, the most studied of the three, is that the catalyzed acyl transfer reactions of esters proceed through a very similar pathway with the major chemical difference being that the nucleophilic acyl group acceptor on the enzyme is the β-SH of a cysteine residue (Sanner and Pihl, 1963; Lowe and Williams, 1965a; Brubacher and Bender, 1966). In addition some evidence has been advanced to show that in certain cases the rate of departure of P_1 from the enzyme surface may be of kinetic importance (Henry and Kirsch, 1967, and references therein). Although papain does not react well with *p*-nitrophenyl acetate,[1] it very efficiently catalyzes the hydrolysis of nitrophenyl esters of *N*-acylamino acids (Lowe and Williams, 1965b; Bender and Brubacher, 1966; Kirsch and Igelström, 1966).

The steady-state analysis of eq 1 leads to the following expressions for the Michaelis–Menten parameters, k_{cat} and K_m.

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \qquad (2)$$

* From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received March 18, 1968.* Supported by U. S. Public Health Service Grant GM 12278 and National Science Foundation Grant GB-4606.

[1] J. F. Kirsch, unpublished experiments.

$$k_{cat}/K_m = \frac{k_3 K_s}{k_2 + k_3} \quad (3)$$

Division leads to eq 4.

$$k_{cat}/K_m = k_2/K_s \quad (4)$$

The last equation indicates that the rate constant for the presteady-state acylation of the enzyme may be predicted by the ratio of k_{cat} to K_m, both of which are derived solely from steady-state measurements.

Except for the experiments with *p*-nitrophenyl acetate described above, only very limited attempts have been made to study the acylation reaction itself. The competitive inhibitor of chymotrypsin, proflavin, which exhibits a change in absorption spectrum when bound to the enzyme (Bernhard *et al.*, 1966), has been employed by Bernhard and Gutfreund (1965) to investigate the trypsin-catalyzed hydrolysis of *N*-α-benzoyl-L-arginine ethyl ester and the reaction of chymotrypsin with *N*-α-acetyl-L-tyrosine ethyl ester. Further studies on the presteady-state reactions of ethyl esters have been reported by Barman and Gutfreund (1966) and Brandt *et al.* (1967). The latter workers determined both the binding constant and the rate constant for acylation of the enzyme for *N*-acetyl-L-tryptophan ethyl ester using this technique. Himoe and Hess (1967) have further reported the results of an investigation of the presteady-state kinetics of the reaction of *N*-acetyl-L-tryptophan *p*-nitrophenyl ester, in which they were unable to observe saturation of chymotrypsin by substrate.

Since the presteady-state kinetics of papain-catalyzed reactions have not been previously investigated and steady-state values of k_{cat} and K_m for the *o*-, *m*-, and *p*-nitrophenyl esters of carbobenzoxyglycine are known (Kirsch and Igelström, 1966), this study was initiated in order to check directly the predictions of eq 4 for the three substrates whose reactivity as measured by k_{cat}/K_m differed by a factor of 20.

Experimental Section

Materials. The *o*-, *m*-, and *p*-nitrophenyl esters of carbobenzoxyglycine were available from a previous study (Kirsch and Igelström, 1966).

Two different preparations of papain were used in these experiments: (1) enzyme crystallized from dry papaya latex (Wallerstein Co.) essentially according to Masuda's (1959) modification of the method of Kimmel and Smith (1954) and (2) Worthington Biochemical Corp., lot no. 6HB, twice recrystallized. The activity of each enzyme preparation was determined by assaying with the *p*-nitrophenyl ester of carbobenzoxyglycine at pH 6.8 and 25° (Kirsch and Igelström, 1966). The enzyme batches had different activity, a fact which makes it difficult to give direct comparisons of kinetic parameters between experiments using different preparations. Concentrations of protein were estimated from the absorbance at 280 mμ (Henry and Kirsch, 1967). Enzyme batch 1 was used for all experiments except for those in which kinetic constants determined in acetonitrile were compared with those measured in ethanol. Acetonitrile was distilled, and water was glass distilled from dilute alkaline potassium permanganate. Rossville absolute ethanol and Nutritional Biochemical Corp. L-cysteine hydrochloride were used without further purification.

Kinetic Measurements. The steady-state parameters, k_{cat} and K_m, were determined at pH 6.8 and 25° for each of the nitrophenyl ester substrates at [S] ≫ [E] (Kirsch and Igelström, 1966). The wavelengths of 400, 415, and 330 mμ were used for spectrophotometrically following the liberation of *p*-, *o*-, and *m*-nitrophenol, respectively. Rates of reaction at [E] ≫ [S] were too rapid for conventional spectrophotometry, and these reactions were followed with a Durrum Instruments Corp. stopped-flow spectrophotometer fitted with a deuterium lamp source and ultraviolet optics. The same wavelengths used in determining the steady-state parameters were used in the acylation experiments. The general procedure for the stopped-flow experiments was to allow a papain incubation mixture containing 3.5×10^{-4} M cysteine, 1.0×10^{-3} M EDTA, and 0.042 M phosphate buffer (pH 6.8) to activate for 45 min at 25° where constant activity is attained (Kirsch and Igelström, 1966). The solution was then introduced into one of the drive syringes. The required substrate was dissolved in 12% (v/v) acetonitrile solution and introduced into the other drive syringe. Thus the mixed solution was 0.021 M in phosphate, 6% (v/v) in acetonitrile, and had a final pH of 6.9. In the experiments where 10% (v/v) ethanol was substituted for 6% (v/v) acetonitrile, the final concentration of ethanol was present in both reactant solutions.

The mixing of the aqueous solutions with those containing 12% acetonitrile resulted in an artifact appearing as an apparent loss of the oscilloscope trace for the early portion of the reaction. This artifact does not interfere with the analysis of zero- or first-order kinetics since these rate constants are independent of the zero time points. The instrument dead time was determined to be 3–5 msec from experiments on the rate of reduction of dichlorophenolindophenol by sodium ascorbate (Strittmatter, 1964).

Oscilloscope traces of the reactions were photographed and the millimeter coordinates were converted into absorbance units with the aid of a computer.[2]

Results

Presteady-State Reactions. The rate constant for acylation of the enzyme by the substrate where [E] ≫ [S] is given by eq 5. Kézdy and Bender (1962) have

$$k_{obsd}/[E] = \frac{k_2}{K_s + [E]} \quad (5)$$

[2] The program TRDATA written by Mr. James Allard converts ruler measurements of photograph coordinates into tables of time *vs.* absorbance and $A_\infty - A_t$. An instruction sheet and listing of the program are available from J. F. K.

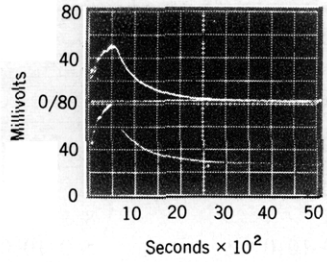

FIGURE 1: Oscilloscope traces of successive determinations of the papain-catalyzed release of *p*-nitrophenol from carbobenzoxyglycine *p*-nitrophenyl ester. Conditions in Table I, footnote *a*. [E] = 6.9×10^{-5} M, [S] = 3.5×10^{-6} M. Each trace represents a total increase in optical density of 0.03. The ordinate scale is proportional to per cent transmission.

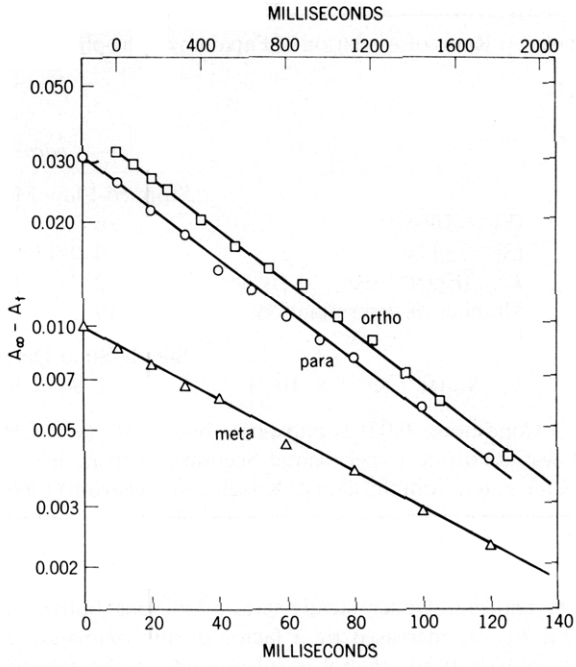

FIGURE 2: Typical first-order plots of the rate of acylation of papain by nitrophenyl esters of carbobenzoxyglycine obtained from stopped-flow measurements under conditions described in Table I, footnote *a*, with [E] ≫ [S]. (○) *p*-Nitrophenyl ester, lower time scale. Plot derived from upper trace in Figure 1; (△) *m*-nitrophenyl ester, lower time scale, [E] = 1.11×10^{-4} M, [S] = 1.0×10^{-5} M; (□) *o*-nitrophenyl ester, upper time scale, [E] = 8.1×10^{-5} M, [S] = 1.0×10^{-5} M.

pointed out the advantage of measuring rates of acylation under these conditions since the subsequent deacylation step measured by k_3 (eq 1) does not affect the kinetics. The conditions of [E] ≫ [S] make it necessary to employ enzyme concentrations far greater than those ordinarily employed in steady-state experiments. In order to ascertain whether the catalytic behavior of papain remained unchanged over such a large range of enzyme concentrations, the zero-order rate of reaction of 5×10^{-5} M ZG*p*NP[3] was measured with enzyme concentrations varying from 4×10^{-7} to 1.15×10^{-5} M employing conventional spectrophotometric methods for the slower reactions, and a stopped-flow apparatus for the faster ones. A linear relationship was obtained within the limits of experimental error (±10%) between the observed zero-order rate constants and [E], thus implying the absence of enzyme association or other concentration-dependent phenomena affecting enzyme activity. The limited solubility of ZG*p*NP precluded the extension of these experiments to the highest concentration of enzyme used in the stopped-flow studies.

Values of k_{obsd} were shown to be independent of cysteine concentration over the range 3.5×10^{-4}–3.5×10^{-3} M in a series of experiments employing [E] = 8.8×10^{-5} M and [ZG*p*NP] = 5×10^{-6} M. The lower concentration of cysteine was therefore sufficient for complete activation, and was used for all stopped-flow studies.

Under conditions of [E] ≫ [S] the rate of appearance of nitrophenol was first order for at least two half-times. Typical reaction traces are shown in Figure 1, and sample first-order plots for each of the three esters are given in Figure 2.

Equation 5 predicts Michaelis–Menten saturation with increasing concentration of enzyme. The observed rates of appearance of *p*-nitrophenol from the acylation of papain by ZG*p*NP were therefore studied as a function of papain concentration. Practical limitations due to the limited solubility of papain and the necessity of working at [E] ≫ [S] in order to maintain pseudo-first-order conditions restricted these experiments to only a twofold variation of enzyme concentration covering the range between 5.0 and 9.9×10^{-5} M. Values of $k_{obsd}/[E]$ were invariant over this span. This result is only consistent with a K_s value considerably greater than 10^{-4} M. Further consideration of K_s is given in the Discussion section.

Because of the uncertainties in K_s and the fact that the highest enzyme concentration used in these studies was about 10^{-4} M, no attempt was made to correct for saturation of the substrate by enzyme, and the figures presented in Table I are given as $k_{obsd}/[E]$ rather than $k_2/K_s + [E]$. Previously determined steady-state values of k_{cat}/K_m are included for comparison.

The Locus of Action of Added Nucleophiles. The addition of ethanol results in an increased rate of formation of *p*-nitrophenol in the papain-catalyzed reactions of *p*-nitrophenyl hippurate under conditions of [S] ≫ [E]. This increased rate is quantitatively accounted for by the appearance of the transesterified product, ethyl hippurate (Henry and Kirsch, 1967). These observations were interpreted in terms of the added alcohol, increasing the rate of the slow step of the reaction which is reflected primarily in the rate of deacylation. The added alcohol cannot participate as a nucleophile in the acylation of the enzyme and it is therefore predicted that replacement of the aprotic solvent, acetonitrile, by ethanol should affect only the rate of deacylation as measured by parallel increases in k_{cat} and K_m but that $k_{cat}/K_m = k_2/K_s$ should be insensitive to this substitution. The data in Table II show that these expectations

[3] ZG*p*NP, ZG*m*NP, ZG*o*NP are, respectively, the *p*-, *m*-, and *o*-nitrophenyl esters of carbobenzoxyglycine.

TABLE I: Rates of Acylation of Papain by Nitrophenyl Esters of Carbobenzoxyglycine.[a]

	Nitrophenyl Ester		
	para	*meta*	*ortho*
	Stopped-Flow Measurements at [E] ≫ [S]		
[E] × 10^5 M	6.9	11.1	8.1
[S] × 10^6 M	4.0–13.0	6.0–10.0	5.0–10.0
k_{obsd}/[E] (M^{-1} sec^{-1} × 10^{-5})[b]	2.75 ± 0.28	1.11 ± 0.11	0.144 ± 0.004
Number of determinations	19	15	18
	Steady-State Determinations at [S] ≫ [E]		
k_{cat}/K_m (M^{-1} sec^{-1} × 10^{-5})[c]	2.94 ± 0.25	1.15 ± 0.08	0.141 ± 0.012

[a] Conditions: 0.021 M phosphate buffer, 6% (v/v) CH_3CN, 3.5 × 10^{-4} M cysteine (pH 6.8), and 25°. Enzyme batch 1 was used (see Experimental Section). [b] Errors are standard deviations based on total number of determinations. [c] Calculated from the data of Kirsch and Igelström (1966).

are realized. Under steady-state conditions both k_{cat} and K_m are increased by a factor of approximately 2 when 10% (v/v) ethanol is substituted for the solvent routinely employed, 6% (v/v) acetonitrile. The ratio k_{cat}/K_m is uninfluenced by the solvent change. The directly measured rate constant for the acylation reaction, k_{obsd}/[E], is similarly unaffected.

Discussion

Relationship between the Steady-State and Acylation Kinetics. The observed rate constant for the acylation of papain by substrate under conditions of [E] ≫ [S] (eq 5) reduces to k_2/K_s when $K_s \gg [E_0]$ and under these circumstances should be equal to k_{cat}/K_m derived from the independently measured steady-state reaction (eq 4). Comparison of the steady-state and acylation parameters given in Table I shows that they are in good agreement for each of the three isomeric nitrophenyl esters as predicted. This agreement for esters of varying reactivity taken together with the observation that added ethanol affects only the deacylation rate as measured by parallel increases in k_{cat} and K_m (eq 2 and 3) and does not change the rate of the acylation reaction as reflected in k_{cat}/K_m or k_{obsd}/[E] (Table II) provides strong direct support for the sequence depicted by eq 1. There is no evidence that the rate of dissociation of the nitrophenol from the acyl-enzyme is of kinetic significance for the reactions of carbobenzoxyglycine, although this does seem to be important in reactions of *p*-nitrophenyl hippurate (Henry and Kirsch, 1967). The fact that no evidence for saturation of substrate by the enzyme was seen in this present study implies that K_s must be at least 10^{-4} M for ZG*p*NP and is possibly much higher.

Determinants of K_s. In our earlier work on the steady-state kinetics of the reactions of esters of carbobenzoxyglycine, it was assumed on chemical grounds that the variation in the contribution of the leaving group to K_s was small compared with that in the acylation rate constant, k_2 (Kirsch and Igelström, 1966). This assumption was in accord with the analysis of the pH *vs.* k_{cat} and K_m profiles of Whitaker and Bender (1965), which showed that the greater reactivity of *N*-α-benzoyl-L-arginine ethyl ester over the corresponding amide resided in k_2 rather than K_s. The subsequent pH variation studies of Bender and Brubacher (1966) which, assuming K_s and k_3 to be independent of pH above 5.5, gave K_s values of 10.7, 0.52, and ≥0.033 mM, and estimates of k_2 of 175, 830, and ≥860 sec^{-1} for the benzyl, methyl, and *p*-nitrophenyl esters, respectively, of carbobenzoxylysine demand the opposite conclusion; *i.e.*, that the reactivity of the ester as measured by its rate of hydrolysis is reflected primarily in the strength of binding

TABLE II: The Effect of Ethanol on the Acylation of Papain by Carbobenzoxyglycine *p*-Nitrophenyl Ester.[a]

Solvent	6% (v/v) Acetonitrile	10% (v/v) Ethanol
	[S] ≫ [E]	
[E] × 10^7 M	3.64	3.35
[S] × 10^5 M	0.8–10.0	1.0–10.0
k_{cat} (sec^{-1})	4.00 ± 0.08	8.00 ± 0.35
K_m (M × 10^5)	2.17 ± 0.13	4.49 ± 0.44
k_{cat}/K_m (M^{-1} sec^{-1} × 10^{-5})	1.84 ± 0.08	1.79 ± 0.11
Number of determinations	24	20
	[E] ≫ [S]	
[E] × 10^5 M	4.58–6.10	4.83–6.40
[S] × 10^6 M	5.0	5.0
k_{obsd}/[E] (M^{-1} sec^{-1} × 10^{-5})	3.00 ± 0.63	3.05 ± 0.51
Number of determinations	15	19

[a] Conditions as in Table I, footnote *a*, with 10% (v/v) ethanol substituted for 6% CH_3CN where indicated. Enzyme batch 2 was used.

to the enzyme rather than in the rate of nucleophilic attack by the enzyme on the substrate. Although it is possible that these last estimates, which are not in accord with the chemical expectations, might be reconciled with them by subdivision of K_s into a binding step followed by acylation and consequent reassignment of k_2 to a different rate process, it is unlikely that the concomitant complexity would be justified by the data presently available. An additional possible mode of resolution of this problem might be found if K_s can be shown to be sensitive to an ionization constant in the alkaline region, as has been demonstrated for reactions of chymotrypsin (Himoe and Hess, 1966; Bender *et al.*, 1966; Himoe *et al.*, 1967). There have as yet been no direct measurements of substrate binding to papain, although Sluyterman (1966) found that the substrate *N*-α-benzoyl-L-arginine ethyl ester decreased the rate of activation of papain by cysteine and determined that the binding constant of this substrate to the inactive enzyme was approximately equal to the independently determined value of K_m. A direct measurement of a dissociation constant for a nitrophenyl ester substrate to a proteolytic enzyme has yet to be made. All present evidence seems to indicate that the acylation reactions of these substrates are well described by simple second-order kinetics (Faller and Sturtevant, 1966; Himoe and Hess, 1967).

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