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A Comparison of Esterolytic Mechanisms of Several Sulfhydryl Proteases†

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A comparison was made of the esterolysis reactions catalyzed by chymopapain, papaya peptidases A and B, several asclepains of both the A and B families, ficin, and bromelain. Michaelis parameters for a series of aryl mesyl glycinates were measured and plotted versus the relevant Hammett σ values. All enzymes exhibited the same response to the substrates used.

INTRODUCTION

A number of sulfhydryl proteases, isolated from two sources, have lately been examined in this laboratory. From *Asclepias syriaca* L. we have separated (*1*) two families of such enzymes, asclepains A and B, which have been partly characterized (*2, 3*). Papaya latex has also yielded three cysteinyl proteolytic enzymes beside papain, namely, chymopapain ((*4*) and references therein) and papaya peptidases A and B (*5*). A familial relationship to papain, bromelain, and ficin was evident in the enzymes from both these sources, not only in the general modes of reaction (*2*), but also in the N-terminal homologies recently established (*3, 6*).

Qualitative investigations using oxidized insulin B chain as substrate have shown, however, that while all the sulfhydryl proteases of papaya latex produce similar maps of peptides (*5*) they exhibit different preferences toward some peptide bonds. Also, while the two families of asclepains behave in similar manners in the hydrolysis of insulin B chain (*2*), they yield two-dimensional peptide maps that are different from those observed with the papaya enzymes. Thus a more detailed comparison of the reactivities of the two groups of proteases discussion here, with those of papain, bromelain, and ficin was of interest.

The behavior of those latter three proteinases in esterolysis has been examined with a variety of substrates ((*7*) and references therein). Among these are a series of substituted aryl glycinates (I),

$$CH_3SO_2NH\ CH_2CO \cdot O \cdot C_6H_4X,$$

for which hydrolytic rates have been measured and related to the Hammet σ parameter (*8–10*). Such studies offer a direct way of comparing general mechanistic similarities (*9, 10*). We here report the results of measurements of Michaelis constants, and of pseudo-first-order rate constants obtained under subsaturating

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conditions, for a series of aryl mesyl glycinates hydrolyzed in the presence of chymopapain, papaya peptidases A and B, several isoenzymes of both asclepains, and samples of ficin and bromelain.

EXPERIMENTAL

Reagents. Unless otherwise noted, all reagents used were of analytical grade. The substrate phenyl mesyl glycinates employed were prepared by reaction of 0.01 mol of the appropriate substituted phenol (Aldrich Chemical Co.) in 30 ml of dichlormethane (Anachemia) containing an equimolar amount of pyridine (redistilled) with 0.01 mol of mesyl glycyl chloride. The solution was refluxed for 2 hr, extracted with 0.1 *M* HCl, then with 2% sodium bicarbonate and dried over sodium sulfate. Crystallization of the phenyl mesyl glycinates was in benzene.

Mesyl glycine was prepared by the method of Helferich and Grunert (*11*) and the acid chloride generated from this using phosphorus pentachloride.

The meta- and para-substituted aryl esters of mesyl glycine were prepared where X was $-CH_3$; $-Br$; $-Cl$; $-F$; $-NO_2$; and $-C(CH_3)_3$. The melting points and elemental analyses obtained (performed by Mr. H. Seguin of this division) were in agreement with those previously reported (*9*) when comparison could be made. Those data from the remaining compounds are collected in Table 1.

Enzyme isolation and purification. The chymopapain and the two papaya peptidases were purified to homogeneity as has been described (*4, 5*) as were the members of the two groups of asclepains used in this study (*1, 2*). Two isoenzymes of bromelain were obtained in the manner previously reported (*12*) and two isoenzymes of ficin were similarly separated from Mann Laboratories crude material. The homogeneities of each of the enzymes employed were checked by disc gel electrophoresis as reported (*1, 2, 5*). The quantities of enzymes employed were then determined from protein concentration measurements.

Kinetics measurements. These were made using conventional spectroscopic procedures in the thermostated cell compartment (20°C) of a Beckman Model 26 instrument. Reaction ws followed in 0.1 *M* acetate buffer of pH 7.0 containing 10^{-4} *M* mercaptoethanol using concentrations of the enzymes which provided convenient rates of reaction. Michaelis–Menten parameters were obtained from double-

TABLE 1

MELTING POINTS AND ELEMENTAL ANALYSES OF ARYL MESYL GLYCINATES

Compound	Melting point (°C)	Theoretical			Experimental		
		C	H	N	C	H	N
m-Br	151	33.35	3.10	4.32	33.96	3.19	4.32
p-Br	159.5				34.30	3.30	4.46
m-Cl	127	38.67	3.58	5.01	38.86	3.63	4.96
p-tert.-Bu	111	51.82	6.31	4.64	51.79	6.38	4.68

reciprocal plots with substrate concentrations (seven were commonly used) ranging from 6×10^{-5} to 1×10^{-3} M. Pseudo-first-order kinetic constants k_ψ were measured at subsaturating concentrations of substrate which were about 10-fold lower than saturating conditions. It may readily be shown that, as determined, $k_\psi' = V_{\max}/K_m$, which provided a check on the Michaelis–Menten parameters measured.

RESULTS AND DISCUSSION

In Table 2 are shown a typical collection of Michaelis–Menten and pseudo-first-order rate constants for the 11 aryl mesyl glycinates employed in this work, reacting in the presence of the asclepain isoenzymes A_4 and B_1, bromelain fraction III, ficin fraction II, papaya peptidases A and B, and chymopapain. As expected, $V_{\max}/K_m$ approximated k_ψ for each substrate, and thus no evidence was found of inhibition during the course of these reactions. When the data for, e.g., asclepain A_4 was plotted conventionally versus the Hammet σ function (*13*), straight lines were obtained with slopes (ρ) of zero for the plot of the Michaelis constant and 0.91 and 1.0, respectively, for those of the $V_{\max}$ and k_ψ data. Comparable results were obtained for the data from papaya peptidase A and B, chymopapain, the two isoenzymes of ficin and of bromelain, three isoenzymes of asclepain A and two isoenzymes of asclepain B. All the data collected may be summarized as $\rho_{K_m} = 0$; $\rho V_{\max} = 1.0 \pm 0.04$; and $\rho k_\psi = 0.96 \pm 0.01$, where average deviations were calculated using results from the 12 different enzyme systems studies. These results are in accord with the relationship $\rho_{k_\psi} = \rho V_{\max} - \rho_{K_m}$, which may readily be derived from the definition of ρ (*13*).

The response to substituents on the aryl mesyl glycinates for the cysteinyl proteases examined in this work is not in accord with that previously found with papain (*8, 9*) and bromelain (*10*), possibly because of differing reaction conditions. While some divergences for relations of σ and the kinetics parameters were elsewhere reported (*9*) with the fluoro-substituted aryl mesyl glycinates, this was not observed in the work described here.

The precise details of the mechanism of papain catalysis are still being investigated. However, there is agreement that the esterolytic reaction route of that enzyme as well as of ficin and bromelain (*7*) is minimally described by Eq. [1], comprising enzyme(E)/substrate(S) binding followed by acylation (k_2) and deacylation (k_3) steps. P_1 is the alcohol, amine, or amino acid, and P_2 the carboxyllic acid product.

$$\mathrm{E} + \mathrm{S} \underset{k_1}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_2} \underset{\underset{\mathrm{P}_1}{+}}{\mathrm{ES}^1} \xrightarrow{k_3} \mathrm{E} + \mathrm{P}_2. \qquad [1]$$

Transient tetrahedral intermediates are probably involved in both acylation and deacylation, the formation of such intermediates being rate determining for esterolysis (*7*). The data reported here, where values of $V_{\max}$ are shown to be

TABLE 2

KINETIC VALUES (K_m ($\times 10^3$ M^{-1}), V_{max} ($\times 10^7$ M^{-1} sec), AND k_ψ ($\times 10^4$ sec), IN ORDER) OBTAINED ON HYDROLYSIS OF ARYL MESYL GLYCINATES

	Asclepains		Bromelain	Ficin			
Substrate	A_4	B_1	III	II	PPA	PPB	CP
m-CH_3	2.22, 2.63, 1.49	1.92, 2.08, 1.07	4.00, 4.35, 1.07	1.70, 1.21, 0.49	2.22, 0.59, 0.50	0.54, 2.00, 3.10	1.18, 21.28, 13.8
p-CH_3	2.08, 2.17, 1.57	1.82, 2.07, 1.00	1.82, 1.43, 0.65	1.92, 1.00, 0.53	— — —	2.50, 0.67, 0.35	2.50, 9.09, 4.17
m-Br	2.50, 6.67, 2.48	5.56, 16.67, 3.23	— — 1.71	— — 0.76	0.71, 0.59, 1.03	1.25, 1.00, 0.80	0.34, 11.49, 33.80
p-Br	2.50, 8.33, 2.67	2.00, 5.00, 2.72	— — 1.32	— — 1.03	1.05, 1.00, 0.84	3.34, 1.67, 0.50	— — —
m-Cl	1.11, 4.00, 3.69	2.50, 6.67, 3.39	1.11, 2.33, 1.67	2.33, 3.70, 1.48	1.11, 0.71, 0.60	1.75, 1.43, 0.81	1.54, 33.3 , 27.9
p-Cl	2.94, 7.69, 3.30	1.33, 3.57, 2.88	2.50, 3.57, 1.66	0.98, 1.37, 1.25	1.67, 0.71, 0.34	1.11, 0.48, 0.41	— — —
m-F	3.33, 5.88, 1.79	3.33, 8.33, 2.80	1.39, 2.50, 1.24	2.86, 4.35, 0.76	1.25, 0.63, 0.81	1.29, 0.74, 0.80	0.83, 3.33, 3.48
p-F	4.00, 6.67, 1.58	2.00, 3.70, 1.93	3.23, 2.86, 0.98	2.04, 1.92, 0.72	2.28, 0.50, 0.26	2.50, 0.59, 0.43	2.00, 3.23, 1.27
m-NO_2	0.53, 5.00, 10.26	0.74, 5.56, 6.84	1.70, 8.01, 4.31	1.68, 6.32, 3.31	1.43, 1.00, 0.81	1.59, 3.20, 1.84	0.99, 32.50, 22.8
p-NO_2	1.75, 12.50, 8.44	1.72, 11.11, 7.45	0.71, 4.76, 5.97	1.75, 8.88, 5.44	1.25, 1.94, 2.25	1.61, 5.11, 3.46	0.98, 37.0 , 29.4
p-*tert*.-Bu	1.33, 1.25, 0.89	2.33, 2.63, 1.42	— — 0.56	— — 0.58	0.78, 0.67, 0.97	0.91, 0.80, 1.07	0.16, 6.06, 31.3

somewhat responsive to substituent effects, are in accord with that analysis of the reaction.

REFERENCES

1. W. J. Brockbank and K. R. Lynn, *Biochem. Biophys. Acta* **578,** 13 (1979).
2. K. R. Lynn, W. J. Brockbank, and N. A. Clevette, *Biochim. Biophys. Acta* **612,** 119 (1980).
3. K. R. Lynn, M. Yaguchi, and C. Roy, *Biochim. Biophys. Acta* **624,** 579 (1980).
4. K. R. Lynn, *J. Chromatogr.* **84,** 423 (1973).
5. K. R. Lynn, *Biochim. Biophys. Acta* **569,** 193 (1979).
6. K. R. Lynn and M. Yaguchi, *Biochim. Biophys. Acta* **581,** 362 (1979).
7. G. Lowe, *Tetrahedron* **32,** 291 (1976).
8. G. Lowe and A. Williams, *Biochim. J.* **96,** 199 (1965).
9. A. Williams, E. C. Lucas, and A. R. Rimmer, *J. Chem. Soc. Perkins Trans. 2,* 621 (1972).
10. H. C. Hawkins and A. Williams, *J. Chem. Soc. Perkins Trans. 2,* 723 (1976).
11. B. Helferich and H. Grünert, *Annalen* **545,** 178 (1940).
12. K. R. Lynn, *Anal. Biochem.* **77,** 33 (1977).
13. R. W. Alder, R. Baker, and J. M. Brown, "Mechanisms in Organic Chemistry." Wiley-Interscience, London, 1971.

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