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D. R. Coulson
Contribution No. 1508, Central Research Department
E. I. du Pont de Nemours and Company
Experimental Station, Wilmington, Delaware 19898
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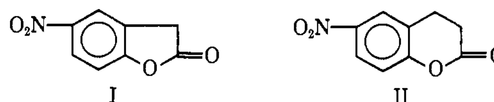
The α-Chymotrypsin-Catalyzed Hydrolysis of Lactones[1]

Sir:

α-Chymotrypsin, a pancreatic protease and esterase, was recently shown to react rapidly and stoichiometrically with aromatic five-membered sultones.[2] The sultones contain a *cis* ester function. Since aliphatic *cis* esters are much more reactive toward hydroxide ion than their *trans* ester analogs,[3] while the predominant configuration of chymotrypsin substrates is the planar *trans* form, it was of interest to determine whether the active site of chymotrypsin could react also with the *cis* carboxylic ester analogs of the sultones. The comparison of the reactivity of chymotrypsin toward equivalent *cis* and *trans* esters could then yield valuable information concerning the mechanism and the specificity of the enzyme-catalyzed reaction.

By analogy with the sultones and because of the favorable spectral properties of the reaction products, lactones I and II were chosen as the substrates.[4] We found that I and II react readily with α-chymotrypsin, producing relatively stable intermediates with concomitant formation of nitrophenol chromophores. Gel filtration on Sephadex G-25 at pH 5 showed that the intermediates had lost their catalytic activity toward N-acetyl-L-tryptophan methyl ester and that the chromophores are covalently attached to the enzyme. These facts indicate that the intermediates are acyl enzymes.

The kinetics of acylation were measured on a Durrum-Gibson stopped-flow spectrophotometer at 390 mμ for I and 332.5 mμ for II (the apparent isosbestic point of the acyl enzyme and the product acid) in the neutral pH range in the presence of excess enzyme. From the data good pseudo-first order rate constants can be calculated (k_{exptl}) which obey the equation $k_{exptl} = k_2E_0/(E_0 + K_s)$.



Thus by plotting $1/k_{exptl}$ *vs.* $1/E_0$ we determine k_2 and K_s (Table I). The chromophore of the acyl enzyme derived from lactone II showed a single ionization constant, pK = 7.9; λ_{max} 410 mμ (ϵ_{max} 14,200). In contrast, the ionization behavior of the phenolic group in the acyl enzyme derived from I is identical with that seen previously for the sulfonyl enzyme formed from the analogous sultone.[2] The pH dependency of the spectrum yields (numbered according to Scheme II of ref 2) pK_1 = 7.31; pK_2 = 7.30; pK_3 = 8.14; pK_4 = 8.15; λ_{max} 390 mμ (ϵ_{max} 13,700).

A qualitative difference in behavior is also observed in the deacylation of the two acyl enzymes. The acyl enzyme derived from II deacylates in a first-order reaction, yielding the product acid. The reaction rate was measured at 415 mμ and the first-order rate constant (k_3) is reported in Table I. Since k_3 is smaller than k_2, deacylation must be the rate controlling step in the overall enzymatic hydrolysis of II. This has been confirmed by measuring the kinetics under turnover conditions ($S_0 > E_0$). We observed Michaelis–Menten-type kinetics, *i.e.*, $dP/dt = k_{cat}E_0S/(S + K_m)$, and the k_{cat} determined by the use of Lineweaver–Burk plots was found to be identical within experimental error with k_3 (Table I).

The deacylation of the acyl enzyme obtained from I is quite different from that derived from II. When deacylation occurs in the presence of a great excess of methyl N-acetyl-L-tryptophanate or N-*trans*-cinnamoylimidazole which rapidly inhibit the enzyme, the sole reaction product is the original lactone in greater than 90% yield as observed at 400 mμ. The first-order rate constant of this reaction (k_4) is reported in Table I. When deacylation occurs without inhibition of the enzyme at low acyl enzyme concentration (<10^{-6} M) a rapid equilibrium is established between the acyl enzyme, the free

Table I

Substrate[a]	$k_2/K_s \times 10^{-3}$ M^{-1} sec^{-1}	$K_m \times 10^7$ M^{-1}	$k_3 \times 10^4$ sec^{-1}	$k_4 \times 10^8$ sec^{-1}	k_2 sec^{-1}	$K_s \times 10^3$ M^{-1}	$k_{sp} \times 10^5$ sec^{-1}
I	9.0		<4	4.7	15.4[b]	1.2[b]	43.2
II	7.8	230	2100				43.5
p-Nitrophenyl phenylacetate (III)	140	0.57[f]	80[c]				8.41
p-Nitrophenyl *m*-nitrophenylacetate (IV)	415	0.013[f]	5.6				11.2
p-Nitrophenyl β-phenylpropionate[d] (V)	560[d]		1100				2
p-Nitrophenyl acetate[e] (VI)	2.8	11	31		3	1.2	1.2

[a] Rate data were determined at pH 7.2 and 25° in 0.05 M phosphate buffer containing 0.2 M KCl except where noted otherwise. [b] These data were obtained from runs done at pH 8.06 and 25° in 0.025 M Ammediol buffer. [c] A plot of the rate constants for the deacylation of phenylacetylchymotrypsin *vs.* pH fits a sigmoid curve with a pK of 7.25. [d] Calculated from F. J. Kézdy, J. Feder, and M. L. Bender, *J. Am. Chem. Soc.*, **89**, 1009 (1967). [e] F. J. Kézdy and M. L. Bender, *Biochemistry*, **1**, 1097 (1962). [f] Calculated from k_3 and k_2/K_s of Table I using the relation $K_m = K_sk_3/k_2$.

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(4) These compounds were prepared by nitration in concentrated sulfuric acid at −15° from the corresponding unsubstituted lactones, which were purchased from Aldrich Chemical Co. Compound III of Table I was prepared from *p*-nitrophenol and the corresponding acid by the methods of Smiles: B. T. Tozer and S. Smiles, *J. Chem. Soc.*, 1897 (1938). Compound IV was prepared from sodium *p*-nitrophenolate and the corresponding acid chloride in dioxane. I had mp 189–189.5°; II had mp 130–130.7°; III had mp 62–63°; IV had mp 112°. The elemental analyses, ir, uv, and nmr spectra, and analyses of the hydrolysis products were in good agreement with the structures indicated.

enzyme, and the lactone. This equilibrium mixture then slowly yields the final acid product by a nonenzymatic hydrolysis of the lactone. At high enzyme concentrations ($>10^{-5}$ *M*) the reaction is further complicated by the acceleration of the spontaneous hydrolysis by the protein.

The rates of α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl phenylacetate (III) and *p*-nitrophenyl *m*-nitrophenyl acetate (IV) have been measured for comparison. These compounds also readily acylate the enzyme as measured by a burst of *p*-nitrophenol at 400 mμ. The kinetics of acylation have been followed on a stopped-flow instrument. The second-order acylation rate constants (k_2/K_s) have been determined. For compound III the rate constants of enzymatic hydrolysis have been calculated from turnover experiments ($S_0 > E_0$). For compound IV the acyl enzyme has been generated *in situ* by mixing stoichiometric amounts of enzyme and substrate and the deacylation rate constant (k_3) determined by measuring the time dependence of the recovery of the enzyme activity (Table I).

Finally, the rate constants for the uncatalyzed hydrolysis (k_{sp}) of all four compounds have been measured at pH 7.2 (Table I).

Our results suggest the following conclusions: (1) α-chymotrypsin is able to react with esters of carboxylic acids which are constrained in the *cis* configuration. Comparison of the acylation rate constants of *cis* and *trans* esters show, however, that the enzyme is unable to display its full specificity for the acyl group in the *cis* compound; *e.g.*, compound V acylates the enzyme some 60 times faster than its *cis* analog II, while in deacylation, when the acyl enzymes from both compounds can assume a *trans* configuration, the rate constants for the two systems are different by less than a factor of 2. Furthermore, the nonenzymatic hydrolysis of I and II is faster than that of their *trans* analogs III and V, although the difference is much less pronounced than in the case of the aliphatic lactones. This then seems to indicate that the enzyme reacts with its substrate whenever possible in the *trans* form[5] and that distortion of the substrate[6] is not a major factor in the acylation reaction of the enzyme. (2) The acyl enzymes derived from I and II are ideally suited for the role of "reporter molecules";[7] they are bound covalently but still reversibly to the very center of the active site. The spectral and kinetic behavior of the acyl enzymes indicate that in the acyl enzyme II the phenol group is located far away from the catalytic site, and from its p*K* we conclude that its environment does not differ much from the solvent. The behavior of the acyl enzyme derived from I shows that the phenol group must be constrained in the neighborhood of the catalytic site. The acid group perturbing the ionization of the phenol is then in all likelihood the histidine-57 of the active center. (3) The deacylation of the acyl enzyme from I indicates that the phenolic group remains in a sterically favorable position for attack of the carbonyl function. This is the first example of which we are aware where a reactive function introduced into an enzyme molecule has been demonstrated to act as an intramolecular nucleophile.

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(8) Predoctoral trainee of the National Institutes of Health.
(9) National Science Foundation Predoctoral Fellow.
(10) Fellow of the Alfred P. Sloan Foundation.

P. Tobias,[8] J. H. Heidema,[9] K. W. Lo, E. T. Kaiser,[10] F. J. Kézdy
Departments of Biochemistry and Chemistry
University of Chicago, Chicago, Illinois 60637
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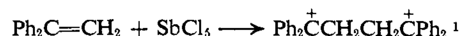
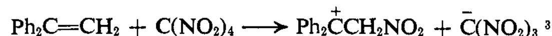
Formation of Carbonium Ions by Cl^+ Transfer from Antimony Pentachloride to Olefins

Sir:

Two distinct routes lead to the formation of carbonium ions from olefins. (1) Electron transfer from an olefin to a suitable acceptor yields radical cations which dimerize into dimeric dicarbonium ions, *e.g.*



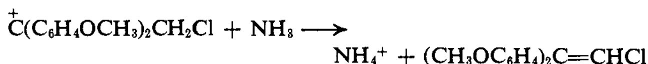
or $(p\text{-}Me_2NC_6H_4)_2C{=}CH_2$ + $(p\text{-}BrC_6H_4)_3N\cdot^+ClO_4^-$ gives the analogous dimethylamino derivative.[2] (2) Transfer of a positive moeity from a suitable donor to olefin converts the latter into carbonium ion, *e.g.*



It seems that $SbCl_5$ may act not only as an electron acceptor[1,4] but also as a Cl^+ donor. A colored species, λ_{max} 540 mμ, is formed when a solution of $(p\text{-}CH_3OC_6H_4)_2C{=}CH_2$ (I) is rapidly mixed with a large excess of $SbCl_5$. The spectrum reveals a shoulder at 500 mμ, and this absorption may appear as a peak if the mixing is poor or if the excess of $SbCl_5$ is small. In fact, only a peak at λ_{max} 500 mμ appears if the olefin is in large excess.

The peak at λ_{max} 540 mμ is *not* due to $[^+C(C_6H_4OCH_3)_2CH_2-]_2$ or $C^+(C_6H_4OCH_3)_2CH_3$. The former dicarbonium ion was prepared by allowing I to react with $(BrC_6H_4)_3N\cdot^+,ClO_4^-$, and the latter carbonium ion was formed by allowing $HOC(C_6H_4OCH_3)_2CH_3$ to react with $SbCl_5$ or by adding trifluoroacetic acid to $(CH_3OC_6H_4)_2C{=}CH_2$. In these preparations the resulting carbonium ions absorb at λ_{max} 500–505 mμ. Moreover, the nmr spectrum of the species absorbing at λ_{max} 540 mμ (shown in Figure 1) reveals a signal due to $>^+C-CH_2-$ protons at δ 5.4 ppm, while the CH_2 or CH_3 protons of the carbonium ions mentioned above absorb at δ 3.4 ppm.

We proved that the species absorbing at λ_{max} 540 mμ is $^+C(C_6H_4OCH_3)_2CH_2Cl$ (II). (1) $(CH_3OC_6H_4)_2C{=}CHCl$ was isolated from the solution absorbing at 540 mμ after quenching with ammonia. Apparently



Its identity with synthetic sample[5] was confirmed by ir

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