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Cycloamylose-Accelerated Cleavage of Acylimidazoles[1]

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Hydrolyses of *N-trans*-cinnamoylimidazole (**1**) and *N*-acetylimidazole (**2**) were accelerated by cyclohexaamylose (α-CA) and cycloheptaamylose (β-CA) at 25°C. The cleavage of the amide bond in **1** at pH 9.0 was accelerated by α-CA and β-CA by 28- and 38-fold, respectively, whereas the cleavage of the amide bond in **2** at pH 7.0 was accelerated by α-CA and β-CA by 50- and 28-fold, respectively. The β-CA-accelerated hydrolysis of **1** proceeded via binding, acylation of β-CA, and deacylation of β-CA *trans*-cinnamate, which is consistent with the pathway used by serine proteases. The deuterium oxide solvent isotope effects for acylation and deacylation steps indicate nucleophilic attack in acylation and general basic attack in deacylation. The present finding of the acceleration by cycloamyloses in the cleavages of amide bonds in **1** and **2** indicates that cycloamyloses are an excellent model for hydrolytic enzymes.

INTRODUCTION

Cycloamyloses have served as models of hydrolytic enzymes (*1*, *2*). There have been many studies of cycloamylose accelerations, e.g., accelerations of hydrolyses of ethyl esters of substituted mandelic acid (*3*), phenyl esters (*4*, *5*), organophosphorus derivatives (*6–9*), and aryl sulfates (*10*).

The hydrolyses of phenyl esters follow the pathway of binding, acylation of cycloamylose, and deacylation of the acyl-cycloamylose, which is consistent with enzymatic reactions (*4*, *5*). However, there is scanty information on cycloamylose-catalyzed cleavages of amides. The only known example of cleavage of an amide by a cycloamylose is the cycloheptaamylose-catalyzed cleavage of the strained β-lactam ring in penicillins (*11*).

Here we report the cyclohexaamylose (α-CA)- and cycloheptaamylose (β-CA)-catalyzed cleavage of amide bonds in *N-trans*-cinnamoylimidazole (**1**) and *N*-acetylimidazole (**2**). The acylimidazoles [formed as intermediates (*12*, *13*)] are hydrolyzed through acid-catalyzed, neutral, and base-catalyzed mechanisms (*14–16*).

EXPERIMENTAL

Materials. α-CA and β-CA were purified by recrystallization from water. Compounds **1** and **2** were recrystallized from cyclohexane and isopropenyl acetate,

[1] This contribution is dedicated to the memory of Dr. S. Morris Kupchan, who was a great scientist and a great friend. The friendship started when we (M. L. B.) roomed together as postdoctoral fellows at Harvard. It continued during his stay at Wisconsin, where he made a big impact on my research. At Virginia, his work was of the caliber that would have elected him to the National Academy of Sciences, except for his untimely death.

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respectively: **1**, mp 133–134°C (lit. *17*, mp 133–133.5°C): **2**, mp 102.5–103.5°C (lit. *18*, mp 104°C). Cycloheptaamylose *trans*-cinnamate (**3**), kindly furnished by Dr. Y. Kurono, was used after purification as described in a previous paper (*19*). 1,4-Diazabicyclo(2.2.2)octane (**4**) was purified by recrystallization from heptane and had a melting point of 158°C (lit. *20*, 159–160°C). All water used in the kinetic studies was doubly distilled.

Kinetics. Cleavages of **1** and **2** were followed at 335 and 245 nm on a Cary Model 14 PM spectrophotometer equipped with a thermostatted cell compartment. The reaction was initiated by the addition of 15 μl of stock solution of **1** or **2** in acetonitrile to 3 ml of thermoequilibrated buffer, followed by thorough mixing of the solution. The initial concentration of **1** or **2** was 10^{-4} *M*, and the concentration of α-CA or β-CA was maintained in excess of 50 times that of the substrate. The hydrolyses **1** and **2**, both in the presence and absence of α-CA and β-CA, followed first-order kinetics. Infinite absorbance values were obtained after at least ten half-lives. The reported rate constants are averages of two determinations which agreed within 3%. For the deuterium oxide experiments, pD was determined using the equation: pD = pH meter reading +0.4 (*21*).

RESULTS AND DISCUSSION

Both α-CA and β-CA accelerated the cleavage of **1** and **2**. The accelerations by cycloamyloses involve inclusion complex formation by **1** and **2** with cycloamyloses, since the observed first-order rate constant k_{obs} was not a linear function of the concentration of added (excess) cycloamylose, but rather it asymptotically approached a maximum value with increasing concentration of cycloamylose. Thus, the rate constant for the substrate complexed with cycloamylose, k_2, and the dissociation constant of the cycloamylose–substrate complex, K_d, were determined by plotting $1/(k_{obs}-k_{un})$ versus $1/[C]_0$, under the condition that $[C]_0 \gg [S]_0$ (*1*, *2*, *4*). Here $[C]_0$ and $[S]_0$ are the initial concentrations of the cycloamylose and the substrate, respectively, and k_{un} is the first-order rate constant in the absence of cycloamylose.

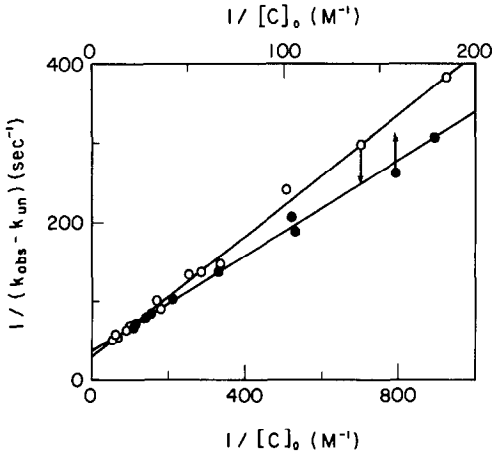

FIG. 1. Plots of $1/(k_{obs}-k_{un})$ versus $1/[C]_0$ for the cleavage of **1** accelerated by α-CA and β-CA; ●, α-CA;○, β-CA; 25°C, pH 9.0 (Borax buffer), $I = 0.2$ *M* (KCl).

Figure 1 depicts the plots of $1/(k_{obs}-k_{un})$ versus $1/[C]_0$ for the cleavage of **1** catalyzed by α-CA and β-CA. In these plots, the slope and the intercept, respectively, give the value of $K_d/(k_2-k_{un})$ and of $1/(k_2-k_{un})$, from which the value of k_2 and K_d were obtained. Table 1 lists the values of k_0, k_2, and K_d, as well as the ratio k_2/k_0. Here, k_0 is the rate constant extrapolated to zero buffer concentration in the absence of cycloamylose, since k_{un} for **1** and **2** depends on buffer concentration. On the other hand, the resulting values of k_2 and K_d were hardly affected by change of buffer concentration. Inclusion complex formation of **1** with α-CA and β-CA were found to accelerate fission of the amide bond by 28- and 38-fold, respectively. Cleavage of **2** was also accelerated by α-CA and β-CA by 50- and 28-fold, respectively.

The K_d values for **2** were much larger than those for **1**, because the acyl group of **2** is less bulky. The comparatively larger deviation in k_2 for the β-CA–**2** complex derives from the large value of K_d and the limited solubility of β-CA.

The reaction schemes for the cycloamylose-accelerated hydrolyses of acylimidazoles were examined for the β-CA–**1** system. A plot of the logarithm of k_{obs} versus pH for the

TABLE 1

VALUES OF k_0, k_2, AND K_d FOR CYCLOAMYLOSE-CATALYZED CLEAVAGE OF ACYLIMIDAZOLES[a]

Substrate	pH	Cycloamylose	$10^3 k_0$ (sec^{-1})	$10^2 k_2$ (sec^{-1})	k_2/k_0	$10^2 K_d$ (M)
1	9.0	α-CA	1.0 ± 0.02	2.8 ± 0.4	28 ± 5	4.3 ± 0.4
		β-CA	1.0 ± 0.02	3.8 ± 0.6	38 ± 6	1.4 ± 0.2
2	7.0	α-CA	0.13 ± 0.01	0.65 ± 0.05	50 ± 8	34 ± 8
		β-CA	0.13 ± 0.01	0.36 ± 0.10	28 ± 10	14 ± 4

[a] 25°C, $I = 0.2$ M (KCl), pH 9.0 (Borax buffer), pH 7.0 (phosphate buffer). All rate constants are values extrapolated to zero buffer concentration.

cleavage of **1** in the presence of 0.015 M β-CA at 25°C exhibited a straight line of slope 1.0 in the pH range investigated (pH 7.4–10.0). This result shows that the cleavage of the amide bond of **1** by β-CA proceeds through intracomplex nucleophilic attack by alkoxide ion of β-CA or through intermolecular nucleophilic attack by hydroxide anion. The pH–k_{obs} profile in the higher pH region could not be obtained because the hydrolysis of **1** in the presence of β-CA was too fast to be measured above pH 10.0.

However, the following experiment definitely shows that the acceleration by β-CA involves nucleophilic attack by the alkoxide ion of β-CA toward the carbonyl carbon atom of **1** included in the cavity of β-CA, resulting in formation of **3** as reaction intermediate. As shown in Fig. 2, absorbance at 335 nm rapidly decreased with time, when the concentration of β-CA was 0.015 M at pH 10.0. Decrease of absorbance at 335 nm corresponds to disappearance of **1**. After 1 min, the absorption spectrum of the solution was identical with that of an authentic sample of **3** (λ_{max} = 279 nm) (*22*) plus that of imidazole.

Thus, it was concluded that the first step of the β-CA-accelerated hydrolysis **1** is the fission of the amide bond by attack of the alkoxide ion of β-CA toward the carbonyl carbon atom of **1**.

After the first step was over, absorbance at 300 nm decreased much more slowly than the first step. This decrease at 300 nm is associated with the hydrolysis of **3** to

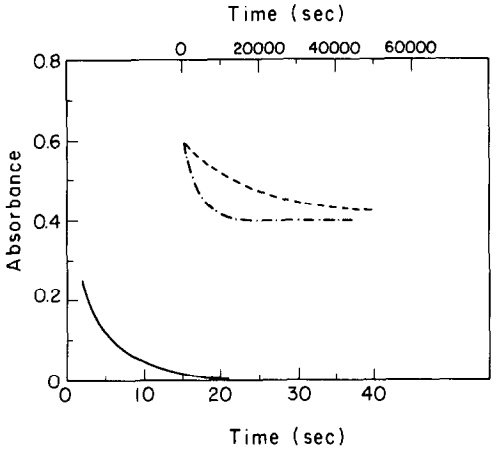

FIG. 2. Hydrolysis of **1** at 25°C, pH 10.0 (carbonate buffer) $I = 0.2\ M$ (KCl) in the presence of 0.015 M β-CA; [**1**] = $2 \times 10^{-5}\ M$; ——, change in absorbance at 335 nm due to disappearance of **1** (lower scale), – – – –, Change in absorbance at 300 nm due to hydrolysis of **3** to β-CA and *trans*-cinnamate anion (upper scale); – · – · –, change in absorbance at 300 nm in the presence of 0.5 M **4** (upper scale). **4** was added at the reaction time of 1 min when the first step of the β-CA-catalyzed hydrolysis of **1** was over.

trans-cinnamate anion and β-CA. The rate constant for this second step was 4.4×10^{-5} sec^{-1}, which is equal to 4.3×10^{-5} sec^{-1} for the hydrolysis of an authentic sample of **3** under the same conditions, within experimental error. It was experimentally confirmed that $5 \times 10^{-4}\ M$ imidazole barely affects the hydrolysis of **3**. After 2 days, during which the second step was completed, the spectrum of the solution was identical with that of *trans*-cinnamate anion ($\lambda_{max} = 270$ nm) plus that of imidazole.

In a previous paper (*19*), the hydrolysis of **3** was largely accelerated by certain amines such as **4**, *n*-butylamine, quinuclidine, piperidine, and triethylamine:, **4** was quite an effective catalyst. Thus, 0.5 M **4** was added to the reaction solution after 1 min to

TABLE 2
D_2O SOLVENT ISOTOPE EFFECTS FOR THE HYDROLYSIS OF CYCLOHEPTAAMYLOSE *trans*-CINNAMATE (**3**) AS A FUNCTION OF pH[a]

pH	$k_{obs}(H_2O)/k_{obs}(D_2O)$
10.0	5.2
10.5	5.2
11.2	5.2
12.2	4.8
12.4	3.8
12.5	2.9
12.7	2.5
12.9	2.3
13.2	2.1
13.4	2.1

[a] 25°C, $I = 0.2\ M$ (KCl).

accelerate the second step of the β-CA-catalyzed hydrolysis of **1**, and the pH of the solution was adjusted to pH 10.0. As shown in Fig. 2, the second step, the hydrolysis of **3**, became much faster in the presence of **4**. The rate constant in the presence of **4** was 2.5×10^{-4} sec^{-1}, which was about 5.7 times that in the absence of **4**. This magnitude of acceleration by **4** is consistent with the result in the previous paper that **3** complexed by **4** hydrolyzed 5.0 times faster than **3** itself at 20°C, $I = 1.0$ M (*19*).

The scheme of binding, formation of acyl-cycloamylose, and deacylation of acyl-cycloamylose, found for the β-CA-accelerated hydrolysis of **1** in the present study, is consistent with the schemes for serine protease-catalyzed hydrolyses of amide compounds as well as with those for cycloamylose-accelerated hydrolyses of esters (*1*, *2*, *4*, *5*).

The D_2O solvent isotope effects for the first step of the β-CA-accelerated hydrolysis of **1** were examined at 25°C, pH (pD) 8.0, 9.0, and 9.5. The rate constants k_{obs} in H_2O were 3.6- and 3.4-fold larger than those in D_2O at the concentrations of β-CA of 0.015 and 0.022 M, respectively. These ratios were independent of pH (pD). These values are almost equal to that (3.2) for the α-CA-accelerated cleavage of *m-t*-butylphenyl acetate, and are attributable to the difference between the pK_a of the hydroxyl group of β-CA in H_2O and that in D_2O (*23*). Thus, there is no kinetically important D_2O effect for the β-CA-accelerated cleavage of the amide bond of **1**. This result is in accord with nucleophilic attack by alkoxide ion of β-CA towards **1**. Without significant D_2O effect, general acid catalysis by the un-ionized hydroxyl group(s) of the host β-CA cannot be important in the cleavage of **1** (*24*).

The D_2O solvent isotope effect for the second step of the β-CA-accelerated hydrolysis of **1**, the hydrolysis of **3**, was examined at 25°C, using an authentic sample of **3**. The ratio of k_{obs} in H_2O to k_{obs} in D_2O at the same pH(pD) depends on pH(pD), as shown in Table 2. Below pH(pD) 11.2, $k_{obs}(H_2O)/k_{obs}(D_2O)$ remains constant at 5.2. Above pH(pD) 11.2 this ratio gradually decreases with pH(pD) up to the limiting value of 2.1.

The limiting value of $k_{obs}(H_2O)/k_{obs}(D_2O)$, 2.1, indicates that the hydrolysis of **3** involves participation of an alkoxide ion of **3** as general base catalyst (*25*), since most of the secondary hydroxyl groups of all **3** molecules are ionizing in this pH(pD) region (*26*). A larger value (5.2) of $k_{obs}(H_2O)/k_{obs}(D_2O)$ below pH(pD) 11.2 is attributable to the difference between pK_a of **3** in H_2O and in D_2O, in addition to the net D_2O solvent isotope effect. It should be noted, however, that the D_2O solvent isotope effect does not rule out the possibility of an overall simultaneous occurrence of alkaline hydrolysis of **3**.

It is probable that both general base catalysis by alkoxide ion and alkaline hydrolysis are taking place in the hydrolysis of **3**, though the relative contributions of these two types of catalyses cannot be determined at present.

In summary, it was found that α-CA and β-CA accelerate the cleavage of amide compounds such as **1** and **2**. The reaction proceeds through binding, acylation, and deacylation, which is consistent with the pathway used by hydrolytic enzymes. Thus, cycloamylose can be an excellent model for hydrolytic enzymes.

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25. Note that nucleophilic attack by alkoxide ion towards the *trans*-cinnamoyl MLB group of **3** gives **3** again, resulting in no net hydrolysis.
26. The hydroxyl groups of cyclohexaamylose benzoate have a pK_a of 12.1 (ref. *5*).