THE EFFECT OF ENZYME ACETYLATION ON THE KINETICS OF THE CARBOXYPEPTIDASE-A-CATALYZED HYDROLYSIS OF HIPPURYL-L-β-PHENYLLACTIC ACID*

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One approach to the elucidation of the active site of an enzyme is to modify the site chemically, observe the effect of the chemical modification on the kinetics of the enzymatic process, and derive therefrom some conclusion about the binding and/or chemical reactions of the site. Many chemical modifications of enzymes have been effected, some of them quite specific and intimately connected with the active site. The most striking of these include chemical modifications which inhibit enzymatic activity completely, such as the classical inactivation of α-chymotrypsin and related enzymes by diisopropylphosphorofluoridate[1] and the inactivation of α-chymotrypsin by L-1-tosylamino-2-phenylethyl chloromethyl ketone.[2] These modifications are usually interpreted in terms of the blocking of a specific chemical functionality of the active site.

In addition to modifications resulting in complete inhibition, there exist a number of chemical modifications which result only in a partial change of enzymatic activity. These effects are more subtle, and their interpretation is not always obvious without detailed investigation. Of many chemical modifications of chymotrypsin resulting in modified but finite reactivity, the following examples illustrate some of the complex behavioral patterns. The methionine residue, three amino acids from the reactive serine of the active site of chymotrypsin, on treatment with hydrogen peroxide was converted to the corresponding sulfoxide. This chemical treatment resulted in a decrease of 10 per cent in V_{max} and an increase of 350 per cent in K_m(app).[3] Iodination of chymotrypsin (actually the above sulfoxide derivative) to the extent of 6.3 iodine atoms per molecule of chymotrypsin resulted in an essentially unchanged V_{max} and a 6-fold increased K_m (app).[4] The methionine residue, three amino acids from the reactive serine of the active site of chymotrypsin, has also been alkylated by *p*-nitrophenyl bromoacetyl-α-aminoisobutyrate (presumably through the intervention of the corresponding acyl enzyme).[5] This specifically derivatized enzyme shows a 10-fold increase in K_m (app) and a 4-fold increase in V_{max} toward a number of specific substrates of α-chymotrypsin.[5, 6] One tentative conclusion from these data is that K_m (app), which is related to binding, appears invariably to be adversely affected by modifications which do not entirely abolish activity. Another tentative conclusion from these data is that V_{max}, which must reflect the catalytic process *per se*, may not be affected by such modifications, or may even increase!

Vallee and co-workers have found that various treatments of carboxypeptidase A such as acylation with carboxylic acid derivatives, photooxidation, iodination, or replacement of the zinc ion of the enzyme by either cadmium or mercury ions *increase the esterase activity* of the enzyme, as measured by a standard assay using hippuryl-DL-β-phenyllactic acid or hippuryl-DL-β-indolyllactic acid, but *decrease the peptidase activity*, as measured by a standard assay using benzyloxycarbonyl-

glycyl-L-phenylalanine or several other peptide substrates.[7-13] Thus, modification of carboxypeptidase A presents some particularly intriguing results. In the light of the work reported on chymotrypsin, it is of interest to examine the modifications of carboxypeptidase A, with a view to finding out whether such modifications exert changes in binding (K_m(app)) or in the catalytic process (V_{max}) or in both these processes.

The particular system reported here is the effect of acetylation of carboxypeptidase A by N-acetylimidazole on the kinetics of the enzymatic hydrolysis of hippuryl-L-β-phenyllactic acid.

Experimental.—Five times crystallized carboxypeptidase A (Mann Research Laboratories, lot no. C2560, water suspension) was solubilized by dialysis in a cold room against 0.05 *M* pH 7.50 Tris-HCl buffer containing 2 *M* sodium chloride. The solution was centrifuged and stored in a refrigerator. Stock solutions (1×10^{-4} *M*) were found to be stable for at least 3 months under these conditions. Acetylation of carboxypeptidase A with N-acetylimidazole followed the procedure of Simpson, Riordan, and Vallee.[10] Two such acetylcarboxypeptidase A preparations are compared with the native enzyme, using a standard assay procedure employing benzyloxycarbonylglycyl-L-phenylalanine as substrate (Table 1). The different activities of the two acetylated enzymes are a function of the extent of acetylation of the enzyme. Enzyme 3 which has 5.3 acetyl groups/mole has an activity on the standard assay which is 8 per cent of that of the native enzyme, in agreement with that reported by Vallee.[10] The kinetics using the acetylated enzymes were carried out within a week of their preparation. Sodium hippuryl-DL-β-phenyllactate (Cyclo Chemical Corp., lot no. M1070, reported to be 99.44% pure racemate) was used without further purification, mp 123–125°. The kinetics of hydrolysis of benzyloxycarbonylglycyl-L-phenylalanine and hippuryl-L-β-phenyllactic acid were determined using a Cary 14 PM recording spectrophotometer equipped with a thermostatted cell compartment. The former reaction was followed in the range of 236–220 mμ, while the latter was followed in the range of 250–270 mμ, with some high concentration runs being followed at 280–285 mμ. For the hydrolysis of hippuryl-L-β-phenyllactic acid, both blank and reaction cuvettes contained 3 ml of a freshly prepared solution of substrate in buffer. After equilibration at 25.0° for 25 min and adjustment of the base line absorbance to zero, 50 μl of buffer was added

TABLE 1

ACTIVITY OF NATIVE AND ACETYLCARBOXYPEPTIDASE A TOWARD BENZYLOXYCARBONYLGLYCYL-L-PHENYLALANINE*

Enzyme	V_0/E_0 (sec^{-1})	Acetyl groups/mole†
1. Carboxypeptidase A	112	0
2. Acetylcarboxypeptidase A‡	36	2.0
3. Acetylcarboxypeptidase A§	9	5.3

* In aqueous solution at 25.0° and pH 7.50. Substrate concentration = 2×10^{-2} *M*.

† The number of acetyl groups/mole for enzyme 2 was determined spectrophotometrically by comparing absorptions at 222.5 and 278 mμ. The number of acetyl groups/mole for enzyme 3 was determined spectrophotometrically by determining the absorption at 278 mμ before and after deacetylation by 0.01 *M* hydroxylamine.[10]

‡ Acetylation with a 120-fold molar excess of N-acetylimidazole for 30 min at 23° in Tris-HCl buffer at pH 7.50. The enzyme was then dialyzed against the same buffer, centrifuged at 10,000 rpm, and stored in the refrigerator.

§ Acetylation with two successive 60-fold molar excesses of N-acetylimidazole (recrystallized from isopropenyl acetate, mp 102–103.5°) over a period of 2 hr at room temperature in 0.02 *M* sodium veronal buffer, μ = 1.0, pH 7.50. The enzyme was then dialyzed against the same buffer, centrifuged at 10,000 rpm, and stored in the refrigerator. The difference in the degree of acetylation appears to be buffer-dependent.

to the blank cuvette and 50 μl of enzyme solution, in buffer, to the reaction cuvette. After mixing, recording was started within 5–10 sec. The reaction was followed to completion and the absorbance data were converted to rate data by use of the molar absorption coefficient determined on the reaction solution. Initial rates were used in the Lineweaver-Burk plots. All kinetic data were treated by the method of least squares.

Results and Discussion.—The rate of hydrolysis of hippuryl-L-β-phenyllactic acid ($0.25–1.0 \times 10^{-2}$ M, racemate used) by carboxypeptidase A was previously found to be zero-order up to approximately 80 per cent of completion, and the rate was found to decrease as the initial substrate concentration increased.[14, 15] The latter finding was confirmed[11] and has recently been extensively investigated over a range of substrate concentration of 2860-fold.[16] From the latter investigation, the system was concluded to involve a complex inhibition by excess substrate, suggesting either that the enzyme binds substrate to form several inactive complexes or that the enzyme exists as several differentially susceptible species. Our results using native carboxypeptidase A further emphasize the sizeable substrate inhibition in the hydrolysis of hippuryl-L-β-phenyllactic acid (Table 2 and Fig. 1). Our data, however, encompass only a substrate range of 250-fold and exhibit only normal substrate inhibition, in contrast to the complex substrate inhibition found in the previous investigation over a wider substrate concentration range.[16] As seen in Table 3, the kinetic constants for the native enzyme determined in this investigation agree reasonably well with those reported previously.[16]

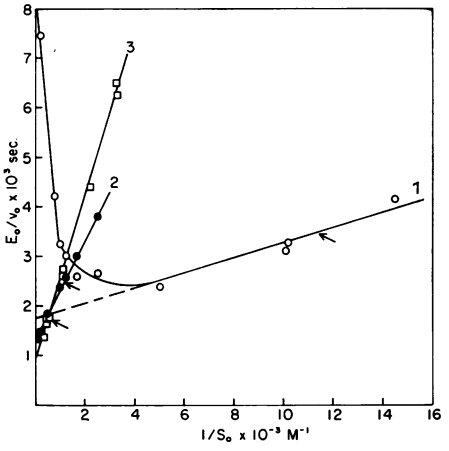

FIG. 1.—The hydrolysis of hippuryl-DL-β-phenyllactic acid by carboxypeptidase A (*1*) and two acetylated carboxypeptidases A (*2* and *3*, see Table 1 for identification) at 25.0°, pH 7.50, Tris-HCl buffer, $I = 0.5$, ← denotes K_m (app) of each reaction. Two low substrate points for the native enzyme fall on the linear portion of the plot but fall outside the scale of the graph.

A striking change occurs in the kinetics of the hydrolysis of hippuryl-L-β-phenyllactic acid when the enzyme is acetylated: the substrate inhibition disappears (Table 2 and Fig. 1). Of course, it cannot be categorically stated that the substrate inhibition is lost entirely. But certainly it is depressed to the extent that it is not seen up to a substrate concentration of $3–6 \times 10^{-3}$ M which is 10-fold higher concentration than the first appearance of substrate inhibition with the native enzyme. A summary of the kinetic constants of the reactions catalyzed by the native and two acetylated enzymes is given in Table 3. Differences in both k_{cat} (V_{max}) and in K_m(app) are seen for the three enzymes. The most extensively acetylated enzyme (enzyme 3) differs from the native enzyme by having a K_m(app) approximately 20-fold higher than that of the native enzyme and a k_{cat} which is approximately 2-fold higher than that of the native enzyme. Thus, the most extensive effect of acetylation is seen in the K_m(app), presumably related to binding, rather than in the k_{cat}, presumably related to the catalytic process. Of course,

TABLE 2

THE KINETICS OF HYDROLYSIS OF HIPPURYL-L-β-PHENYLLACTIC ACID BY NATIVE AND ACETYLATED CARBOXYPEPTIDASE A*

Native (Enzyme 1)		Acetylcarboxypeptidase A (Enzyme 2)		Acetylcarboxypeptidase A (Enzyme 3)	
$S_0 \times 10^4$† (M)	$V_0/E_0 \times 10^{-3}$ (sec^{-1})	$S_0 \times 10^3$ (M)	$V_0/E_0 \times 10^{-2}$ (sec^{-1})	$S_0 \times 10_0$ (M)	$V_0/E_0 \times 10^{-2}$ (sec^{-1})
49.2	1.34	5.97	7.55	3.03	7.29
12.6	2.38	4.92	6.83	2.42	5.80
9.90	3.09	3.98	6.70	2.42	6.07
7.92	3.32	1.99	5.44	1.82	5.65
5.94	3.85	0.997	4.41	0.909	3.64
3.96	3.76	0.794	3.90	0.909	3.87
1.98	4.20	0.597	3.34	0.454	2.28
0.990	3.23	0.398	2.63	0.303	1.60
0.984	3.06			0.303	1.54
0.690	2.42				
0.400	1.74				
0.197	1.07				

* In aqueous solution at 25.0°; I = 0.5; pH = 7.50; Tris-HCl buffer.
† Substrate concentrations are expressed in terms of the L-component of the DL mixture since the D-isomer is neither hydrolyzed nor inhibitory.[15]

TABLE 3

KINETIC CONSTANTS OF THE HYDROLYSIS OF HIPPURYL-L-β-PHENYLLACTIC ACID BY NATIVE AND ACETYLATED CARBOXYPEPTIDASE A*

Enzyme†	Activity on standard peptidase assay	k_{cat} (sec^{-1})	K_m (app) (M)
1. Native‡	100	578	8.8×10^{-5}
2. Acetylcarboxypeptidase	32	797	8.2×10^{-4}
3. Acetylcarboxypeptidase	8	1090	1.78×10^{-3}

* In aqueous solution at 25.0°; I = 0.5; pH = 7.50; Tris-HCl buffer.
† The numbers of the enzymes correspond to those in Table 1.
‡ The most recently reported kinetic constants for the native enzyme are k_{cat} = 467 sec^{-1} and K_m (app) = 2.55×10^{-5} M.[16] The latter value is recalculated on the basis that only the L portion of the substrate hydrolyzes.[15]

neither of these numerical changes expresses the fact pictorially evident in Figure 1 that the most profound change upon acetylation of the enzyme is the loss (or displacement to an experimentally inaccessible region) of substrate inhibition.

The standard assays employed previously for the characterization of acetylcarboxypeptidase A used benzyloxycarbonylglycyl-L-phenylalanine at a fixed concentration of 0.02 M to measure peptidase activity and used hippuryl-L-β-phenyllactic acid at a fixed concentration of 0.01 M to measure esterase activity.[10] As is seen in Table 1, enzyme 3 exhibits a peptidase activity on this arbitrary scale of 8 per cent of the native enzyme, agreeing exactly with that reported by Vallee and co-workers.[10] As may be seen in Figure 1, the concentration of substrate used in the standard esterase assay falls in a region of very pronounced substrate inhibition with the native enzyme, but in a region of essentially no substrate inhibition with the acetylated enzymes. Using Figure 1 to make very small extrapolations to 0.01 M (DL), it is found that at this concentration, enzyme 3 is 600 per cent as active as the native enzyme, in reasonable agreement with the 714 per cent reported by Vallee and co-workers.[10] Also, the average number of acetyl groups introduced into each molecule of enzyme 3 is 5.3, while that found previously was 4.3. Thus, it appears both kinetically and by analysis of acetyl groups that enzyme 3 is quite similar to that reported previously and therefore that the results with this enzyme are pertinent to the acetylcarboxypeptidase discussed earlier.

Enzyme 2, which is not as extensively acetylated as either enzyme 3 or the pre-

viously reported acetylcarboxypeptidase, exhibits a behavior intermediate between that of the native enzyme and that of the extensively acetylated enzyme, as might be expected. An enzyme which is even more extensively acetylated than enzyme 3 might show even more accentuated changes in k_{cat} and K_m(app), but it is clear from the data of enzymes 2 and 3 that the general pattern of the acetylation effect of Table 3 will hold up. (Professor B. L. Vallee informs us that he has prepared an acetylcarboxypeptidase whose activity in the standard peptidase assay is 1% or less of that of the native enzyme.)

This kinetic analysis has no clear-cut mechanistic implications. Obviously, the structural requirements of the active site have been changed by the extensive acetylation, presumably of two tyrosine residues.[10] One may interpret this result by saying that the tyrosines participate in the binding of the substrate to the active site. But this need not be the case if acetylation simply introduces steric hindrance to the site.

The small effect of acetylation on k_{cat} is also difficult to interpret mechanistically. Acetylcarboxypeptidase A may be considered to be a superenzyme, with respect to native carboxypeptidase A in the hydrolysis of hippuryl-L-β-phenyllactic acid. But the acetyl enzyme does not appear to be efficient with respect to a peptide substrate.

The closest analogy to the increased k_{cat} and increased K_m(app) in the acetylcarboxypeptidase-A-catalyzed hydrolysis of hippuryl-L-β-phenyllactic acid is found in the reactions of chymotrypsin bearing an acetylaminoisobutyrate substituent on its methionine 191.[5] This enzyme exhibits a 4-fold increased k_{cat} and a 10-fold increased K_m(app) toward a variety of the specific substrates of chymotrypsin.[6] The increased K_m(app) may be explained by hypothesizing that the acetylaminoisobutyrate substituent is an internal competitive inhibitor of the substrate. The increased k_{cat} may be interpreted in terms of an increased fixation of the substrate leading to an enhanced reactivity. Perhaps the same phenomena occur in the acetylcarboxypeptidase-A-catalyzed hydrolysis of hippuryl-L-β-phenyllactic acid.

Summary.—A kinetic analysis of the hydrolysis of hippuryl-L-β-phenyllactic acid by carboxypeptidase A and two acetylated carboxypeptidases A has been carried out. The substantial substrate inhibition in the reaction catalyzed by carboxypeptidase A is not even seen in the reactions catalyzed by the acetylated enzymes. The Michaelis-Menten parameters, k_{cat} and K_m(app) of the most extensively acetylated enzyme are increased 2-fold and 20-fold, respectively, over that of the native enzyme, indicating an inhibitory effect on the binding but an enhancement of the catalytic process.

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*OPTICAL ROTATORY DISPERSION OF CHLOROPHYLL IN SOLUTION AND IN CHLOROPLAST SUBUNITS**

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Preparations of isolated pigmented lamellar fragments from spinach chloroplasts exhibit many of the photochemical activities associated with photosynthesis.[1-3] In the electron microscope these lamellae appear to be made up of a highly regular two-dimensional array of subunits which have been called quantasomes.[1, 4] Within the quantasomes, which correspond to a molecular weight of 2×10^6, is a complex complement of pigments, redox agents, colorless lipid, and structural protein. Although little is known about the environment and interrelationships of the pigment molecules and other cofactors, there is evidence that a small fraction of the chlorophyll is oriented in a way that produces a dichroic absorption maximum at 690–700 mμ.[5, 6] Other spectrophotometric and photochemical evidence suggests that the immediate environment of the pigment molecules in quantasomes is very similar, if not identical, to that present in intact chloroplasts or in whole cells.[2]

Several recent studies have shown that the optical rotatory dispersion (ORD) can, in favorable circumstances, be a useful measure of the interaction among pigment molecules or chromophores in an optically active environment. Blout and Stryer found that symmetric dye molecules bound to helical polypeptides exhibit pronounced Cotton effects in the regions of the dye absorption bands.[7, 8] Furthermore, it has been shown theoretically and experimentally that chromophoric molecules with intrinsic optical activity can undergo profound changes in the observed Cotton effects when the absorbing species are brought into close association.[9, 10] In order to obtain further information on the environment of chlorophyll and other pigment molecules in photosynthetic systems, we have examined the ORD of suspensions of spinach quantasome aggregates (lamellar fragments). To aid in the interpretation of the complex Cotton effects observed, we have also measured the ORD spectra of chlorophyll *a* in two different solvents and over a wide range of concentrations.