Inhibition in Ester Hydrolyses Catalyzed by Carboxypeptidase A

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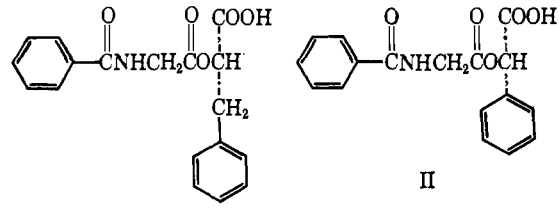
Abstract: Substrate inhibition has been found to occur in the carboxypeptidase A catalyzed hydrolysis of O-(N-benzoylglycyl)-L-mandelate (II) at pH 7.5 and 25°. The kinetics of the hydrolytic reaction can be adequately treated by means of a rate expression that contains terms which are first and second order in the substrate concentration. Schemes giving rise to this rate expression, that invoke an inhibitory site to which the substrate can bind in addition to the catalytic site, can be postulated. In earlier kinetic work substrate activation was observed in the carboxypeptidase-catalyzed hydrolysis of O-(N-benzoylglycyl)glycolate. Furthermore, N-carbobenzyloxyglycine (CBZ-Gly) when added as an effector was shown to suppress substrate activation by binding to the activator site of the enzyme. Now the behavior of CBZ-Gly in the carboxypeptidase-catalyzed hydrolysis of cinnamoyl-DL-β-phenyllactate (III), a reaction which does not appear to involve substrate activation at pH 7.5 and 25°, has been demonstrated to provide a remarkable contrast. In this case CBZ-Gly acts as a competitive inhibitor with a K_i value of 0.016 *M*. The finding that CBZ-Gly can act as an inhibitor or an activator in reactions catalyzed by carboxypeptidase leads to an important new problem which must be solved: what is the relationship of the activator and inhibitor sites?

In a recent paper by Quiocho and Richards[4] a very general mechanism for carboxypeptidase A catalyzed reactions involving a monomeric form of the enzyme and multiple substrate binding sites was proposed. A rate equation which fits this mechanism may be written in the form of the general polynomial

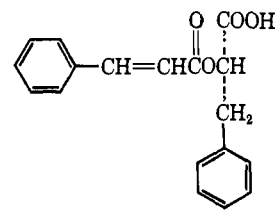
$$\frac{-d(S)}{dt} = v = \frac{A_1(S) + A_2(S)^2 + A_3(S)^3 + \ldots . A_n(S)^n}{1 + B_1(S) + B_2(S)^2 + B_3(S)^3 + \ldots . B_n(S)^n} \quad (1)$$

where (S) represents the substrate concentration and $A_1 \ldots . A_n$, $B_1 \ldots . B_n$ are appropriate combinations of rate constants (equilibrium constants) for the individual steps. As Quiocho and Richards have pointed out,[4] their general mechanism accommodates the phenomena both of substrate inhibition as well as substrate activation.[5] During the course of our work on the kinetics of the esterase action of carboxypeptidase A we have employed the same general equation which Quiocho and Richards have presented in order to account for our experimental observations. In the present paper and in a series of forthcoming reports from our laboratory we shall discuss our results on native carboxypeptidase A, other metallocarboxypeptidases, and the chemically modified enzyme in this framework.

The carboxypeptidase A catalyzed hydrolysis of an ester substrate, O-(N-benzoylglycyl)-DL-3-phenyllactate (I), at pH 7.5 and 25° is known to be complicated by substrate inhibition.[6-9] Only the L isomer of the ester is hydrolyzed, and the D antipode apparently has no effect upon the kinetic constants of the hydrolytic reaction.[10]



I (L isomer) II



III (L isomer)

Comparisons of the kinetics of the hydrolysis of I and of the peptide substrate, N-(N-carbobenzyloxyglycyl)-L-phenylalanine, catalyzed by both native and chemically modified forms of carboxypeptidase A have provided the basis of mechanistic hypotheses concerning the hydrolytic action of this enzyme.[11-13] Bender, *et al.*, studied the effect of acetylation of carboxypeptidase A on the kinetic constants for substrate I and found that the most striking effect caused by this modification was the release of substrate inhibition leading to the observation of simple Michaelis–Menten kinetics for the acetylated enzyme.[9] Since this result bears upon the mechanistic interpretations which might be attached to the kinetic data for carboxypeptidase A it is important to understand the mode of substrate inhibition of such reactions.

We have found substrate inhibition in investigating the enzymatic hydrolysis of the analogous ester sub-

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(12) J. F. Riordan and B. L. Vallee, *Biochemistry*, **2**, 1460 (1963).
(13) B. L. Vallee, *Federation Proc.*, **23**, 8 (1964).

strate, O-(N-benzoylglycyl)-L-mandelate (II), and we present here a detailed analysis of the carboxypeptidase A catalyzed hydrolysis of II at pH 7.5 and 25°.[14]

Another problem which we will discuss concerns the relationship between activation and inhibition phenomena observed for carboxypeptidase A. Two reports in the recent literature have dealt with the effect of N-carbobenzyloxyglycine (CBZ-Gly) on hydrolytic reactions catalyzed by this enzyme. In the case of the carboxypeptidase A catalyzed hydrolysis of the ester O-(N-benzoylglycyl)glycolate, the suppression of substrate activation by the addition of CBZ-Gly, acting as a modifier, was described.[5]

A related investigation has shown that CBZ-Gly is an activating product in the carboxypeptidase A catalyzed hydrolysis of the peptide substrate, N-carbobenzyloxyglycyl-L-phenylalanine.[15] Both these studies have been concerned with the influence of CBZ-Gly on reactions in which substrate activation was observed. The interesting question thus arises as to what the effect of CBZ-Gly will be on the hydrolysis of a substrate whose kinetic behavior is uncomplicated by the phenomenon of substrate activation (or of substrate inhibition), and we shall address ourselves to this question.

Experimenal Section

O-*trans*-Cinnamoyl-DL-β-phenyllactic Acid. This compound was prepared as follows. A solution of 10 g (0.062 mole) of DL-β-phenyllactic acid (Sigma Chemical Co., Lot No. P518-059) in 90 ml of benzene was heated at reflux in a 200-ml, three-necked flask. Magnetic stirring was provided, and 10.5 g (0.062 mole) of freshly distilled cinnamoyl chloride (Eastman White Label) in 30 ml of benzene was added dropwise from a pressure-equilibrated dropping funnel. Dry nitrogen was continuously swept through the system to prevent contamination by atmospheric moisture. After 3 hr, the refluxing reaction mixture was allowed to cool to room temperature, and the solvent was removed on a rotary evaporator leaving behind a yellow oil. The oil was dissolved in a 1:1 mixture of 1,2-dichloroethane and carbon tetrachloride and the solution concentrated on a hot plate and chilled. The product crystallized as white crystals melting between 90 and 100° (18.5 g, 92.7% crude yield). Recrystallization from carbon tetrachloride and drying *in vacuo* at 100° produced the pure compound, mp 129.5–131.5°.

Anal. Calcd for $C_{18}H_{16}O_4$: C, 72.96; H, 5.44. Found: C, 73.34; H, 5.39.

Due to the low solubility of O-*trans*-cinnamoyl-DL-β-phenyllactic acid in water, the substrate was converted to its sodium salt before use. The salt was obtained by very slow titration of a water suspension of the acid with an equivalent amount of 0.1 *N* NaOH. After titration the clear solution of the sodium salt was concentrated on the rotary evaporator and chilled. The pure sodium salt was then precipitated, collected by filtration, washed with ice water, and thoroughly dried. Enzymatic hydrolyses of the salt were followed to completion by automatic titration,[16] and the end points which were obtained corresponded exactly to those expected for complete hydrolysis of the active L component of the racemic salt. Since only the L isomer was hydrolyzed by carboxypeptidase, the concentrations given in this paper for substrate solutions always refer to the concentration of this isomer. Concentrations of stock solutions of the substrate salt (~0.005 *M*, unbuffered, and 0.5 *M* in NaCl) were checked continually to ensure that no spontaneous hydrolysis or other change had taken place. It was found that such solutions, which had a pH of 5–6, were stable for months at room temperature. Periodic control runs showed no change in the behavior of the hydrolysis kinetics of these solutions for as long as they were used.

N-Carbobenzyloxyglycine. This material was purchased from Aldrich Chemical Co. and was recrystallized from benzene, mp 120–120.5° (lit.[17] mp 120°). Stock solutions of the sodium salt of this acid were prepared by neutralization with sodium hydroxide and were stored at 4°.

O-(N-Benzoylglycyl)-L-mandelic acid was prepared in the manner described previously,[16] mp 167.0–168.1°. Stock solutions (0.2 or 0.4 *M*) of the sodium salt of this acid in water (ionic strength was adjusted to 0.5 with sodium chloride) at pH 5 were prepared and stored at 4° for use in kinetic runs. Under these conditions nonenzymatic hydrolysis of this substrate was negligible over at least a 3-week period.

Carboxypeptidase A. The enzyme preparation used in this study was purchased from Sigma Chemical Co. (Lot No. 123B-1590) and was stated to have been obtained from bovine pancreas by the method of Anson[18] as modified by Putnam and Neurath[19] and has been designated carboxypeptidase Aγ by Bargetzi, *et al.*[20] Stock solutions (2×10^{-5} *M*) of this enzyme were prepared by dialyzing a water suspension of crystals at 4° against 0.05 *M* 2-amino-2-hydroxymethyl-1,3-propanediol (Tris)–hydrochloric acid buffer containing 1 *M* sodium chloride. The solution was centrifuged, filtered, recentrifuged as described previously,[16,21] and stored in a refrigerator at 4°. Stock solutions thus prepared were stable with respect to their absorbance and activity toward II for at least 3 months under these conditions.

Buffers. Tris–hydrochloric acid buffers at pH 7.5 used to make stock enzyme solutions and for enzyme dialysis were prepared from Tris (Matheson Coleman and Bell, highest purity, mp 170–171°), 2 *M* hydrochloric acid (Fisher standardized reagent), sodium chloride (Baker and Adamson, reagent grade), and water that was distilled and then passed through a mixed-bed, ion-exchange column (Continental Demineralization Service). Solutions for kinetic runs on the automatic titrator were made up with demineralized water and were 0.500 *M* in sodium chloride. Solutions for kinetic runs on the recording spectrophotometer were made up with 0.05 *M* Tris buffer, were 0.500 *M* in sodium chloride, and had a measured pH of 7.48.

pH Measurements. These measurements on the buffer and on reaction mixtures were carried out on a Beckman Research pH meter standardized against fresh Fisher Certified standard buffers. Checks of the pH both before and after enzymatic hydrolysis on reaction mixtures in spectrophotometer cuvettes in no case revealed a variation in hydrogen ion concentration greater than a fraction of 1%.

Enzyme Concentrations. The protein content of stock solutions of carboxypeptidase A was determined by measuring the absorbance of an aliquot at 278 mμ on a Cary Model 14 recording spectrophotometer using the same buffer (containing no protein) as a reference. A value of 6.42×10^4 l. mole^{-1} cm^{-1} for the extinction coefficient at this wavelength was used to calculate the molar concentration of the enzyme.[22]

Kinetic Measurements. Hydrolyses of O-cinnamoyl-DL-β-phenyllactic acid were followed spectrophotometrically on a Cary 14 recording spectrophotometer. The cell compartment and cell jacket itself were thermostated at 25.00 ± 0.05°. For each run the reaction was followed for at least two half-lives, and then 20–25 points, read from the trace at equal time intervals, were subjected to analysis by the IBM 7094 computer.

In a typical run, a quartz cuvette was filled with exactly 3 ml of the pH 7.48 Tris buffer. Then an appropriate small volume of the buffer was withdrawn with a Hamilton microsyringe and replaced with the same volume of substrate stock solution (or substrate plus inhibitor) so that the total volume was still 3.00 ml. The cuvette was then placed in the thermostated cell compartment of the spectrophotometer and allowed to equilibrate for at least 15 min. The initial absorbance reading was then taken at an appropriate wavelength. Following this, 5 μl of the enzyme stock solution was introduced with a microliter pipet, and the solution was stirred. The chart drive on the instrument was activated exactly 20 sec after the introduction of enzyme. The concentration

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(22) R. T. Simpson, J. F. Riordan, and B. L. Vallee, *Biochemistry*, 2, 616 (1963).

of enzyme ($\sim 10^{-3}$ *M*) was too small to noticeably affect the observed optical density of the solution at the wavelengths employed.

Hydrolyses of O-(N-benzoylglycyl)-L-mandelic acid were followed by pH-Stat titration employing a Radiometer Type TTTlb titrator in conjunction with a Type SBR2c titrigraph and a Type GK2021c combined glass and calomel electrode. The reaction cell was thermostated at 25.00 ± 0.02°, and all reactions were run in a purified nitrogen atmosphere.

In a typical run the instrument was first standardized against a buffer of pH 7.40 ± 0.02. The titrating mechanism was set at pH 7.50 and sufficient 0.500 *M* sodium chloride to give a final volume of 5.00 ml was added to the reaction vessel. Then an appropriate volume of 0.2 or 0.4 *M* sodium O-(N-benzoylglycyl)-L-mandelate solution was added, and the mixture was equilibrated to pH 7.50 and 25.00° for 10 min, while being magnetically stirred. At this point enough 1.931×10^{-5} *M* stock carboxypeptidase A (5–500 μl) to give more than 90% hydrolysis within 1 hr was added, and the titrating mechanism was activated. The 0.500-ml syringe buret contained 1.000×10^{-2} to 0.500 *M* sodium hydroxide (Fisher standardized reagent) so that reactions could be followed to completion. End points were determined for about 50% of the kinetic runs, and they all agreed within 1% with the values expected for complete hydrolysis of II to N-benzoylglycine anion and L-mandelate.[23]

Data Analysis. Initial velocities of reactions were calculated with a high-speed computer using a program described elsewhere.[24] The data were fitted by least squares to orthogonal polynomials of the form $y = a_0 + a_1x + \cdots + a_mx^m$,[25,26] where $y - a_0$ is a measure of the concentration of the product formed, x is directly proportional to the time, and the a's are constants. The initial velocity is directly proportional to a_1. The program corrects the data for the product buffering effect and for the base-catalyzed hydrolysis of the substrate in the manner described earlier.[16,24] Since the pK_a's of both substrate and products are less than 5 and since the second-order rate constant for hydroxide ion catalysis of hydrolysis of II was found to be 62.2 ± 1.0 l. mole^{-1} min^{-1} at 25°, both of these corrections were negligible at pH 7.5. Kinetic parameters were calculated from the initial velocities by means of a computer program based upon a least-squares treatment.[27]

Results

Initial rate data for the hydrolysis of II catalyzed by carboxypeptidase A at pH 7.5 and 25° are given in Table I.

We have applied eq 2 to our results allowing A_1, B_1, and B_2 to be independent of each other. This equation is obtained from eq 1 making the assumption that n can be 2 and that A_2 is negligibly small. The

$$v_0/(E_0) = \frac{[A_1/(E_0)](S_0)}{1 + B_1(S_0) + B_2(S_0)^2} \qquad (2)$$

values obtained for the coefficients are as follows: $A_1/(E_0) = 1.09 \times 10^7\ M^{-1}\ \text{min}^{-1}$, $B_1 = 1.26 \times 10^3\ M^{-1}$, and $B_2 = 3.13 \times 10^5\ M^{-2}$.

The standard deviation of values of $v_0/(E_0)$ computed with these coefficients is 6.4%.[27] Since the standard deviation of experimental values of $v_0/(E_0)$ is approximately the same (5–6%), applying the principle of "Occam's razor,"[28] it would seem that eq 2 fits our data satisfactorily and that it is unnecessary in the case of O-(N-benzoylglycyl)-L-mandelate to postulate any further higher order binding of the substrate. Figure 1 shows calculated and experimentally obtained points in a plot of $v_0/(E_0)$ *vs.* (S_0) for the hydrolysis of this compound.

Table I. Initial Rate Data for Hydrolysis of O-(N-Benzoylglycyl)-L-mandelate by Carboxypeptidase A at pH 7.5 and 25° [a]

(S_0), *M*	$10^{-3}v_0/(E_0)$, min^{-1} [b]	(S_0), *M*	$10^{-3}v_0/(E_0)$, min^{-1} [b]
0.000300	2.20	0.00500	3.31
0.000400	2.84	0.0100	2.27
0.000500	3.12	0.0150	1.76
0.000700	3.92	0.0200	1.49
0.00100	4.57	0.0300	1.08
0.00140	4.51	0.0400	0.815
0.00200	4.76		

[a] Most values are averages for two to four runs. [b] The standard deviations of these values were consistently found to be 5–6%.

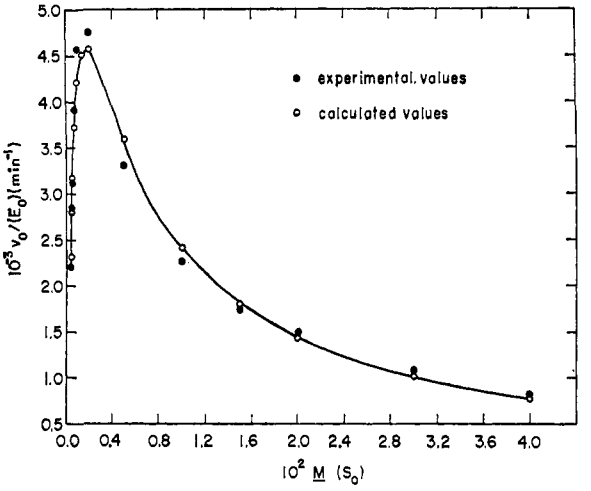

Figure 1.

A rather general scheme which will give rise to the rate expression given in eq 2 is shown in eq 3 below where P_1 and P_2 are L-mandelate[23] and N-benzoylglycine, respectively, and SE and SES are catalytically inactive complexes.

$$\begin{array}{ccc} E + S & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & ES \xrightarrow{k_{cat}} E + P_1 + P_2 \\ {\scriptstyle k'_{Is}}\downarrow\uparrow{\scriptstyle k'_{-Is}} & & {\scriptstyle k_{Is}}\downarrow\uparrow{\scriptstyle k_{-Is}} \\ SE & \rightleftharpoons & SES \end{array} \qquad (3)$$

If it is assumed that the complexes in this scheme are in equilibrium with one another (*i.e.*, that k_{cat} is much smaller than k_{-1}, k_{Is}, and k'_{-Is}), the rate expression in eq 4 is obtained, where $K_m = k_{-1}/k_1$, $K_{Is} = k_{-Is}/k_{Is}$, and $K'_{Is} = k'_{-Is}/k'_{Is}$.

$$v_0/(E_0) = \frac{(k_{cat}/K_m)(S_0)}{1 + (1/K_m + 1/K'_{Is})(S_0) + (S_0)^2/K_mK_{Is}} \qquad (4)$$

(23) No direct evidence for the configuration of product mandelate was obtained. It was found, however, that O-acetyl-L-mandelate was hydrolyzed by carboxypeptidase A to mandelate which was at least 90% of the L configuration.[16] Limited polarimetric studies of the enzymatic hydrolysis of II also indicated that one of the products was L-mandelate. Furthermore, N-benzoylglycine was not significantly hydrolyzed by 10^{-5} *M* carboxypeptidase A under these conditions. Considering these results and the end point stoichiometry, it therefore seems likely that the above assignment of products is correct.

(24) F. W. Carson, Ph.D. Thesis, University of Chicago, 1965.

(25) K. A. Booman and C. Niemann, *J. Am. Chem. Soc.*, 78, 3642 (1956).

(26) D. T. Elmore, A. E. Kingston, and D. B. Shields, *J. Chem. Soc.*, 2070 (1963).

(27) S. Awazu and E. T. Kaiser, unpublished work. In the investigation reported by Quiocho and Richards,[4] kinetics were done using carboxypeptidase in the solid state or at constant concentrations of the enzyme and the parameter A_1 was constant. However, in our work A_1 was not a constant since various concentrations of the enzyme were employed, and instead the term $A_1/(E_0)$ was calculated since it was constant.

(28) B. Russell, "A History of Western Philosophy," Simon and Schuster, New York, N. Y., 1945, p 472.

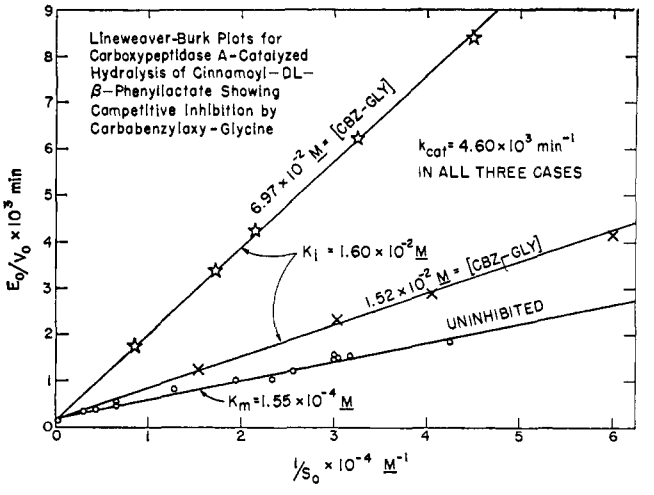

Figure 2.

In order to calculate values for the kinetic parameters in eq 4, it is necessary to make some further assumptions. One possible assumption is to say that K'_{Is} is infinite. The scheme in eq 3 then becomes that shown in eq 5, and the rate expression in eq 4 is reduced to eq 6. The kinetic parameters calculated from eq 6

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_{cat}} \mathrm{E} + \mathrm{P_1} + \mathrm{P_2} \qquad (5)$$

k_{1s} ⇓ ⇑ k_{-1s}

SES

$$v_0/(\mathrm{E}_0) = \frac{(k_{cat}/K_m)(\mathrm{S}_0)}{1 + (1/K_m)(\mathrm{S}_0) + (\mathrm{S}_0)^2/K_m K_{Is}} \qquad (6)$$

are $k_{cat} = 8.65 \times 10^3$ min^{-1}, $K_m = 7.94 \times 10^{-4}$ mole/l., and $K_{Is} = 4.02 \times 10^{-3}$ mole/l.[29]

A reasonable alternative assumption[30] which can be made in regard to eq 4 is that $K_{Is} = K'_{Is}$ since the modes of inhibitory binding of S to E or to ES might be very similar. This hypothesis leads to a dual solution for the kinetic parameters. One solution is that $k_{cat} = 3.2 \times 10^4$ min^{-1}, $K_m = 2.93 \times 10^{-3}$ mole/l., and $K_{Is} = 1.09 \times 10^{-3}$ mole/l., and the other is that $k_{cat} = 1.19 \times 10^4$ min^{-1}, $K_m = 1.09 \times 10^{-3}$ mole/l., and $K_{Is} = 2.93 \times 10^{-3}$ mole/l.

Clearly, on the basis of the presently available data it is not possible to assign unique values for the kinetic constants in the carboxypeptidase A catalyzed hydrolysis of O-(N-benzoylglycyl)-L-mandelate. However, our results do show that this enzymatic reaction can be adequately analyzed in terms of schemes in which an inhibitory site exists to which the substrate can bind in addition to the catalytic site.

Relatively few carboxypeptidase substrates are known which have uncomplicated behavior and exhibit neither substrate activation nor substrate inhibition in their hydrolyses.[16] However, we have found that at pH 7.5 and 25.0° the kinetics of the carboxypeptidase A catalyzed hydrolysis of O-cinnamoyl-DL-β-phenyllactate (III) fit a fairly simple Michaelis–Menten scheme. Under these conditions and at the concentrations of substrate and enzyme used there was no evidence for either substrate activation or inhibition. In terms of eq 1 it appears that our data can be satisfactorily fit by eq 7 which arises from the assumption that n is 1 and that the empirical parameters A_1 and B_1 represent the Michaelis–Menten parameters shown.

$$v_0/(\mathrm{E}_0) = \frac{[A_1/(\mathrm{E}_0)](\mathrm{S}_0)}{1 + B_1(\mathrm{S}_0)} = \frac{(k_{cat}/K_m)(\mathrm{S}_0)}{1 + (1/K_m)(\mathrm{S}_0)} \qquad (7)$$

The course of the hydrolysis of O-cinnamoyl-DL-β-phenyllactate (III) was followed spectrophotometrically. Only the L isomer underwent hydrolysis in accord with the known specificity requirements of carboxypeptidase A as an esterase.[16,24] The pH of reaction solutions was buffered to a value of 7.48 with 0.05 M Tris and the ionic strength was adjusted to 0.5 with NaCl.

Figure 2 shows plots of $(\mathrm{E}_0)/v_0$ *vs.* $1/(\mathrm{S}_0)$[31] for the carboxypeptidase A catalyzed hydrolysis of III, both in the presence and absence of CBZ-Gly. The substrate concentration was varied up to about 5×10^{-3} M (10^5 times the enzyme concentration) with no observed deviation from linearity in the Lineweaver–Burk plot. The value of K_m obtained in the absence of CBZ-Gly is 1.55×10^{-4} M. As can be seen from the figure, the intercepts on the $(\mathrm{E}_0)/v_0$ axis observed in the absence of CBZ-Gly and in the presence of 0.016 and 0.0697 M CBZ-Gly are the same, indicating that in this case CBZ-Gly is acting as a competitive inhibitor with a K_i value of 0.016 M. The apparent k_{cat} values are 4.60 $\times 10^3$ min^{-1}. O-Cinnamoyl-β-phenyllactate is thus an excellent substrate for the action of carboxypeptidase A. From our results it is apparent that the kinetic behavior of CBZ-Gly in a reaction in which no substrate activation is observed provides a striking contrast with its effect on the kinetics of the hydrolysis of compounds showing substrate activation.[5,27]

In conclusion, a considerable amount of evidence has accumulated supporting the hypothesis that there are multiple binding sites for substrates and modifiers in the carboxypeptidase molecule, in addition to the catalytic site,[4,16,24] and the kinetic behavior of carboxypeptidase has been shown to be similar to that which is generally observed for enzymes which are recognized to be allosteric.[5] Furthermore, the suggestion has

(29) These kinetic results differ somewhat from those previously reported[14] for the same reaction in 0.03 to 2% v/v dimethylformamide. The difference was not due to the organic solvent, which had an almost negligible inhibitory effect on the reaction, but apparently to some trace contaminant in the sodium chloride solutions in which reactions were run. The previously reported data could also be analyzed by the equations given in the present paper, and this analysis showed that the conclusions of our earlier paper remain unchanged.

(30) Besides eq 3 and 5 other schemes can obviously be proposed which involve additional assumptions. One such scheme is shown in eq i below. Here ES′ represents the acyl enzyme, N-benzoylglycylcarboxypeptidase, which might be formed from the Michaelis complex, ES, with concomitant expulsion of P_1, L-mandelate. According to this hypothesis substrate inhibition occurs when the substrate binds to ES′ (forming SES′) and prevents the further breakdown of ES′ to give P_2, N-benzoylglycine, and to regenerate carboxypeptidase. On the basis of the kinetic results in hand we cannot distinguish between the schemes shown in eq 3, 5, or i since there is no evidence which conclusively establishes whether or not the acyl enzyme, N-benzoylglycylcarboxypeptidase, is formed in the course of the hydrolysis of II.

SES′

⇕

$$\mathrm{E} + \mathrm{S} \rightleftharpoons \mathrm{ES} \longrightarrow \mathrm{ES'} + \mathrm{P_1} \qquad (i)$$

⇓

$\mathrm{E} + \mathrm{P_2}$

(31) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

been made that the cooperative phenomena found in the reactions of carboxypeptidase could be associated with the monomeric state of the enzyme.[4,5] The findings reported here demonstrate that CBZ-Gly can act as an inhibitor as well as an activator in carboxypeptidase-catalyzed hydrolyses and pose an intriguing new problem. What is the relationship between the sites on the carboxypeptidase molecule which CBZ-Gly occupies when it acts as an activator or as an inhibitor? Also, what is the relationship between the sites for substrate inhibition and substrate activation? In this connection it is important to mention that the value of K_i which we have found for the action of CBZ-Gly as an inhibitor is very close to the enzyme–CBZ-Gly dissociation constant determined for this compound as an activator,[5,15,27] and this suggests that the inhibitor and activator sites on carboxypeptidase to which CBZ-Gly binds may be closely related, if not the same.[32]

Acknowledgments. This work was supported by a grant from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.

(32) There has been a report that 9-aminoacridine can act as an activator in the chymotrypsin-catalyzed hydrolysis of methyl hippurate whereas other related compounds such as acridine itself and 2-aminoacridine act as competitive inhibitors. See: R. A. Wallace, R. L. Peterson, C. Niemann, and G. E. Hein, *Biochem. Biophys. Res. Commun.* 23, 243 (1966).

Mass Spectrometry of Porphyrins. II.[1] Characterization of Petroporphyrins

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Received December 10, 1966

Abstract: The nonhomogeneity of petroporphyrins extracted from petroleum sources has been previously established by low-voltage mass spectrometry. To extend these findings, the asphaltenes of Agha Jari, Baxterville, Belridge, Boscan, Burgan, Mara, Melones, Rozel Point, Santiago, and Wilmington petroleums, as well as of a gilsonite, Athabasca tar sand, and Colorado oil shale (Green River), were selected for study. The results of this survey confirm the presence of two major and one minor homologous series of petroporphyrins: the major series are a monocycloalkano series (DPEP) with visible spectra similar to that of deoxophylloerythroetioporphyrin, and a series of alkylporphyrins (etio) with visible spectra indicative of either incomplete β substitution or of bridge substitution. The ratios of the two series as well as the width of the envelope of molecular weights present were found to vary rather widely with the source. A high DPEP/etio series ratio coupled with a narrow envelope of molecular weights is suggested as indicative of a nonmarine origin of the petroporphyrins. An alkylbenzporphyrin structure is suggested for the minor series on the basis of the mass and electronic spectral evidence. The possibility is pointed out that petroporphyrins present in petroleum of marine origin with carbon skeletons of greater than 34 carbons may be derived from photosynthetic bacteria which contain chlorophylls with carbon skeletons of up to 38 carbons.

In an earlier paper, we showed that petroporphyrins obtained from certain asphaltenes were not homogeneous.[1] This interesting observation prompted a further investigation to determine if the distribution and types of porphyrins in a wider variety of asphaltenes and other bituminous materials varied significantly. We report here the results of such a survey on ten petroleum asphaltenes and three other bituminous materials selected to cover a range of geologic types and ages.

Results

Table I shows the sources, geologic ages, and yields of the petroporphyrins selected for study. The petroporphyrins were obtained by extraction of asphaltenes with methanesulfonic acid (MSA) in the manner described previously.[1,2] In cases where the starting material was solid (*i.e.*, Athabasca tar sand, gilsonite, and Green River Colorado oil shale) direct extractions were performed. The petroporphyrins were readied for spectroscopic analysis by a two-step purification: (1) transfer to an organic phase (methylene chloride) without neutralization, and (2) conversion to the free base and column chromatography over silica gel. That these purification steps were adequate is demonstrated by the quality of the mass spectra; extraneous peaks not due to porphyrins are, generally, less than 5% of the main porphyrin peaks.

In Figure 1a, b, and c are shown portions of the low-voltage (12 ev) mass spectra of the porphyrins extracted from the Baxterville, Rozel Point, and Melones petroleums. These were selected to demonstrate the differences in type and distribution of porphyrins present in asphaltenes. These spectra are typical of those obtained on other petroporphyrin samples.

In Table II are listed the normalized peak intensities of the major porphyrin peaks in the low-voltage mass spectra of the petroporphyrins from ten asphaltenes and three other bituminous materials. In obtaining the mass spectra an ionizing voltage of 12 ev was used so that essentially only parent molecular ions appear in these spectra (see Experimental Section). Two homologous series of porphyrins, the etioporphyrin (etio) series corresponding to a molecular weight of 310

(1) For the first paper of the series see: E. W. Baker, *J. Am. Chem. Soc.*, **88**, 2311 (1966). This work was presented in part at the 153rd National Meeting of the American Chemical Society, Division of Petroleum Chemistry, Symposium on Asphalts and Metals in Petroleum, Miami Beach, Fla., April 1967.

(2) J. G. Erdman, U. S. Patent No. 3,190,829 (June 22, 1965).