Biochimica et Biophysica Acta, 527 (1978) 98—107

BBA 68584

THE pH-DEPENDENCE OF THE NON-SPECIFIC ESTERASE ACTIVITY OF CARBOXYPEPTIDASE A

JOHN W. BUNTING and SHAIKH H. KABIR

Department of Chemistry, University of Toronto, Toronto, Ontario M5S 1A1 (Canada)

(Received March 20th, 1978)

Summary

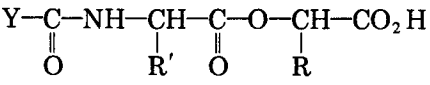
The hydrolysis of the following 6 esters by bovine pancreatic carboxypeptidase A (peptidyl-L-amino-acid hydrolase, EC 3.4.12.2) has been investigated over the range pH 5—10 at 25°C, ionic strength 0.2: $CH_3CO_2CHRCO_2H$ ($R = C_6H_5$ (ester 3), $C_6H_5CH_2$ (ester 4)), $4\text{-}NO_2C_6H_4CO_2CHRCO_2H$ ($R = C_6H_5$ (ester 5), $C_6H_5CH_2$ (ester 6), $CH_3(CH_2)_2$ (ester 7)), $CH_3CH_2CO_2CH(CH_2C_6H_5)$-$CO_2H$ (ester 9). For each ester the pH dependence of k_{cat}/K_m indicates that substrate binding is controlled by an acid of $pK_{EH} = 9.2 \pm 0.2$ in the free enzyme, and although k_{cat}/K_m decreases in acidic solutions no simple dependence on an enzymic ionization is apparent. For esters 3, 5 and 7 the dependence of k_{cat} on pH is 'bell-shaped' and is controlled by $pK_{EH_2S} = 6.73$, 6.72, 6.23, respectively and $pK_{EHS} = 9.3 \pm 0.2$ for each ester. For esters 4 and 6 the 'bell-shaped' k_{cat} ($pK_{EH_2S} = 7.38$, 6.28, respectively) is modified by a significant increase in k_{cat} in the vicinity of pH 10. This latter phenomenon is also shown by ester 9, although data are only accessible over the range pH 7—10 for this latter ester due to pronounced product inhibition in acidic solutions. The common pH-dependences observed for the enzymic hydrolyses of these non-specific ester substrates are compared with literature data for specific ester and peptide substrates, and possible assignments for the various enzymic pK_a values are discussed.

Introduction

Two general classes of ester substrates are known for bovine pancreatic carboxypeptidase A (peptidyl-L-amino-acid hydrolase, EC 3.4.12.2). One class

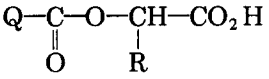
Supplementary data to this article, giving details of hydrolysis of non-specific ester substrates, are deposited with, and can be obtained from Elsevier Scientific Publishing Company, BBA Data Deposition, P.O. Box 1345, 1000 BH Amsterdam, The Netherlands. Reference should be made to No. BBA/DD/087/68584/527 (1978) 98.

consists of depsipeptides of the type 1 which contain an amide bond corresponding to the penultimate C-terminal



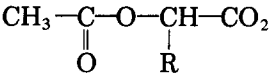
type 1

amide bond of the natural peptide substrates. This resemblance to the biological substrates for this enzyme leads to the classification of ester substrates of the type 1 as specific ester substrates. The importance of an interaction of the amide unit with the enzyme in the productive binding of substrates of type 1 has been demonstrated [1]. This interaction probably involves the formation of a hydrogen-bond between the hydrogen atom of this amide group and an enzymic acceptor. Other ester substrates (type 2) in which Q lacks such an amide bond, and therefore cannot participate in such an hydrogen-bonding interaction, are then referred to as non-specific ester substrates.



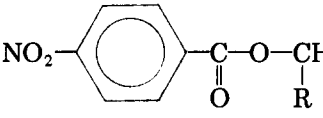
type 2

While the above division of esters into specific and non-specific substrates is based on structure, important empirical differences between these two classes of substrates are also observed experimentally. In particular, the limited data that are currently available seem to indicate striking differences in the pH-dependences of the rates of enzymic hydrolysis of non-specific and specific ester substrates [2—8]. The first non-specific ester substrate for this enzyme to be studied in detail was *O*-acetyl-L-mandelic acid (ester 3). Carson and



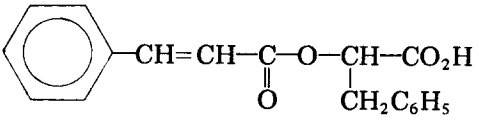
(ester 3, R = C_6H_5 ; ester 4, R = $C_6H_5CH_2$)

Kaiser [3] report bell-shaped pH-dependences of the enzymic hydrolysis of this ester for both k_{cat} (pK_a = 7.2, 7.9) and k_{cat}/K_m (pK_a = 6.9, 7.5) controlled by enzymic pK_a values as indicated. The enzymic hydrolysis of *O*-*p*-nitrobenzoyl-L-mandelic acid (ester 5) appeared, on the basis of a less-detailed



(ester 5, R = C_6H_5 ; ester 6, R = $C_6H_5CH_2$; ester 7, R = $CH_3(CH_2)_2$)

study [6], to diplay similar pH-dependences, however, quite different pH-profiles have been reported for k_{cat} and k_{cat}/K_m for the enzymic hydrolysis of cinnamate esters [4,7] (e.g. ester 8). While similar enzymic pK_a values control the

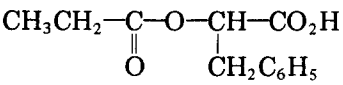


ester 8

binding of cinnamate ester and depsipeptide ester substrates, quite different pH-dependences for k_{cat} are observed for these two groups of ester substrates [4,5,7,8]. Typically, for hippurate esters (ester 1: Y = C_6H_5) k_{cat} is essentially pH-independent in the region pH 5—10 [8].

Thus the data currently available appear to indicate different pH-dependences for the enzymic hydrolysis of specific and non-specific ester substrates and also variations within the class of non-specific esters (cinnamates vs. acetates and *p*-nitrobenzoates). Since pH-rate profiles provide essential experimental data for the interpretation of mechanisms of enzymic hydrolysis, we felt a more detailed investigation of the pH-dependence of the non-specific esterase activity of carboxypeptidase A was required. It is also essential that the relationship between the enzymic hydrolysis of cinnamate esters and other esters be established on a firm basis in the light of the recent report [9] of the observation of the catalytically important acyl-enzyme intermediate during the hydrolysis of a *p*-chlorocinnamate ester by this enzyme.

We report detailed pH-rate profiles for the carboxypeptidase A-catalyzed hydrolysis of three *p*-nitrobenzoate esters (esters 5, 6 and 7), two acetate esters (esters 3 and 4) and a propanoate ester (ester 9), and make a detailed comparison of the current data with literature data



ester 9

for the enzymic hydrolysis of cinnamate esters, specific esters and peptides.

Materials and Methods

Substrates

The *p*-nitrobenzoate esters were available from a recent study [10]. *O*-acetyl-L-mandelic acid (ester 3) and *O*-acetyl-DL-3-phenyllactic acid (ester 4) were synthesized via this same general route [10].

O-acetyl-L-mandelic acid (ester 3) had m.p. 83—84°C after recrystallization from a benzene/chloroform mixture (literature values [11—13] m.p. 97—99°C, 96.8°C, 96.5—98°C for samples obtained by desiccation of the monohydrate); $[\alpha]_D^{25}$ = +156.8° (C 2.04, acetone) (literature [11] $[\alpha]_D^{25}$ = +153° (C 2.044, acetone)). Analysis: Calcd. for $C_{10}H_{10}O_4$: C, 61.85; H, 5.15. Found: C, 62.06; H, 5.13.

O-acetyl-DL-3-phenyllactic acid (ester 4): m.p. 85—86°C. Analysis: Calcd. for $C_{11}H_{12}O_4$: C, 63.46; H, 5.76. Found: C, 63.78; H, 5.70.

O-propanoyl-DL-3-phenyllactic acid (ester 9): a mixture of DL-3-phenyllactic acid (5 g) and propanoyl chloride (30 ml) was refluxed for 3 h. Excess acid chloride was removed by vacuum distillation and the oily residue was titrated with 0.1 M NaOH to pH 8 in 50% aqueous acetone. Removal of solvent gave the sodium salt as a white solid which was recrystallized several times from ethyl acetate/hexane; m.p. 225—227°C. Analysis: Calcd. for $C_{12}H_{13}O_4Na$: C, 59.0; H, 5.36. Found: C, 58.67; H, 5.32.

Enzyme solutions

Carboxypeptidase A_γ (Code COA) was obtained as a toluene-preserved sus-

pension from Worthington Biochemical Corp. Dialysis and preparation of enzyme stock solutions were performed as previously described [1,14].

Kinetic Studies

The enzymic hydrolysis of each ester was followed at a series of substrate concentrations on a Radiometer Corp. pH-state at pH 7.5, 25°C, ionic strength 0.2 (NaCl), using standard 0.01 M KOH solution as titrant. Initial velocities at each substrate concentration were calculated from the recorded curves of volume of titrant against time. The data, were plotted in the form of Lineweaver-Burk plots, inspected for linearity, and fitted to the Michaelis equation by a computer program based on Wilkinson's regression method [15]. Observed velocities at pH $\geqslant$ 9.5 were corrected for non-enzymic hydrolysis of the ester, while for pH $\leqslant$ 5.5 corrections for incomplete dissociation of the acetic acid product were necessary. All solutions at pH $\leqslant$ 6.0 contained 10^{-4} M zinc nitrate to prevent dissociation of zinc ion from the enzyme [16]. Data obtained from racemic esters are reported in terms of the concentrations of the L-isomers only. We have previously established [10] that the presence of the D-isomer does not significantly influence k_{cat} and K_m for these non-specific ester substrates.

Results

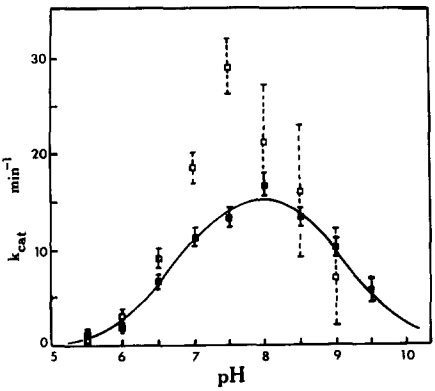
For each substrate examined, the initial velocity of enzymic hydrolysis was measured as a function of substrate concentration (8—12 points) at intervals of 0.5 pH units over as much of the range pH 5—10.5 (25°C, ionic strength 0.2) as was experimentally accessible. The pH-dependences of k_{cat} and K_m were then calculated for each of the esters investigated.

Our data for k_{cat} for *O*-acetyl-L-mandelic acid (ester 3) are compared in Fig. 1 with the data previously reported by Carson and Kaiser [3] for this ester at 25°C and ionic strength 0.55. Although there is a general similarity in k_{cat} values at most pH values, significant differences are observed at pH 7.0 and 7.5. In particular, at this latter pH our value for k_{cat} is only 50% of that reported earlier and we find that at ionic strength 0.5, k_{cat} is even smaller (see footnote p. 98). There is also a considerable difference between the K_m values observed in our work and those reported earlier for ester 3. At pH 7.5 we find K_m = 10.8 mM (ionic strength 0.2) and 12.5 mM (ionic strength 0.5) whereas Kaiser and Carson [3,11] report K_m = 60—82 mM at ionic strength 0.55. Our observed decrease in k_{cat} but little change in K_m upon going from ionic strength 0.2 to 0.5 is similar to the variation in these parameters reported previously [10] for the same ionic strength change with ester 6 as substrate.

The observed pH-dependence of k_{cat} for ester 3 in Fig. 1 has been fitted to Eqn. 1 with the parameters k_2, K_{EH_2S} and K_{EHS} being evaluated by a modification of Wilkinson's regression method [15]. The

$$k_{cat} = \frac{k_2}{1 + [H^+]/K_{EH_2S} + K_{EHS}/[H^+]} \quad (1)$$

curve in Fig. 1 has been calculated using the parameters for this ester in Table I and Eqn. 1, and gives an acceptable fit to the experimental data.



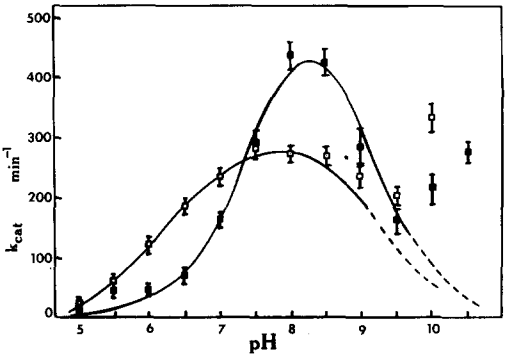


Fig. 1. The pH-dependence of k_{cat} for the hydrolysis of *O*-acetyl-L-mandelic acid (ester 3) by carboxypeptidase A at 25° C, ionic strength 0.2; (■) current data; (□) data from ref. 3 (ionic strength 0.55). Curve is calculated from Eqn. 1 using the appropriate parameters from Table I.

Fig. 2. The pH-dependence of k_{cat} for the hydrolysis of *O*-acetyl-L-3-phenyllactic acid (■) and *O*-*p*-nitrobenzoyl-L-3-phenyllactic acid (□) by carboxypeptidase A at 25° C, ionic strength 0.2. Curves are calculated from Eqn. 1 using the appropriate parameters from Table I.

The pH-dependences of k_{cat} for esters 5 and 7 are similar to that shown for ester 3 in Fig. 1 and are fitted by Eqn. 1 and the appropriate parameters from Table I. Each of the three *O*-acyl-3-phenyllactic acid substrates (esters 4, 6 and 9) display pH-dependences for k_{cat} that are similar to one another, but which differ from those for esters 3, 5 and 7 in displaying unexpectedly large values for k_{cat} in the most basic solutions investigated. For esters 4 and 6 we have evaluated k_2 and K_{EH_2S} from the data at pH $\leqslant$ 8.5, and have drawn the curves in Fig. 2 from Eqn. 1 using these values (Table I) and an assumed value for $pK_{EHS} = 9.3$.

For each of the 6 esters investigated in the current study, the pH-dependence of k_{cat}/K_m for pH $\geqslant$ 7 can be represented by Eqn. 2. Values of k_2/K_S and K_{EH} have been evaluated and are collected in Table I.

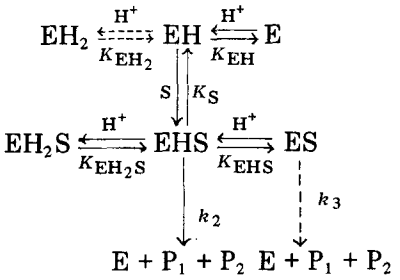
$$k_{cat}/K_m = \frac{k_2/K_s}{1 + K_{EH}/[H^+]} \qquad (2)$$

In the more acidic pH range, all of these esters show a gradual decrease in k_{cat}/K_m as the pH decreases, however, in no case is this decrease simply explicable by introducing another enzymic ionization constant. In all cases the decrease in k_{cat}/K_m with pH is far too gradual to be fitted by an enzymic ionization of $pK_{EH_2} \approx 6$.

Discussion

From the data in Table I it is clear that for each of the 6 esters investigated in the present study an enzymic acid of $pK_{EH} = 9.2 \pm 0.2$ controls productive substrate binding. For both acetate and *p*-nitrobenzoate esters, k_{cat} displays a dependence on two dissociation constants in the enzyme-substrate complex; pK_{EH_2S} varies from 6.23—7.38 and is very dependent on the particular substrate present, while pK_{EHS} is approximately 9.3 and is relatively independent of the

nature of the substrate. These observations require a minimum kinetic scheme as indicated by the solid arrows in Scheme 1. These solid arrows generate Eqns. 1 and 2. The broken arrows in Scheme 1 are the minimum necessary additions if



Scheme 1

one also attempts to account for the observed decrease in k_{cat}/K_m in acidic solutions and the enhanced values of k_{cat} in basic solutions for phenyllactic acid derivatives. However, as indicated above, the variation of k_{cat}/K_m in acid solution is more complicated than can be satisfactorily represented by a simple dissociation constant K_{EH_2}.

The general forms of pH-dependences observed for k_{cat} and K_m for *O*-acetyl-L-mandelic acid (ester 3) in the present study are similar to those previously observed for this ester [3]. However, as noted above, the absolute values that we have obtained for these parameters are considerably different from those reported earlier. Carson and Kaiser report $pK_{EH_2} = 6.9$, $pK_{EH} = 7.5$, $pK_{EH_2S} = 7.2$ and $pK_{EHS} = 7.9$ (at ionic strength 0.55) which are considerably different from the pK_a values that we find for this ester (Table I). Inspection of earlier data (Fig. 1) indicates quite large experimental errors for pH > 7.5, so that curve fitting in this region can only be considered as quite tentative. Furthermore, the values of the parameters K_{EH_2S} and K_{EHS} that are obtained upon curve fitting are very dependent on the values of k_{cat} at pH 7.0 and 7.5, and it is in this region where the greatest deviations exist between our current data and the earlier data.

In a previous report [6] from our laboratory on the hydrolysis of ester 5 the following values were reported: $pK_{EH_2} = 6.95$, $pK_{EH} = 7.9$, $pK_{EH_2S} = 7.5$, $pK_{EHS} = 8.3$. Differences between these values and the data for this ester in Table I are attributable to one or more of the following three factors: (i) the lower overall quality of the earlier data; (ii) the more limited pH range previously investigated; (iii) the presence in the solvent of 5% dimethyl sulfoxide which has been shown [6] to have considerable effect on both k_{cat} and K_m.

Based on the consistent pattern that is apparent in Table I over the 6 esters included in the present study, we are confident that our current data for the pH-dependence of the enzymic hydrolysis of both ester 3 and ester 5 are more reliable than the earlier data for these two esters. The literature data [4] for the pH-dependence of the hydrolysis of the cinnamate ester 8 are included in Table I and show the same general pattern as is observed for the other non-specific ester substrates in this table, although a discrete value for pK_{EH_2} is

TABLE I

PARAMETERS FOR pH-DEPENDENCE OF HYDROLYSIS OF NON-SPECIFIC ESTERS BY CARBOXYPEPTIDASE A

Measurements at 25°C, ionic strength 0.2. Parameters are those for Eqns. 1 and 2.

Ester	k_2 (min^{-1})	pK_{EH_2S}	pK_{EHS}	k_2/K_S ($M^{-1} \cdot min^{-1}$)	pK_{EH_2}	pK_{EH}
3	17.1 ± 1.1	6.73 ± 0.08	9.13 ± 0.08	$(1.19 \pm 0.05) \cdot 10^3$		9.29 ± 0.10
4	540 ± 40	7.38 ± 0.09	(9.3) *	$(3.3 \pm 0.1) \cdot 10^5$		9.21 ± 0.09
5	100 ± 3	6.72 ± 0.05	9.32 ± 0.06	$(7.9 \pm 0.7) \cdot 10^4$		9.11 ± 0.21
6	292 ± 11	6.28 ± 0.06	(9.3) *	$(9.8 \pm 0.6) \cdot 10^5$		9.40 ± 0.15
7	137 ± 12	6.23 ± 0.15	9.43 ± 0.18	$(1.76 \pm 0.16) \cdot 10^5$		9.47 ± 0.26
8 **	4200	6.2	approx. 9.0	$2.4 \cdot 10^7$	6.5	9.4
9	approx. 700			$(3.8 \pm 0.4) \cdot 10^4$		9.15 ± 0.22

* Value assumed for curve in Fig. 2.

** Data from ref. 4 at 25°C, ionic strength 0.5.

observable for this ester. Ester 8 also resembles the *O*-acyl-3-phenyllactic acids that we have investigated in displaying an enhanced k_{cat} value in basic solutions. It is tempting to conclude that this enhancement of k_{cat} in basic solutions is specific to *O*-acyl-3-phenyllactic acid substrates. However, it should be realized that a slight shift in this phenomenon to more basic solutions (by 0.5 pH units say) for other esters would make it difficult to observe experimentally, since non-enzymic hydrolysis of these relatively poor carboxypeptidase A substrates becomes very rapid for pH > 9.5.

Our current data demonstrate that the pK_a values that control the activity of carboxypeptidase A in the hydrolysis of esters 3 and 5 are similar to those involved in the enzymic hydrolysis of other substrates [4—8,16,21]. This removes one of the apparent anomalies which have complicated the interpretation of the hydrolytic activity of this enzyme in mechanistic terms. We had previously suggested [6] that the unusual enzymic pK_a values that appeared to control the enzymic hydrolysis of esters 3 and 5 could be used as a criterion to distinguish non-specific from specific ester substrates. Clearly, this is not the case. However, there does remain an empirical difference in the pH-dependences of the enzymic hydrolysis of specific and non-specific esters as defined above in structural terms (ester 1 and ester 2 respectively). Thus, whereas esters defined as non-specific ester substrates display bell-shaped pH profiles for k_{cat} (controlled by pK_{EH_2S} and pK_{EHS}), specific ester substrates typically display [8] a pH-independent k_{cat} over the range pH 5—10.

Consideration of pK_{EH_2} and pK_{EH}

The enzymic acid of pK_{EH} 9.2 ± 0.2 that is observed to control productive binding of non-specific ester substrates in the present study is presumably the same acid of $pK_a \approx 9$ that has been observed to control the binding to this enzyme of specific esters [5,6,8] and peptides [16,21] and also thiol esters [22]. In fact, that $pK_{EH} \approx 9$ has now been established for all carboxypeptidase A substrates for which the pH-dependence of enzymic hydrolysis has been investigated. Thus, the identification of this enzymic acid of $pK_{EH} \approx 9$, which when present in its conjugate base form prevents substrate binding to the enzyme, is

clearly of prime importance in understanding the very first step in the mechanism of carboxypeptidase A catalysis i.e. formation of the Michaelis complex.

Based on the structure of the enzyme as determined by X-ray crystallography [23,24], there seem to be only three likely candidates in the active site region for an acid $pK_a \approx 9$. These are the phenolic hydroxyl groups of Tyr-198 and Tyr-248, and the zinc-bound water molecule. Suh and Kaiser [7] have established that nitration or diazo-coupling of the aromatic ring of Tyr-248 has no effect on pK_{EH} for the enzymic hydrolysis of *p*-chlorocinnamoyl-L-3-phenyllactic acid, so that Tyr-248 can be confidently eliminated. Auld and Vallee [16,21] have noted that the acid of $pK_{EH} \approx 9$ has thermodynamic parameters that are most consistent with tyrosine ionization, although metal-bound water ionization cannot be definitely ruled out. If such a tyrosine is in the active site it must by Tyr-198. There has been a tendency to ignore the role of Tyr-198 since it is somewhat distant from the metal ion which is usually assumed to be central to the catalytic activity. The possibility should also be considered that pK_{EH} arises from an enzymic acid that is remote from the active site, but which controls substrate binding via an important conformational change in the conjugate base form of the enzyme i.e.

$$EH \underset{K_{EH}}{\overset{H^+}{\rightleftharpoons}} E \rightleftharpoons E'$$

This latter interpretation most readily rationalizes the presence of $pK_{EH} \approx 9$ for both specific ester and peptide substrates, even though there is now an impressive amount of evidence for significantly different productive binding sites for these two classes of substrate [24—28].

Unlike specific ester and peptide substrates, the binding of acetate and *p*-nitrobenzoate esters to carboxypeptidase A shows no clear dependence on an enzymic acid of $pK_{EH_2} \approx 6$, although there is a general decrease in k_{cat}/K_m for each ester in the region pH 5—6.

Consideration of pK_{EH_2S} and pK_{EHS}

While complications in the pH profiles, for k_{cat} can arise from changes in rate determining step if an acyl-enzyme intermediate is involved, there is now considerable evidence that at room temperature such is not the case for commonly studied carboxypeptidase A substrates [8,10,26,29,30]. Significantly, in the one case in which direct evidence for an acyl-carboxypeptidase intermediate is available, it is clearly shown that at room temperature deacylation of *p*-chlorocinnamoyl-carboxypeptidase A is much faster than its formation via acylation of the enzyme with *p*-chlorocinnamoyl-L-3-phenyllactic acid [9]. It thus seems that pK_{EH_2S} and pK_{EHS} refer to ionizations within the enzyme-substrate complex rather than in an acyl-enzyme intermediate, and this reasonable assumption will be made in the following discussion.

The pH profiles for k_{cat} for non-specific ester substrates are summarized in terms of pK_{EH_2S} and pK_{EHS} in Table I, and are quite different from those observed for specific ester [6,8] and peptide [16,21] substrates. Thus for peptide substrates k_{cat} is dependent on the presence of the conjugate base of an enzymic acid of $pK_{EH_2S} \approx 6.3$ but is not influenced by $pK_{EHS} \approx 9.3$. For

specific ester substrates, k_{cat} is typically pH-independent over the complete pH 5—10 range.

Presumably pK_{EH} and pK_{EHS} represent the ionization of the same enzymic acid in the free enzyme and the enzyme-substrate complex, respectively. Since pK_{EHS} does not influence k_{cat} for specific ester and peptide substrates, the enzymic acid responsible for pK_{EHS} must be masked in some way in these enzyme-substrate complexes. If pK_{EH} did in fact arise from the ionization of metal-bound water, then clearly this ionization would be effectively blocked in the enzyme-substrate complex if the substrate were coordinated to the metal ion in place of the water molecule. However, such an explanation cannot hold for both specific ester and peptide substrates in view of the apparent requirement for distinct binding sites for these two classes of substrates.

From studies of the reversible inhibition of the specific esterase activity of carboxypeptidase A [28], these is evidence for an enzymic conformational change upon the binding of specific ester substrates. If pK_{EH} is attributable to an acidic group remote from the active site, such a conformational change may result in the suppression of its ionization in the enzyme-substrate complex by its transposition from an exposed to a buried site. Alternatively, the conformational change proposed above upon formation of the pK_{EH} conjugate base in the free enzyme ($E \rightleftharpoons E'$), may no longer be energetically feasible in the enzyme-substrate complex, and consequently pK_{EHS} ionization would have no influence upon k_{cat} for specific esters. Similar arguments could be made for peptide substrates. One might expect that with the smaller non-specific ester substrates, there would be less resistance in the active site region to conformational changes promoted by a remote ionization. Although these ideas are somewhat speculative, it should be noted that there is considerable direct evidence for the conformational flexibility of carboxypeptidase A [23,24,31].

The value of pK_{EH_2S} in Table I is very dependent on the nature of both the acyl and alcohol moiety of the substrate. This pronounced dependence on substrate structure suggests that the enzymic acid associated with pK_{EH_2S} is located very close to the substrate in the enzyme-substrate complex. We have recently presented evidence that suggests that the carboxylate anions of non-specific esters and peptides bind to a common enzymic binding site, but the carboxylate group of specific ester substrates binds to a different site [10]. Such a situation is also compatable with an enzymic acid of pK_{EH_2S} 6—7 influencing the hydrolysis of non-specific ester and peptide substrates, but this acidic group not participating in specific ester hydrolysis. The enzymic acid responsible for pK_{EH_2S} is usually assumed to be the γ-carboxyl group of Glu-270, although there is little direct evidence for this assignment.

Acknowledgement

We appreciate the continued support of this work by the National Research Council of Canada. We thank Dr. Samuel S.-T. Chu for the synthesis of *O*-propanoyl-DL-3-phenyllactic acid.

References

1 Bunting, J.W. and Murphy, J. (1974) Can. J. Chem. 52, 2640—2647
2 Riordan, J.F. and Vallee, B.L. (1963) Biochemistry 2, 1460—1468

3 Carson, F.W. and Kaiser, E.T. (1966) J. Am. Chem. Soc. 88, 1212—1223
4 Hall, P.L., Kaiser, B.L. and Kaiser, E.T. (1969) J. Am. Chem. Soc. 91, 485—491
5 Auld, D.S. and Holmquist, B. (1972) Fed. Proc. Fed. Am. Soc. Exp. Biol., 31, 435 Abs.
6 Bunting, J.W., Murphy, J., Myers, C.D. and Cross, G.G. (1974) Can. J. Chem. 52, 2648—2659
7 Suh, J. and Kaiser, E.T. (1976) J. Am. Chem. Soc. 98, 1940—1947
8 Bunting, J.W. and Chu, S.S.T. (1976) Biochemistry 15, 3237—3244
9 Makinen, M.W., Yamamura, K. and Kaiser, E.T. (1976) Proc. Natl. Acad. Sci. U.S. 73, 3882—3886
10 Bunting, J.W. and Kabir, S.H. (1977) J. Am. Chem. Soc. 99, 2775—2780
11 Kaiser, E.T. and Carson, F.W. (1964) J. Am. Chem. Soc. 86, 2922—2926
12 Angus, W.R. and Owen, R.P. (1943) J. Chem. Soc. 227—230
13 McKenzie, A. and Humphries, H.B.P (1909) J. Chem. Soc. 95, 1105—1114
14 Murphy, J. (1974) Ph.D. Thesis, University of Toronto
15 Wilkinson, G.N. (1961) Biochem. J. 90, 324—332
16 Auld, D.S. and Vallee, B.L. (1971) Biochemistry, 10, 2892—2897
17 Glasoe, P.K. and Long, F.A. (1960) J. Phys. Chem. 64, 188—190
18 Bender, M.L. and Hamilton, G.A. (1962) J. Am. Chem. Soc. 84, 2570—2576
19 Bender, M.L., Clement, G.E., Kezdy, F.J. and Heck, H.d'A. (1964) J. Am. Chem. Soc. 86, 3680—3690
20 Kaiser, B.L. and Kaiser, E.T. (1969) Proc. Natl. Acad. Sci. U.S. 64, 36—41
21 Auld, D.S. and Vallee, B.L. (1970) Biochemistry 9, 4352—4359
22 Kaiser, E.T., Chan, T.W. and Suh, J. (1974) in Protein-Metal Interactions, (Friedman, M., ed.), pp. 49—80, Plenum Press, New York
23 Lipscomb, W.N., Reeke, Jr., G.N., Hartsuck, J.A., Quiocho, F.A., Bethge, P.H., Ludwig, M.L., Steitz, T.A., Muirhead, H. and Coppola, J.C., (1968) Brookhaven Symp. Biol., 21, 24—90
24 Lipscomb, W.N., Reeke, Jr., G.N., Hartsuck, J.A., Quiocho, F.A. and Bethge, P.H. (1970) Phil. Trans. R. Soc. Lond. Ser. B. 257, 177—214
25 Riordan, J.F., (1973) Biochemistry 12, 3915—3923
26 Auld, D.S. and Holmquist, B., (1974) Biochemistry, 13, 4355—4361
27 Lange, L.G., Auld, D.S. and Riordan, J.F. (1974) Biochemistry 13, 1983—1986
28 Bunting, J.W. and Myers, C.D. (1975) Can. J. Chem. 53, 1993—2004
29 Breslow, R. and Wernick, D. (1976) J. Am. Chem. Soc. 98, 259—261
30 Breslow, R. and Wernick, D.L. (1977) Proc. Natl. Acad. Sci. U.S. 74, 1303—1307
31 French, T.C., Yu, N.-T. and Auld, D.S. (1974) Biochemistry 13, 2877—2882