pH Dependence of the Hydrolysis of Hippuric Acid Esters by Carboxypeptidase A†

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ABSTRACT: The pH dependence (pH 4.5–10.5) of the hydrolysis of seven hippuric acid esters ($C_6H_5CONHCH_2CO_2CR_1R_2CO_2H$: **1a:** $R_1 = R_2 = H$; **1b:** $R_1 = R_2 = CH_3$; **1c:** $R_1 = H$, $R_2 = p\text{-}ClC_6H_4$; **1d:** $R_1 = H$, $R_2 = C_2H_5$; **1e:** $R_1 = H$, $R_2 = (CH_3)_2CHCH_2$; **1f:** $R_1 = H$, $R_2 = C_6H_5$; **1g:** $R_1 = H$, $R_2 = C_6H_5CH_2$) by bovine carboxypeptidase A has been investigated, and the pH dependence of the substrate activation of **1a–c** and the substrate inhibition of **1d–g** have been compared. For all seven esters the catalytically productive binding of the first substrate molecule depends on enzymatic pK_a values of 6.0 and 9.1. For **1d, 1e,** and **1g** the rate of hydrolysis (k_2^{app}) of this complex is pH independent, whereas for **1f** k_2^{app} depends on a pK_a of 5.9. The rate of hydrolysis (k_3^{app}) of the 1:2 enzyme–substrate complex (ES_2) is pH independent for **1d–g,** but for **1a–c** k_3^{app} depends on the following pK_a values: **1a,** 6.1 and 9.1; **1b,** 5.4; **1c,** 6.6. The pH dependences of k_2^{app} for **1f** and k_3^{app} for **1c** are rationalized by the presence of catalytically nonproductive species. Equivalent ES_2 species are believed to be productive for **1c–g;** however, the productive ES_2 species for **1b** must be quite different.

In recent investigations (Bunting and Murphy, 1974; Murphy and Bunting, 1974, 1975) we have been attempting to quantitatively describe the substrate activation and inhibition effects that complicate the analysis of the steady-state kinetics of the hydrolysis of many esters by bovine carboxypeptidase A. Esters (**1**) of hippuric acid are especially prone to these phenomena. Thus, for **1a–c,** substrate activation produces sigmoidal curves for the dependence of initial velocity of enzymatic hydrolysis on substrate concentration (Murphy and Bunting, 1974). On the other hand, if R_1 is H and R_2 is C_2H_5 or a larger hydrocarbon unit, substrate inhibition is observed for the L enantiomers of the ester substrates (**1**) (Murphy and Bunting, 1975).

$C_6H_5CONHCH_2CO_2CR_1R_2CO_2H$

1a, $R_1 = R_2 = H$
b, $R_1 = R_2 = CH_3$
c, $R_1 = H$; $R_2 = p\text{-}ClC_6H_4$
d, $R_1 = H$; $R_2 = C_2H_5$
e, $R_1 = H$; $R_2 = (CH_3)_2CHCH_2$
f, $R_1 = H$; $R_2 = C_6H_5$
g, $R_1 = H$; $R_2 = C_6H_5CH_2$

We have shown (Murphy and Bunting, 1974, 1975) that both substrate activation and substrate inhibition[1] with these hippuric acid esters is consistent with the formation of a 1:2 enzyme–substrate complex (ES_2) according to Scheme I.

Scheme I

$$\begin{array}{ccccc} E & \overset{+S}{\underset{K_1}{\rightleftharpoons}} & ES & \xrightarrow{k_2} & E + P_1 + P_2 \\ {\scriptstyle +S}\uparrow\downarrow{\scriptstyle K_2} & & {\scriptstyle +S}\uparrow\downarrow{\scriptstyle K_3} & & \\ SE & \overset{+S}{\underset{K_4}{\rightleftharpoons}} & ES_2 & \xrightarrow{k_4} & E + S + P_1 + P_2 \end{array}$$

Assuming that the equilibria are established rapidly relative to k_2 and k_3, this scheme generates eq 1 for the dependence of initial velocity (v) of enzymatic hydrolysis on the concentrations of enzyme (E) and substrate (S) provided that $S \gg E$.

$$\frac{v}{E} = \frac{\dfrac{k_2 S}{(1 + K_1/K_2)} + \dfrac{k_3 S^2}{K_3(1 + K_1/K_2)}}{\dfrac{K_1}{(1 + K_1/K_2)} + S + \dfrac{S^2}{K_3(1 + K_1/K_2)}} \quad (1)$$

$$\frac{v}{E} = \frac{k_2^{app} S + k_3 S^2/K_{SS}^{app}}{K_S^{app} + S + S^2/K_{SS}^{app}} \quad (2)$$

Equation 2 is a modified form of eq 1, and in general only the four parameters k_2^{app}, k_3, K_S^{app}, and K_{SS}^{app} can be evaluated from the experimental rate data. It is clear that substrate activation will arise from Scheme I if $k_3 > k_2^{app}$, whereas substrate inhibition will be observed for $k_3 < k_2^{app}$. Provided that K_1/K_2 is considerably less than 1, these two criteria are simply satisfied by $k_3 > k_2$, and $k_3 < k_2$, respectively. However, substrate activation can also be generated even if $k_3 < k_2$, provided that K_1/K_2 is sufficiently large that $k_3 > k_2^{app}$ ($= k_2/(1 + K_1/K_2)$). This latter condition suggests that the observation of substrate activation or inhibition is then related to the relative binding strengths of substrate to two different sites on the enzyme, rather than to a change in the relative values of k_2 and k_3 as the alcohol unit of the ester, **1,** is varied. In an earlier paper (Murphy and Bunting, 1974), considerations of relative k_3 values for a number of hippurate esters led us to postulate that both of the above substrate activation mechanisms may occur independently for individual hippurate ester substrates.

To further explore these relationships between substrate activation and inhibition, we have now measured the pH dependence of enzymatic hydrolysis for a series of seven hippurate esters: three (**1a–c**) that display substrate activation and four (**1d–g**) that display substrate inhibition. In initiating these studies, we felt that a knowledge of the pH dependence of the four parameters in eq 2 would provide further insight into the relationship between substrate activation and substrate inhibition, and would possibly allow a distinction to be drawn be-

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[1] In addition to substrate activation, the esters **1b** and **1c** also display substrate inhibition at high concentrations of substrate (Murphy and Bunting, 1974). This phenomenon has not been quantitatively analyzed and is not under consideration in the present paper.

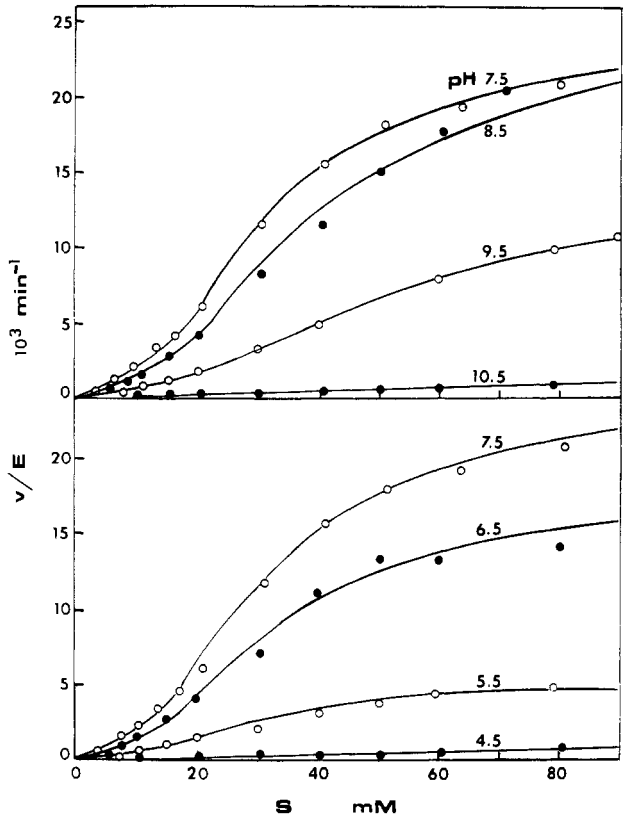


FIGURE 1: Dependence of initial velocity of enzymatic hydrolysis of **1a** on substate concentration at pH values as indicated (25 °C, ionic strength 0.5). Data for pH 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 not shown. Curves calculated as described in text.

tween the two modes of substrate activation previously postulated. At the very least, the data collected in such a study would be a valuable supplement to the relatively meager data that is currently available (Riordan and Vallee, 1963; Carson and Kaiser, 1966; Hall et al., 1969; Auld and Holmquist, 1972; Bunting et al., 1974) on the pH dependence of the esterase activity of carboxypeptidase A.

Materials and Methods

Synthetic routes to the substrates used in this study have been described previously (Murphy and Bunting, 1974, 1975). The sodium salt of *O*-hippuryl-L-3-phenyllactic acid was purchased from Bachem Inc. Fine Chemicals, Marina Del Rey, Calif., and was found to be only 75% L isomer based on complete enzymatic hydrolysis. Stock solutions of each substrate were prepared as previously described (Bunting and Murphy, 1974).

The initial velocities of enzyme-catalyzed ester hydrolysis were determined on a Radiometer pH-stat at 25 °C, ionic strength 0.5 (substrate + NaCl) by automatic titration with standard potassium hydroxide solutions. All reactions at pH $\leq$ 6.0 were carried out in the presence of 10^{-4} M zinc nitrate to counteract dissociation of the zinc ion from the enzyme (Auld and Vallee, 1971). Corrections to the apparent velocities were necessary at both low and high pH to give the true initial velocities of enzyme-catalyzed hydrolysis. At pH $\leq$ 5.5, incomplete dissociation of the carboxylic acid products results in less than a stoichiometric liberation of hydrogen ions per ester molecule hydrolyzed. From the total base uptake at complete hydrolysis at each pH, the percentage (p) of protons released upon complete ester hydrolysis can be calculated. The apparent initial velocity is then multiplied by the factor $100/p$ to give the true initial rate of hydrolysis. Typical values for p are given for *O*-hippurylglycolic acid (**1a**): pH 4.5, p = 80%; pH 5.0, p = 90%; pH 5.5, p = 94%. At pH $\geq$ 9.5, the rate of nonenzymatic base-catalyzed ester hydrolysis becomes significant relative to the rate of the enzyme-catalyzed hydrolysis. The second-order rate constant, k_{OH}, can be determined from measurements of the base-catalyzed rate of ester hydrolysis in the absence of enzyme. Subtraction of the value of $k_{OH}S$ at each substrate concentration from the apparent initial velocity then gives the true initial enzymatic rate. The corrections in basic solution were less than 50% of the observed rate for all data reported in this work.

Calculations of data for esters **1c–g** are based on the L isomer only. We have previously shown (Murphy and Bunting, 1975) that the only influence of the D isomer in these reactions is its ability to replace the L isomer at the inhibitory substrate binding site. The enzyme appears to have identical affinities for the binding of the L and D isomers at this inhibitory site. Under these circumstances, the value of K_{SS}^{app} in eq 2 that is obtained for the L isomer in the racemic mixture is exactly half of the true K_{SS}^{app} value for the L isomer alone. Thus, the K_{SS}^{app} values in Figure 7 are twice as large as the experimentally observed values for the L isomer in the racemic ester. The influence of the D isomer of **1c** is uncertain, although it is not a substrate as only 50% hydrolysis is observed at equilibrium. The reported parameters for this ester are based on the L isomer alone.

Results

The dependence of the initial velocity on substrate concentration for the hydrolysis of *O*-hippurylglycolic acid (**1a**) by carboxypeptidase A is indicated in Figure 1 as a function of pH over the range pH 4.5–10.5. At all substrate concentrations (10^{-3}–10^{-1} M) the maximum velocity is observed at pH 7.5, with a monotonic increase in velocity with increasing pH in the range pH 4.5–7.5 being followed by a monotonic decrease in velocity with further increase in pH up to pH 10.5. At all pH values in Figure 1, the dependence of v/E on S is sigmoidal. This is most obvious when the data are plotted in the form of Lineweaver–Burk plots; such plots are nonlinear and typical of substrate activation. Even at the lowest substrate concentrations investigated nonlinearity is observed, and so values of k_{cat}/K_m for the nonactivated enzymatic hydrolysis cannot be evaluated. Upper limits for k_{cat}/K_m can of course be estimated at each pH, and in all cases such limits are less than the approximate value of 6×10^4 M^{-1} min^{-1} for k_{cat}/K_m at pH 7.5 (Murphy and Bunting, 1974).

It has been shown (Murphy and Bunting, 1974) that, for substrates displaying substrate activation according to Scheme I, eq 2 reduces to eq 3 at high substrate concentrations (i.e., $k_2^{app} \ll k_3S/K_{SS}^{app}$ and $S \ll K_S^{app}$). Equation 3 may be rearranged to eq 4 and 5, which give convenient linear forms for plotting data so as to allow estimation of k_3 and the product $K_S^{app}K_{SS}^{app}$.

$$\frac{v}{E} = \frac{k_3S^2/K_{SS}^{app}}{K_S^{app} + S^2/K_{SS}^{app}} \quad (3)$$

$$\frac{E}{v} = \frac{1}{k_3} + \frac{K_S^{app}K_{SS}^{app}}{k_3S^2} \quad (4)$$

$$\frac{S^2E}{v} = \frac{S^2}{k_3} + \frac{K_S^{app}K_{SS}^{app}}{k_3} \quad (5)$$

When the higher substrate concentration data of Figure 1 are plotted according to eq 4 and 5 the ordinate intercepts are quite small and so are difficult to estimate accurately. In particular,

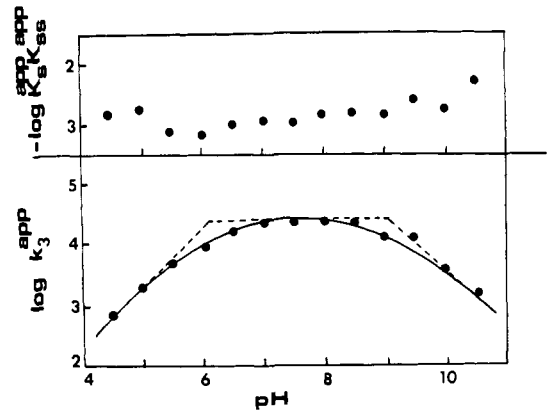


FIGURE 2: The pH dependence of log k_3^{app} and log $K_S^{app}K_{SS}^{app}$ for **1a**. Curve calculated using eq 8 with $k_3 = 2.6 \times 10^4$ min^{-1}, p$K_{EH_2S_2}$ = 6.1, and pK_{EHS_2} = 9.1.

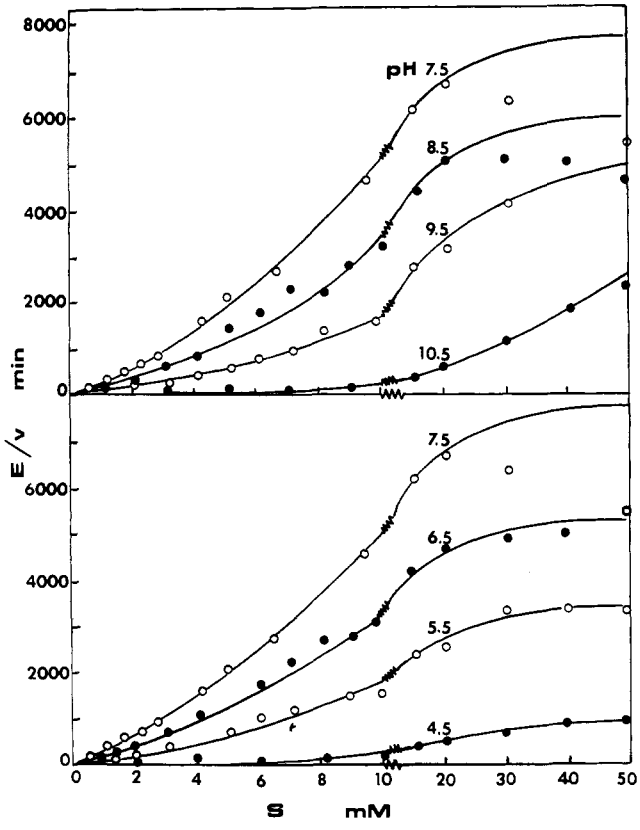


FIGURE 3: Dependence of initial velocity of enzymatic hydrolysis of **1b** on substrate concentration at pH values as indicated (25 °C, ionic strength 0.5). Data for pH 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 not shown. Curves calculated as described in text.

relatively small errors in the ordinate intercept (i.e., $1/k_3$) of eq 4 lead to quite large errors in the estimated value for k_3. We have therefore preferred to use the slopes of both linear plots ($K_S^{app}K_{SS}^{app}/k_3$) of eq 4 and $1/k_3$ of eq 5 for parameter estimation. The values of k_3 and $K_S^{app}K_{SS}^{app}$ obtained by this treatment are plotted in Figure 2. The curve through the data for k_3^{app} is based on pK_a values of 6.1 and 9.1. The curves in Figure 1 are drawn using eq 3 and the values of these parameters from Figure 2. The fit to the experimental data appears to be quite good at all substrate concentrations, and so indicates that $k_2^{app}S$ is relatively unimportant compared to k_3S^2/K_{SS}^{app}. Actually, a closer inspection of the comparison between eq 3 and the experimental data indicates that the predicted

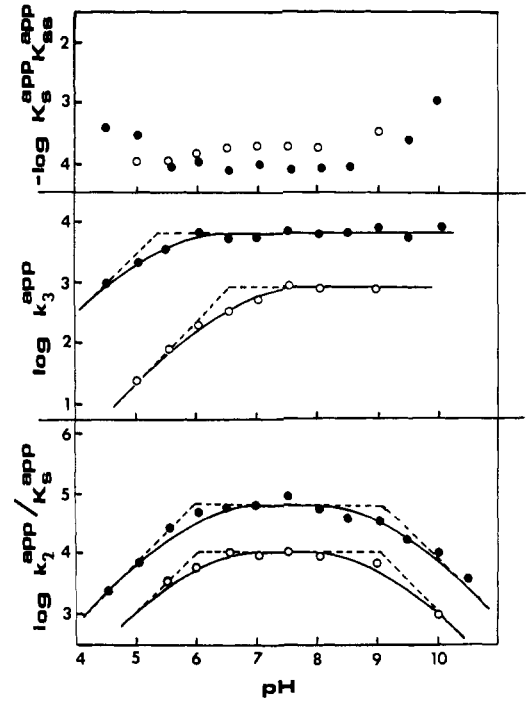


FIGURE 4: The pH dependence of log (k_2^{app}/K_S^{app}), log k_3^{app} (min^{-1}) and log $K_S^{app}K_{SS}^{app}$ for: **1b** (●) curve for k_2^{app}/K_S^{app} (M^{-1} min^{-1}) calculated using pK_{EH_2} = 6.0 and pK_{EH} = 9.1, curve for k_3^{app} calculated using eq 8 with $k_3 = 7.0 \times 10^3$ min^{-1}, pK_{EH_2S} = 5.4, pK_{EHS_2} = ∞; and for **1c** (○) curve for k_2^{app}/K_S^{app} (M^{-1} min^{-1}) calculated using pK_{EH_2} = 6.0 and pK_{EH} = 9.1, curve for k_3^{app} calculated using eq 8 with k_3 = 800 min^{-1}, p$K_{EH_2S_2}$ = 6.6, and pK_{EHS_2} = ∞.

velocity from eq 3 begins to show systematic deviations from the experimental data at substrate concentrations less than about 0.01 M, such that at S = 0.001 M the calculated velocity is only about 30% of the experimentally observed value.

The dependence of the initial velocity of enzymatic hydrolysis on substrate concentration at various pH values for the ester substrate *O*-hippuryl-2-methyllactic acid (**1b**) is shown in Figure 3. The overall dependence of velocity on pH for this ester is similar to that observed for **1a** in Figure 1. At all pH values, substrate activation at low substrate concentration is followed by substrate inhibition that becomes important at high substrate concentrations. We have not attempted a detailed analysis of this latter phenomenon because of the uncertainties that currently surround its interpretation (Murphy and Bunting, 1974). The data at substrate concentrations up to those at which the maximum velocity occurs have been fitted to eq 3 in the same way as described above for **1a**. Substrate activation for **1b** does not appear to be as pronounced as for **1a** and Lineweaver-Burk plots are linear at the lowest substrate concentrations investigated so that $k_{cat}/K_m = k_2^{app}/K_S^{app}$ for nonactivated enzymatic hydrolysis can be evaluated at each pH for **1b**. Values of k_2^{app}/K_S^{app}, k_3, and $K_S^{app}K_{SS}^{app}$ for **1b** at each pH are plotted in Figure 4. The curve fitted to the data for k_2^{app}/K_S^{app} is based on pK_a values of 6.0 and 9.1, while k_3^{app} depends on a pK_a value of 5.4. The lines in Figure 3 are based on eq 3 and the values for the parameters in Figure 4. A detailed comparison of the calculated and experimental velocities at very low substrate concentrations shows much greater deviations than for **1a**. This is a result of the observed linearity of Lineweaver-Burk plots in this region, and indicates that little ES_2 is present at these substrate concentrations.

The dependence of the initial velocity of enzymatic hy-

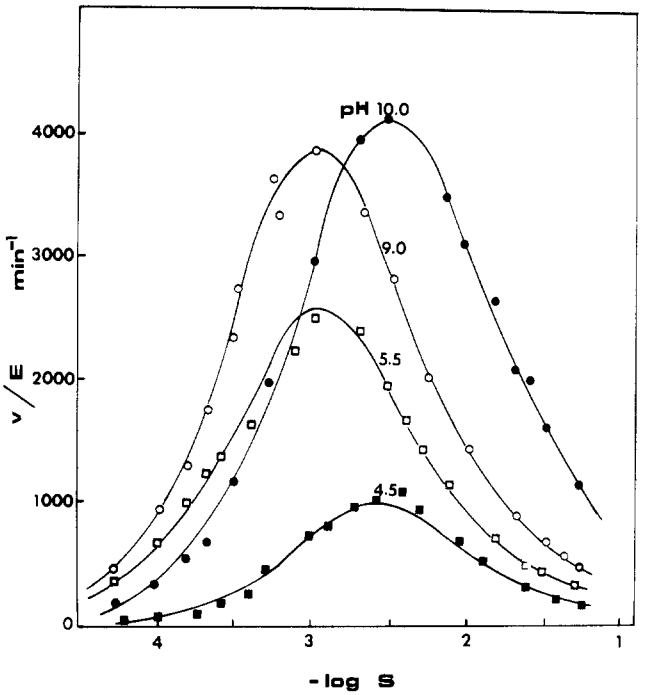


FIGURE 5: Dependence of initial velocity of enzymatic hydrolysis of **1d** on substrate concentration (as log S) at pH values as indicated (25 °C, ionic strengths 0.5). Data for pH 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.5, 10.5 not shown. Curves calculated as described in text.

drolysis on substrate concentration at various pH values for *O*-hippuryl-L-*p*-chloromandelic acid (**1c**) is similar to that shown for **1b** in Figure 3. The general influence of pH is similar to that observed for **1a** and **1b;** however, a shorter pH range is observable because of the smaller velocities of hydrolysis of **1c.** Above pH 9, the nonenzymatic base-catalyzed hydrolysis becomes rapid relative to the enzymatic hydrolysis, and it is difficult to obtain accurate rates of enzymatic hydrolysis at most substrate concentrations for pH > 9. Values of k_2^{app}/K_S^{app}, k_3, and $K_S^{app}K_{SS}^{app}$ were evaluated as described above and are plotted in Figure 4. The curve through the data for k_2^{app}/K_S^{app} is based on pK_a values of 6.0 and 9.1, while k_3^{app} depends on a pK_a value of 6.6.

The pH dependences of substrate inhibition in the hydrolysis of L-2-hippuroxybutanoic acid (**1d**), L-2-hippuroxyisocaproic acid (**1e**), *O*-hippuryl-L-mandelic acid (**1f**), and *O*-hippuryl-L-3-phenyllactic acid (**1g**) are similar, and the data for **1d** in Figure 5 are typical. The data on each curve in Figure 5, and all related sets of data were fitted to eq 1 by the iteration procedure that has been previously described (Bunting and Murphy, 1972). In the case of the mandelic acid derivative (**1f**), unique sets of parameters could not be obtained at pH > 7.5 by fitting the experimental data to eq 2. This problem has also been encountered previously, and the reasons for multiple solutions have been discussed (Murphy and Bunting, 1974, 1975). In such cases only values for k_2^{app}/K_S^{app} can be confidently estimated.

Values of k_2^{app}/K_S^{app} for each of **1d–1g** are plotted as a function of pH in Figure 6. It is clear that the same pH dependence is observed for each of these esters and for **1b** and **c** (Figure 4), with two enzymatic acid dissociation constants controlling the binding of each substrate. Each curve in Figure 6 is based on pK_a values of 6.0 and 9.1, which give satisfactory agreement between the calculated curve and experimental data for each ester.

The pH dependences of k_2^{app}, k_3^{app}, and K_{SS}^{app} for **1d–g**

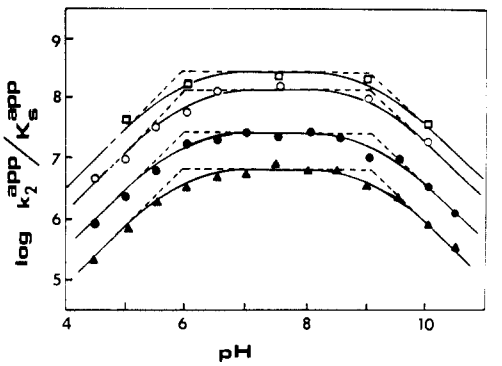


FIGURE 6: The pH dependence of log (k_2^{app}/K_S^{app}) for **1d** (●), **1e** (○), **1f** (▲), and **1g** (□). Curves calculated using pK_{EH_2} = 6.0, and pK_{EH} = 9.1. (Units k_2^{app}/K_S^{app}: M^{-1} min^{-1}).

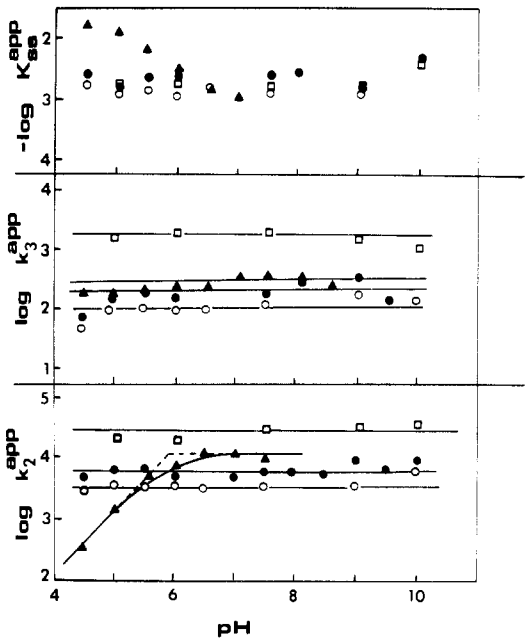


FIGURE 7. The pH dependence of log k_2^{app} (min^{-1}), log k_3^{app} (min^{-1}), and log K_{SS}^{app} for **1d** (●), **1e** (○), **1f** (▲), and **1g** (□). Curve for k_2^{app} for **1f** depends on p$K_{EH_2S_2}$ = 5.9.

are compared in Figure 7. For each of these esters k_3^{app} appears to be pH independent over the entire pH range investigated. For three of these esters, k_2^{app} is also pH independent; however, the mandelic acid derivative (**1f**) is unusual, and displays a dependence on the conjugate base form of an acidic group of pK_a 5.9. Since it is not possible to unequivocally evaluate k_2^{app} at pH > 7.5 for this ester, it is not possible to determine whether a group of p$K_2 \sim 9$ may also be important in controlling k_2^{app} for this ester. The various pK_a values derived in this work for the esters **1a–g** are collected in Table I.

In Table II, the pH independent k_3 values obtained from curve-fitting in Figures 2, 4, and 7 are compared with the corresponding second-order rate constants for the nonenzymatic base-catalyzed hydrolysis of the same esters. Comparison of the k_3/k_{OH} ratios gives a direct estimate of the catalytic efficiencies of the various ES_2 species in ester hydrolysis.

Discussion

It is clear from Table I that two enzymatic acid ionizations control the binding of the first substrate molecule to car-

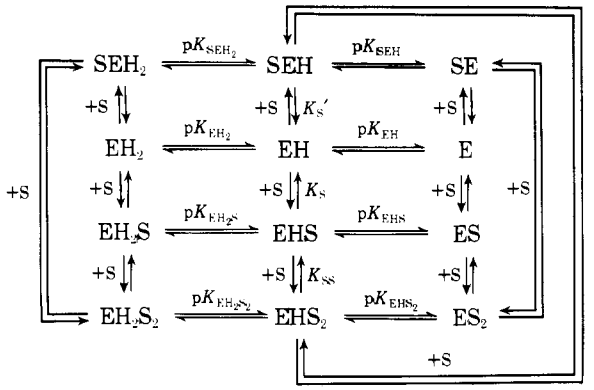
TABLE I: pH Dependence of the Hydrolysis of Hippuric Acid Esters (**1**) by Carboxypeptidase A.[a]

Ester	k_2^{app}/K_S^{app}		k_2^{app}		k_3^{app}	
	pK_{EH_2}	pK_{EH}	pK_{EH_2S}	pK_{EHS}	$pK_{EH_2S_2}$	pK_{EHS_2}
1a	6.0[b]	9.1[b]	[c]	[c]	6.1	9.1
1b	6.0	9.1	[c]	[c]	5.4	
1c	6.0	9.1	[c]	[c]	6.6	[c]
1d	6.0	9.1	pH independent		pH independent	
1e	6.0	9.1	pH independent		pH independent	
1f	6.0	9.1	5.9	[c]	pH independent	
1g	6.0	9.1	pH independent		pH independent	

[a] At 25 °C, ionic strength 0.5. [b] From $k_3^{app}/K_S^{app}K_{SS}^{app}$; see text. [c] Not experimentally observable.

boxypeptidase A for each hippurate ester. This suggests that Scheme I should be modified to include three ionization states of each enzyme species. Scheme II is the most general case of

Scheme II



$$EHS \xrightarrow{k_2} EH + P_1 + P_2$$

$$EHS_2 \xrightarrow{k_3} EH + S + P_1 + P_2$$

such a situation, where H represents a proton and the relative charges of the various species are omitted for the sake of clarity. This scheme is based on the assumption that only one of each of the 1:1 and 1:2 enzyme-substrate complexes can react to produce hydrolysis products. On the assumption that all equilibria (both substrate binding and ionization) are established rapidly relative to k_2 and k_3, Scheme II generates the rate eq 6, where $\alpha_{EH_2} = 1 + ([H^+]/K_{EH_2}) + (K_{EH}/[H^+])$, etc.

$$\frac{v}{E} = \frac{\dfrac{k_2 S}{\alpha_{EH_2S} + \dfrac{K_S}{K_S'}\alpha_{SEH_2}} + \dfrac{k_3 S^2}{K_{SS}\left(\alpha_{EH_2S} + \dfrac{K_S}{K_S'}\alpha_{SEH_2}\right)}}{\dfrac{K_S \alpha_{EH_2}}{\alpha_{EH_2S} + \dfrac{K_S}{K_S'}\alpha_{SEH_2}} + S + \dfrac{S^2 \alpha_{EH_2S_2}}{K_{SS}\left(\alpha_{EH_2S} + \dfrac{K_S}{K_S'}\alpha_{SEH_2}\right)}} \quad (6)$$

At constant pH, eq 6 has the same form as eq 2, with the four parameters being described by eq 7-10.

$$k_2^{app} = \frac{k_2}{\alpha_{EH_2S} + \dfrac{K_S}{K_S'}\alpha_{SEH_2}} \quad (7)$$

$$k_3^{app} = \frac{k_3}{\alpha_{EH_2S_2}} \quad (8)$$

TABLE II: Comparison of k_3 and k_{OH} for Hydrolysis of Hippuric Acid Esters (**1**).

Ester	k_3 (min^{-1})	k_{OH} $M^{-1}\,min^{-1}$	k_3/k_{OH} (M)
1a	2.6×10^4	129[a]	200
1b	7.0×10^3	1.4[a]	5000
1c	800	102[a]	7.8
1d	220	25[a]	8.8
1e	110	14[b]	7.9
1f	300	71[a]	4.2
1g	1800	30[b]	60

[a] From Murphy and Bunting (1974). [b] This work.

$$K_S^{app} = \frac{K_S \alpha_{EH_2}}{\alpha_{EH_2S} + \dfrac{K_S}{K_S'}\alpha_{SEH_2}} \quad (9)$$

$$K_{SS}^{app} = \frac{K_{SS}\left(\alpha_{EH_2S} + \dfrac{K_S}{K_S'}\alpha_{SEH_2}\right)}{\alpha_{EH_2S_2}} \quad (10)$$

Of the four parameters, only k_3^{app} depends on the ionization constants of only one of the four types of enzyme-substrate species in Scheme II. Thus, k_3^{app} is the only single parameter whose pH dependence can in general be used to define catalytically important enzymic ionization constants ($K_{EH_2S_2}$ and K_{EHS_2}).

There are, however, two combinations of parameters that may be used to define α_{EH_2} (i.e., K_{EH_2} and K_{EH}). Thus, $k_2^{app}/K_S^{app} = k_2/(K_S\alpha_{EH_2})$ and $k_3^{app}/(K_S^{app}K_{SS}^{app}) = k_3/(K_S K_{SS}\alpha_{EH_2})$. Now, k_2^{app}/K_S^{app} is of course equivalent to k_{cat}/K_m for the binding of the first substrate molecule to the enzyme, and the pH dependence of this ratio reflects the ionization constants of enzymatic functional groups that control the binding of the substrate molecule in the catalytically productive k_2 binding site. The pH dependence of this ratio (Table I) gives a clear indication that the same two enzymatic functional groups ($pK_{EH_2} = 6.0$ and $pK_{EH} = 9.1$) control the binding of the first molecule of each ester in its catalytically productive mode. The pH dependence of $k_3^{app}/K_S^{app}K_{SS}^{app}$ is indicated in Figure 8 for three substrates and is typical of all seven esters. This function allows both an alternative evaluation of pK_{EH_2} and pK_{EH} for these esters and also gives values for these pK_a values for **1a** as substrate, for which k_2^{app}/K_S^{app} is not directly accessible (see Results). Figure 8 confirms that for **1a** productive binding of the first substrate molecule is controlled by the same pK_a values that are found for the other esters. The agreement between pK_{EH_2} and pK_{EH} evaluated in

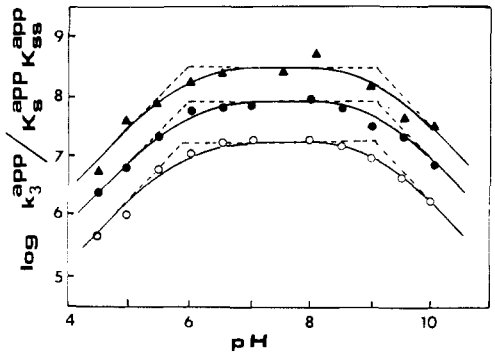


FIGURE 8: Dependence of log ($k_3^{app}/K_S^{app}K_{SS}^{app}$) for **1a** (●), **1b** (○), and **1d** (▲). Each curve calculated using $pK_{EH_2} = 6.0$ and $pK_{EH} = 9.1$.

these two different ways is satisfying, and gives further confidence in the interpretation of the current data in terms of Scheme II.

The pH independence of k_2^{app} for **1d, 1e,** and **1g** (Figure 7) suggests that α_{EH_2S} and $K_S\alpha_{SEH_2}/K_S'$ are not pH dependent in the pH range investigated. For α_{EH_2S}, this requires that K_{EH_2S} and K_{EHS} should lie outside the range pH 5–10, and $\alpha_{EH_2S} = 1$ at all pH values in this range. The value of $K_S\alpha_{SEH_2}/K_S'$ may also be pH independent because K_{SEH_2} and K_{SEH} lie outside this same pH range, or alternatively if $K_S \ll K_S'$, then $K_S\alpha_{SEH_2}/K_S'$ may be too small relative to α_{EH_2S} at all pH values to make a significant contribution to k_2^{app}. This latter condition of course implies that productive binding of substrate to enzyme to give EHS is much stronger than nonproductive binding to give SEH, and is consistent with approximately ordered binding of successive substrate molecules to the catalytically productive and inhibitory substrate binding sites on the enzyme. Such approximately ordered binding of two substrate molecules has previously been established at pH 7.5 for hippurate esters that display substrate inhibition (Murphy and Bunting, 1975).

The pH profile for k_2^{app} for *O*-hippuryl-L-mandelic acid (**1f**) showing a dependence on the conjugate base of an enzymatic acid of pK_a 5.9 is unexpected in the light of the pH independence of k_2^{app} for the esters **1d, e,** and **g.** It should be noted that for **1f,** K_S^{app} is larger than for any of these other three esters, and at pH 7.5, $K_S^{app} \approx K_{SS}^{app}$. Thus, for **1f** it seems likely that the inequality $K_S \ll K_S'$ may not hold and so the contribution of $K_S\alpha_{SEH_2}/K_S'$ to k_2^{app} may not be insignificant as suggested above for **1d, e,** and **g.** Assuming that $\alpha_{EH_2S} = 1$ by analogy with these other esters, the equation for k_2^{app} for **1f** then becomes

$$k_2^{app} = \frac{k_2}{1 + \frac{K_S}{K_S'}\alpha_{SEH_2}} \quad (11)$$

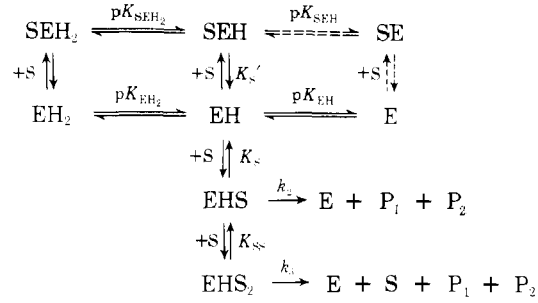
For pH $\leq$ 7.5, which is the only region in which reliable data are obtainable for k_2^{app} for **1f,** this reduces to

$$k_2^{app} = \frac{k_2}{1 + \frac{K_S}{K_S'}\left(1 + \frac{[H^+]}{K_{SEH_2}}\right)} = \frac{k_2/(1 + K_S/K_S')}{1 + [H^+]/K_{SEH_2}^{app}} \quad (12)$$

where $K_{SEH_2}^{app} = K_{SEH_2}(1 + K_S'/K_S)$. This equation predicts the observed pH dependence of k_2^{app} on pH with the observed pK_a being equivalent to $pK_{SEH_2}^{app}$. It is significant that this unusual pH dependence of k_2^{app} for **1f** is not also reflected in k_3^{app}. Thus, k_3^{app} is pH independent for **1f** as is also the case for the other esters in Figure 7.

In the light of the experimentally observed influence of pH on the parameters for the enzymatic hydrolysis of the esters **1d–f,** Scheme II may be modified as in Scheme III. The ob-

Scheme III



served pH independence of k_2^{app} and k_3^{app} indicates that the groups responsible for pK_{EH_2S}, pK_{EHS}, $pK_{EH_2S_2}$, and pK_{EHS_2} in Scheme II are catalytically inessential to k_2 and k_3. Of the four esters displaying substrate inhibition in the present study, the species SEH_2 and SEH are only important for the case of **1f.** The necessity for the inclusion of SE could not be determined in the present study because of the difficulty of establishing unique parameters for **1d** at pH > 7.5.

Scheme III indicates that no catalytically important ionizations occur in the catalytically productive EHS and EHS_2 species over the pH range examined in this work. This indicates that the pK_{EH_2} and pK_{EH} ionizations in the free enzyme are suppressed in EHS and EHS_2 by the presence of the substrate molecule(s). This could result either from a direct physical interaction of the substrate with the enzymatic functional groups that undergo these ionizations, or by the substrate molecules causing a sufficiently large steric or medium effect on these ionizations to result in the displacement of the pK_a values outside of the experimentally observed pH range. Alternatively, the pK_{EH_2} and pK_{EH} ionizations may not appear in k_2 and k_3 because these ionizations occur in regions of EHS and EHS_2 that are relatively remote from the reaction site. This latter interpretation would then suggest that the enzymatic ionizations associated with pK_{EH_2} and pK_{EH} actually control conformational changes in the enzyme prior to substrate binding, rather than actually occurring within the active site region to which the substrate directly binds.

The binding of peptide substrates to carboxypeptidase A has also been shown to be controlled by two enzymatic functional groups of $pK_a \sim 6$ and ~ 9, and the pH profiles for k_2^{app}/K_S^{app} in Figures 4 and 6 are identical in shape to those reported for k_{cat}/K_m for peptides (Auld and Vallee, 1970, 1971). Similar pK_a values have also been reported to control the binding of larger ester substrates of the type Cbz-$(Gly)_n$-L-*O*-Phe (Auld and Holmquist, 1972). It therefore seems clear that the same functional groups on the enzyme control the binding of peptides and related ester substrates. Definitive assignments of these two pK_a values to specific enzymatic functional groups are not possible at the present time (see Bunting et al., 1974, for an analysis of this problem). In view of the evidence that has recently been accumulating for different productive binding sites on this enzyme for ester and peptide substrates (Riordan, 1973; Auld and Holmquist, 1974; Lange et al., 1974; Bunting and Myers, 1975), it is somewhat curious that the same pK_a values should control the binding of both classes of substrate. In this regard, the possibility cannot be dismissed that these pK_a values actually arise from functional groups that control a crucial enzymatic conformational change that is associated with substrate binding.

The pH dependence of k_{cat} for peptide substrates displays

the requirement for the conjugate base of an enzymatic acid of $pK_2 \sim 6.1$ (Auld and Vallee, 1970, 1971) and is formally similar to that shown in Figure 7 for k_2^{app} for **1f.** In view of the above analysis of the unusual k_2^{app}–pH profile for **1f** in terms of nonproductive binding of the first substrate molecule, the question arises whether a similar situation may not also be the case for peptides. Thus, the $pK_a \sim 6.1$ that influences k_{cat} for peptides may not necessarily arise, as has generally been assumed from a species that takes a direct chemical role in the mechanism of peptide hydrolysis.

A further interpretation of the pH independent k_2^{app} and k_3^{app} values of Figure 7 is also possible. Suppose a relatively long-lived intermediate is present in these enzymatic hydrolyses, such that the decomposition of this intermediate is rate determining and is not influenced by any enzymatic functional groups with a pK_a value between 5 and 10. Then, even if the groups responsible for pK_{EH_2S} (or $pK_{EH_2S_2}$) and pK_{EHS} (or pK_{EHS_2}) in Scheme II are involved in the formation of such an intermediate, they would not be kinetically apparent, provided that formation of this hypothetical intermediate was faster than its decomposition to the ultimate hydrolysis products at all pH values. Such a situation would arise if the conjugate base of the pK_{EH_2S} (or $pK_{EH_2S_2}$) ionization were to nucleophilically attack the carbonyl group of the ester resulting in displacement of the alcohol unit and formation of an acyl-enzyme intermediate. Provided that deacylation were much slower than acylation, pK_{EH_2S} (or $pK_{EH_2S_2}$) would not be kinetically apparent in k_2^{app} (or k_3^{app}). A detailed theoretical derivation of the kinetic consequences of the pH dependence of reactions involving an acyl-enzyme intermediate has been provided on a number of occasions for the case of formation of only 1:1 enzyme–substrate complexes (Peller and Alberty, 1959; Krupka and Laidler, 1960; Alberty and Bloomfield, 1963; Stewart and Lee, 1967; Kaplan and Laidler, 1967).

Auld and Holmquist (1974) have established that, for the ester substrate Dns-(Gly)$_3$-L-*O*-Phe, the steady-state K_m parameter is the true binding constant, K_S. Such a result is inconsistent with rate-determining decomposition of an acyl-enzyme intermediate, since in this case K_m is predicted to be considerably less than K_S. In view of the structural resemblence between the present substrates (especially **1g**) and the above ester, it seems reasonable to also rule out the presence of rate-determining decomposition of acyl-enzyme intermediates in the current study. Furthermore, since k_2^{app} and k_3^{app} vary tenfold between **1e** and **g,** these parameters cannot reflect rate-determining decomposition of the same hippuryl-enzyme intermediate.

The above arguments also rule out the rate-determining decomposition of other possible (ES)′ species (e.g., tetrahedral intermediates). Such acyl-enzymes, tetrahedral intermediates, etc., may be important intermediates in the enzyme-catalyzed reaction, but the above considerations require that the formation of such an intermediate should be rate determining. The pH independence of both k_2^{app} and k_3^{app} for the hippurate esters **1d–g,** (allowing for the exception noted above for k_2^{app} for **1f**) then suggests that any enzymatic functional group in EHS that is involved in the hydrolysis of these esters does not have a pK_a value in the range 5–10.

The pH independence of k_3^{app} for those substrates that display only substrate inhibition is in marked contrast to the observed pH dependences of k_3^{app} for those substrates that display substrate activation. According to eq 8 derived from Scheme II, k_3^{app} depends only on $\alpha_{EH_2S_2}$ and based on this scheme the experimental pK_a values that are observed to control k_3^{app} reflect ionizations of enzymatic functional groups in the catalytically productive EHS_2 species. Thus, the observation of catalytically important pK_a values for the reaction of EHS_2 in the case of **1a–c,** but the absence of such values for **1d–g** suggests that the EHS_2 species that are responsible for substrate activation are not equivalent to the EHS_2 species that lead to substrate inhibition. Furthermore, the variety of pH dependences observed for k_3^{app} among **1a, 1b,** and **1c** suggests that substrate activation may also arise from more than one type of EHS_2 species. It is possible that the $pK_{EH_2S_2}$ values of 6.1, 5.4, and 6.6 observed to control k_3^{app} for **1a–c,** respectively, may all arise from the same enzymatic acid whose pK_a is influenced by the particular alcohol unit that is present in the substrate.

The unusually large k_3/k_{OH} ratio for **1b** relative to the other hippurate esters in Table II also suggests that the EHS_2 species for **1b** is not equivalent to the analogous kinetically important species for the other esters. In fact, the k_3/k_{OH} ratios for the three cases of substrate activation show a considerably wider variation than for the four esters that display substrate inhibition.

The similar k_3/k_{OH} ratios for **1c** and those esters displaying substrate inhibition had earlier led us to postulate (Murphy and Bunting, 1974) that equivalent EHS_2 species were involved in the phenomena of substrate activation for **1c** and substrate inhibition for **1d–g.** Based on Schemes II or III, this hypothesis would require that k_3^{app} for **1c** should show a similar pH dependence to k_3^{app} for **1d–g,** which is at variance with the observation that k_3^{app} depends on a pK_a of 6.6 for **1c,** whereas this parameter is pH independent for **1d–g.** However, this difference in pH dependence for k_3^{app} is similar to the variation discussed above for the pH dependence of k_2^{app} for **1f** relative to **1d, 1e,** and **1g,** and which was reconciled in terms of the formation of significant amounts of the nonproductive SEH_2 and SEH species for **1f.** Now, if Scheme III were modified to allow the inclusion of the nonproductive 1:2 enzyme–substrate complexes, S_2EH_2 and S_2EH and S_2E formed by addition of a second nonproductively bound substrate molecule to SEH_2, SEH, and SE, then the dependence of the initial steady-state velocity on substrate concentration would be expressed by eq 13.

$$\frac{v}{E} = \frac{\dfrac{k_2 S}{1 + \dfrac{K_S}{K_{S'}}\alpha_{SEH_2}} + \dfrac{k_3 S^2}{K_{SS}\left(1 + \dfrac{K_S}{K_{S'}}\alpha_{SEH_2}\right)}}{\dfrac{K_S \alpha_{EH_2}}{1 + \dfrac{K_S}{K_{S'}}\alpha_{SEH_2}} + S + \dfrac{S^2\left(1 + \dfrac{K_S K_{SS}}{K_{S'}K_{SS'}}\alpha_{S_2EH_2}\right)}{K_{SS}\left(1 + \dfrac{K_S}{K_{S'}}\alpha_{SEH_2}\right)}} \tag{13}$$

For **1c** pK_{S_2EH} is not important in the region in which data is available for k_3^{app}, and so

$$k_3^{app} = \frac{k_3 \Big/ \left(1 + \dfrac{K_S K_{SS}}{K_{S'}K_{SS'}}\right)}{1 + \dfrac{[H^+]}{K_{S_2EH_2}^{app}}} \tag{14}$$

where $K_{S_2EH_2}^{app} = K_{S_2EH_2}(1 + (K_S K_{SS}/K_{S'}K_{SS'}))$.

Clearly, if $K_S K_{SS}/K_{S'}K_{SS'} \ll 1$, k_3^{app} will appear to be pH independent (see eq 13). However, if this inequality does not hold, k_3^{app} will appear to depend on $pK_{S_2EH_2}^{app}$. Thus, equivalent productive EHS_2 species may lead to a pH independent k_3^{app} (for **1d–g**) when this inequality holds, but to a pH-dependent k_3^{app} for **1c,** when $K_S K_{SS}/K_{S'}K_{SS'} \ll 1$ is not the case. Actually, for **1c** it is known that $K_S/K_{S'} \gg 1$ (Murphy and

Bunting, 1974), so that K_{SS}/K_{SS}' may be considerably less than 1 and yet still lead to $K_S K_{SS}/K_S' K_{SS}'$ being significant relative to 1. Thus, the presence of even small amounts of S_2EH_2 and S_2EH relative to the concentration of EHS_2 may lead to a dependence on $pK_{S_2EH_2}^{app}$ for k_3^{app}. It is clear that $(1 + K_S K_{SS}/K_S' K_{SS}')$ cannot be very much greater than 1 for **1c,** otherwise the apparent k_3/k_{OH} ratio would be much less than the range of values observed for **1d–g.**

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Pre-Steady-State Kinetic Evidence for a Cyclic Interaction of Myosin Subfragment One with Actin during the Hydrolysis of Adenosine 5′-Triphosphate

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ABSTRACT: A single cycle of adenosine 5′-triphosphate (ATP) hydrolysis by a complex of actin and myosin subfragment one (acto-S-1) was studied in a stopped-flow apparatus at low temperature and low ionic strength, using light scattering to monitor the interaction of S-1 with actin and fluorescence to detect the formation of fluorescent intermediates. Our results show that the addition of a stoichiometric concentration of ATP to the acto-S-1 causes a cycle consisting of first, a rapid dissociation of the S-1 from actin by ATP; second, a slower fluorescence change in the S-1 that may be related to the initial phosphate burst; and third, a much slower rate limiting recombination of the S-1 with actin. This latter step equals the acto-S-1 steady-state adenosine 5′-triphosphatase (ATPase) rate at both low and high actin concentrations, and like the steady-state ATPase levels off at a V_{max} of 0.9 s^{-1} at high actin concentration. Therefore, the release of adenosine 5′-diphosphate and inorganic phosphate is not the rate-limiting step in the acto-S-1 ATPase. Rather, a slow first-order step corresponding to the previously postulated transition from the refractory to the nonrefractory state precedes the rebinding of the S-1 to the actin during each cycle of ATP hydrolysis.

It is now generally accepted that contraction of skeletal muscle is caused by the relative sliding of the thick myosin filaments past the thin actin filaments, and considerable evidence suggests that this sliding process is driven by the cyclic interaction of cross bridges extending from the myosin filament with actin and ATP (Huxley, 1969; Huxley, 1974). Clearly in vivo, by its very nature, this interaction must involve not only the association but also the dissociation of the cross bridge from actin during each cycle of ATP hydrolysis. The first evidence that dissociation and reassociation of the actin and myosin occurred each time ATP was hydrolyzed in vitro came from the work of Lymn and Taylor (1971; Taylor, 1972). Their pre-steady-state experiments showed that when ATP was added to a complex of actin and heavy meromyosin (HMM), dissociation of the actin–HMM complex by ATP occurred before the P_i burst, or before the initial burst of ATP hydrolysis on the HMM head. On this basis, they proposed the cycle shown in Scheme I for ATP hydrolysis (where A and M represent actin and myosin, and T, D, and P_i represent ATP, ADP, and phosphate, respectively). The major rate-limiting step in this cycle was a relatively slow release of products occurring

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‡ Abbreviations used are: S-1, myosin subfragment-one; acto-S-1, a complex of actin with S-1; HMM, heavy meromyosin; ATP, adenosine 5′-triphosphate; P_i, inorganic phosphate; OD, optical density.