Effect of pH on the Interaction of Benzoate and D-Amino Acid Oxidase†

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ABSTRACT: The kinetic and equilibrium dissociation constants of the reversible binding of benzoate to hog kidney D-amino acid oxidase (DAAO) were studied at 19 °C over the pH range 5.3–10.5 by means of a stopped-flow apparatus and spectrophotometric titrations. A simple bimolecular reaction of the form second order–first order was observed; a two-step reaction was not seen. Analysis of the pH dependence of the bimolecular rate constants and equilibrium dissociation constants is consistent with three ionizable groups which are important for benzoate binding. The pK values of the enzyme-related ionizations are 6.3, 9.2, and 9.6. Analysis of the change in extinction coefficient at 360 nm indicates the pK of 9.6 can be assigned to the 3-imino group of the enzyme-bound flavin. The effect of benzoate on the apparent pK for the ionization of the 3-imino group of the enzyme-bound FAD has been reexamined. The presence of benzoate causes an apparent shift of this ionization from a pK value of 9.6 to 10.7.

The participation of some basic residue in the binding of substrates or competitive inhibitors to D-amino acid oxidase (DAAO)[1] has long been implicated by the finding that a substrate carboxylate group is essential for binding with the protein (Yagi, 1962; Dixon and Kleppe, 1965; Massey and Ganther, 1965). The existence of a positively charged group in the enzyme close to the isoalloxazine ring of the FAD prosthetic group has also been proposed to explain the lower pK found for the ionization of the 3-imino group of FAD bound to the enzyme than for free FAD (Massey and Ganther, 1965). This same group has also been proposed to be responsible for the primary binding of sulfite to the enzyme, leading to a facilitation of the nucleophilic attack of sulfite to the N(5) position of the flavin (Massey et al., 1969; Müller and Massey, 1969). From the pH profile of sulfite addition, a pK of 8.3 was proposed for this group. As the equilibrium addition of sulfite to the N(5) position of the flavin was found to be reversed by the addition of the competitive inhibitor, benzoate, it seems reasonable to assume that all of these phenomena are dependent on the presence of the same positively charged enzyme group.

Interest in the reaction mechanism of flavoprotein oxidases has been renewed considerably by the recent findings that β-chloro-substituted substrates will undergo anaerobic elimination reactions with several enzymes, including D- and L-amino acid oxidases (Walsh et al., 1971, 1973a; Voet et al., 1972) and lactate oxidase (Walsh et al. 1973b). The reactions suggest that an early step in enzyme catalysis is the abstraction of a proton from the α-carbon atom of the substrate by a protein base. The findings of deuterium-isotope effects in the rate of anaerobic reduction of the enzyme and in catalytic turnover using α-deuterated substrates are also consistent with this concept (Yagi et al., 1970, 1973, 1974; Walsh et al., 1973a). In studies with D-amino acid oxidase of the intramolecular transfer of ^{3}H between the α carbon of β-chloroalanine and the β-carbon atom of the product pyruvate, the degree of transfer suggested strongly that the protein base contained only a single proton in its protonated form, and was therefore likely to be a histidinyl residue (Walsh et al. 1973a). Previous kinetic studies with L-amino acid oxidase also suggested a histidinyl residue as being important in proton abstraction from substrate (Page and Van Etten, 1971). As it would seem unlikely that the same base would be involved both in substrate binding (via the carboxylate group of the substrate) and in proton abstraction from the α-carbon atom, it was therefore of interest to determine if more than one protein ionization could be detected as important in binding of the competitive inhibitor, benzoate. Accordingly, a study was made of the strength of binding of benzoate to D-amino acid oxidase as a function of pH. A reinvestigation was also made of the effect of benzoate binding on the ionization of the 3-imino group of the enzyme-bound FAD.

Experimental Procedure

Materials. D-Amino acid oxidase (DAAO; D-amino acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3) was isolated as the benzoate complex from hog kidney and subsequently converted to the benzoate-free holoenzyme by the method of Brumby and Massey (1966). Purified enzyme was stored in 0.02 M potassium pyrophosphate, pH 8.50, at −20 °C. All reagents were commercial reagent-grade chemicals and were used without further purification. Water, doubly distilled in an all-glass apparatus, was used for all solutions.

Methods. Spectroscopic measurements were made at 19 °C with a Cary 17 or 118 spectrophotometer equipped with thermostated cell compartments. The concentration of DAAO was estimated from the absorbance at 460 nm and the determined extinction coefficient of 11 150 M^{-1} cm^{-1} at pH 8.50, 19 °C (Massey and Ganther, 1965).

To obtain benzoate difference spectra, 0.90 mL of an enzyme solution (2–5 × 10^{-5} M) was added to each of two matched 1-mL quartz cells. The cells were placed in the Cary spectrophotometer and, at thermal equilibrium, the base line

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[1] Abbreviations used are: Bz, benzoate anion; BzH, benzoic acid; DAAO, D-amino acid oxidase; $[P]_0$ and $[Bz]_0$, initial concentrations of all forms of D-amino acid oxidase and benzoate; [P], [Bz], [PBz], equilibrium concentrations of the corresponding forms; [EH] and [EHBz], equilibrium concentrations of the appropriate enzyme forms and complex; FAD, flavin adenine mononucleotide; FADH, reduced FAD.

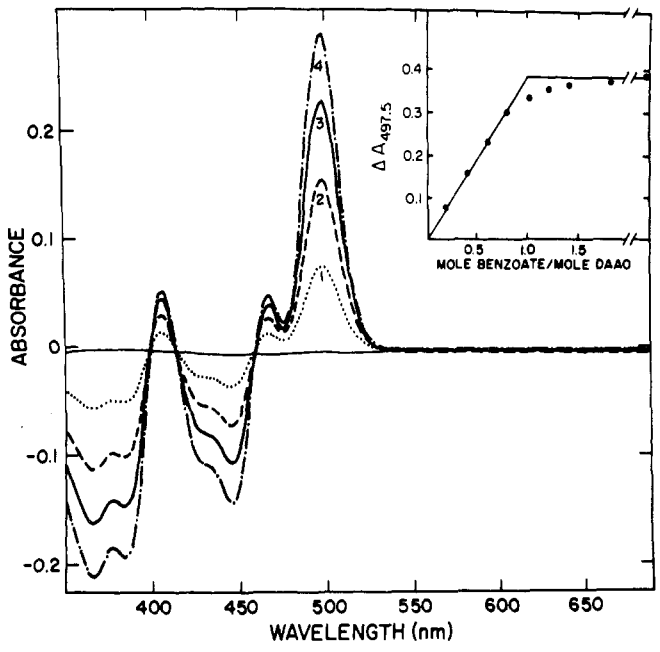


FIGURE 1: Difference spectra on titration of D-amino acid oxidase with sodium benzoate. Absorbancy measurements were made as described under Experimental Procedure. DAAO was at 1.23×10^{-4} M with respect to enzyme-flavin. Curves 1-4, at benzoate concentrations of 2.52×10^{-5}, 5.04×10^{-5}, 7.5×10^{-5}, and 1×10^{-4} M. The difference spectra shown are not corrected for dilution (2.1%). Insert: the increase in absorbance at 497.5 nm vs. the mole ratio of benzoate to enzyme-flavin (corrected for dilution).

absorption was determined from 350 to 650 nm. Additions of 5-15 μL of a sodium benzoate solution were delivered to the sample cell from appropriate volume S. Hagenstam Type B micropipettes. An equal volume of water was delivered to the reference cell. The contents of each cell were mixed and the difference spectrum was recorded. The spectral changes were rapid; no slow, time-dependent changes were observed.

Rapid-mixing experiments were made with a Gibson and Milnes (1964) stopped-flow spectrophotometer equipped with a Bausch and Lomb monochrometer. The photomultiplier output was monitored with an internally calibrated logarithmic operational amplifier connected to a transient recorder and a Honeywell 550 X-Y recorder. The details of construction will be described elsewhere (G. Ford and D. Ballou, in preparation).

Buffer solutions used were 0.1 M sodium pyrophosphate with the pH adjusted to the desired value by the addition of acetic acid (pH 5.3-9.2) or sodium carbonate (pH 9.2-10.5).

Measurements of pH at 19 °C were made with a Radiometer Model 25 pH meter and a GK2321C combination electrode. The electrode was standardized with Sargent Welch Scientific buffer solutions of pH 7.02 and 10.06 (19 °C). Measurements were made before and after each experiment. The pH change was always less than 0.05 pH unit.

Results

The difference spectrum of DAAO at pH 8.50 and 19 °C after sequential additions of sodium benzoate is shown in Figure 1. In the insert, the change in absorbancy at 497.5 nm is plotted against the molar ratio of benzoate to enzyme-bound FAD. As reported previously (Massey and Ganther, 1965), the binding has a stoichiometry of 1:1 with respect to flavin. The same stoichiometry was obtained in experiments from pH 5.65 to 10.5. The dissociation constant, K_d, was calculated by

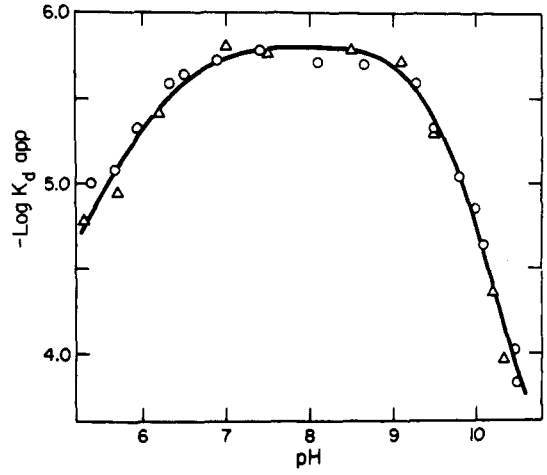


FIGURE 2: The effect of pH on the apparent dissociation constant of the benzoate-DAAO complex. Circles represent values obtained from direct spectrophotometric titrations as in Figure 1; triangles are the values for the ratio $k_{-1,app}/k_{1,app}$ at various pH values. The solid line is calculated using the parameters of mechanism II, as listed in Table II, and eq 5.

determining [PBz] for the mixtures of DAAO and benzoate near equivalency according to eq 1.

$$K_d = \frac{([P]_0 - [PBz])([Bz]_0 - [PBz])}{[PBz]} \quad (1)$$

This determination was repeated at 15 pH values from pH 5.65 to 10.5 and the relationship between $-\log K_d$ and pH is illustrated in Figure 2.

The kinetics for the reaction studies was found to be that for reversible second-order-first-order reactions.[2]

$$P + Bz \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} P\text{-}Bz \quad (2)$$

The second-order rate constant for this reaction was determined under pseudo-first-order conditions ([PBz] = 0, [P] = $[P]_0$, [Bz] = $[Bz]_0$ at $t = 0$ and $[Bz]_0 \gg [P]_0$). As noted under Experimental Procedure, the rapid-reaction experiments were performed with a logarithmically recording X-Y plotter. The production of the PBz complex was monitored at 497.5 nm. A plot of ln ($[P]_0 - [P]$) vs. time should be linear if reaction 2 is the appropriate mechanism and should have a slope, k_{obsd}, equal to ($k_{1,app}[Bz]_0 + k_{-1,app}$). A secondary plot, such as shown in Figure 3, of k_{obsd} vs. $[Bz]_0$ should give a straight line of slope equal to $k_{1,app}$ and intercept equal to $k_{-1,app}$.

Unfortunately, k_{obsd} is often very much larger than $k_{-1,app}$, producing large errors in this graphical determination of $k_{-1,app}$ (cf. Figure 3). For this reason, $k_{-1,app}$ was determined by the displacement method (Gutfreund, 1972). In this technique, an equilibrium solution of DAAO and benzoate is rapidly mixed with a ligand or substrate which competes for the binding site for benzoate. In these studies, the substrate D-alanine or the inhibitor anthranilate was used. The reactions are given below

$$P + Bz \underset{k_{-1,app}}{\overset{k_{1,app}}{\rightleftharpoons}} P \cdot Bz$$

$$P + X \xrightarrow{k_2} P \cdot X \rightarrow$$

where X is D-alanine or anthranilate. If $k_{-1,app} \ll k_2[X]$ and

[2] We use the symbols $\rightleftarrows$ for reactions occurring at measurable rates and $\rightleftharpoons$ for reactions in which equilibrium is assumed to be maintained (King, 1964).

TABLE I: Data for the Binding of Benzoate by DAAO at 19 °C.

pH	Exptl rate constants with SD		K_d = [P][Bz]/[PBz]	
	$k_{1,app}$ (M^{-1} s^{-1})	$k_{-1,app}$ (s^{-1})	From kinetic data (M)	From spectrophotometric determination (M)
5.30	(2.31 ± 0.27) × 10^6	19.30 ± 2.40	8.35 × 10^{-6}	
5.65				(8.40 ± 0.20) × 10^{-6}
5.70	(1.27 ± 0.16) × 10^6	14.20 ± 0.50	1.12 × 10^{-5}	
5.95				(4.70 ± 0.60) × 10^{-6}
6.20	(9.45 ± 0.60) × 10^5	3.60 ± 0.25	3.81 × 10^{-6}	
6.32				(2.54 ± 0.47) × 10^{-6}
6.50				(2.28 ± 1.09) × 10^{-6}
6.89				(1.88 ± 0.47) × 10^{-6}
7.00	(4.40 ± 0.11) × 10^5	0.68 ± 0.06	1.55 × 10^{-6}	
7.41				(1.62 ± 0.16) × 10^{-6}
7.50	(2.50 ± 0.08) × 10^5	0.42 ± 0.05	1.68 × 10^{-6}	
8.11				(1.91 ± 0.16) × 10^{-6}
8.50	(3.15 ± 0.08) × 10^5	0.40 ± 0.20	1.27 × 10^{-6}	
8.65				(1.97 ± 0.51) × 10^{-6}
9.10	(2.11 ± 0.03) × 10^5	0.40	1.90 × 10^{-6}	
9.28				(2.50 ± 0.74) × 10^{-6}
9.50	(1.02 ± 0.03) × 10^5	0.50 ± 0.03	4.90 × 10^{-6}	(4.65 ± 0.07) × 10^{-6}
9.80				(9.10 ± 0.43) × 10^{-6}
9.98				(1.40 ± 0.30) × 10^{-6}
10.10				(2.28 ± 0.22) × 10^{-6}
10.20	(3.15 ± 0.12) × 10^4	1.36 ± 0.25	4.32 × 10^{-5}	
10.34	(1.83 ± 0.12) × 10^4	1.94 ± 0.38	1.06 × 10^{-4}	
10.45				(9.60 ± 0.50) × 10^{-5}
10.50				(1.46 ± 0.12) × 10^{-4}

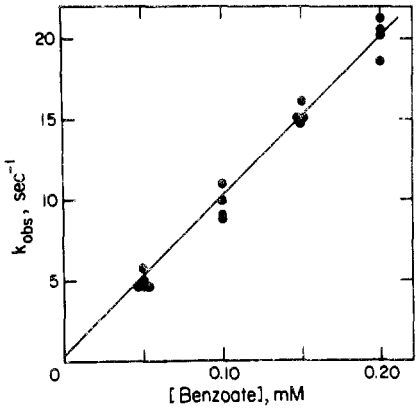


FIGURE 3: Observed first-order rate constants for benzoate-DAAO complex formation, plotted as a function of benzoate concentration. $[DAAO]_0$ = 1.88 × 10^{-5} M at pH 9.50, 19 °C. Each point represents a single determination. The solid line was drawn by least-squares analysis and has a slope of (1.02 ± 0.03) × 10^5 M^{-1} s^{-1} and intercept of 0.12 ± 0.40 s^{-1}.

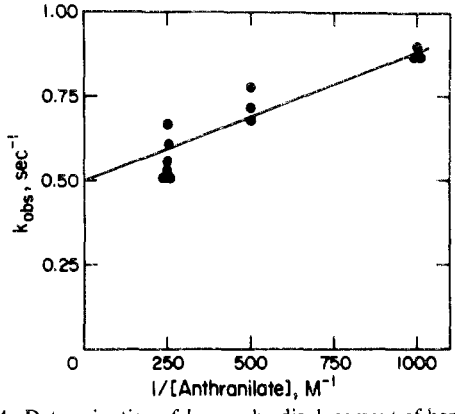


FIGURE 4: Determination of $k_{-1,app}$ by displacement of benzoate from the benzoate-DAAO complex by anthranilate. [DAAO] = 12.6 μM, pH 9.50, 19 °C; [Bz] = 2 × 10^{-4} M. Each point represents a single determination. The solid line was drawn by least-squares analysis and has an intercept of 0.50 ± 0.03 s^{-1}.

$k_2[X] \gg k_{1,app}[Bz]$, the spectrophometric change due to the dissociation of P·Bz can be interpreted in terms of the rate constant $k_{-1,app}$ for the dissociation of this complex. These inequalities were always maintained in the experiments reported here. For example, for the data depicted in Figure 4, the values for these expressions are 0.50 s^{-1} ≪ 100–400 s^{-1} and 100–400 s^{-1} ≫ 20.4 s^{-1}, respectively. The values for $k_{-1,app}$ obtained with anthranilate agreed well with those obtained with D-alanine.

The data for $k_{1,app}$, $k_{-1,app}$, and K_d are given in Table I. Plots of the log of $k_{1,app}$ and $k_{-1,app}$ vs. pH are shown in Figures 5 and 6, respectively. Figure 2 contains −log K_d values obtained from spectrophotometric titrations as well as from kinetic experiments, in which the dissociation constant was calculated from the relationship: $K_d = k_{-1,app}/k_{1,app}$. The excellent agreement in the values obtained by the two methods supports the validity of the $k_{-1,app}$ determinations.

Discussion

The kinetic parameters determined here were based on a model of reversible second-order–first-order kinetics. One feature of this model is the linearity of a plot of k_{obsd} vs. ligand concentration as depicted in Figure 3 (Strickland et al., 1975). Similar plots were always linear in experiments between pH 5.30 and 10.34. However, Nishikimi et al. (1971) observed a hyperbolic relationship between k_{obsd} and [Bz] for DAAO at 15 °C, 0.017 M pyrophosphate, pH 8.2, leading them to propose a two-step mechanism for the enzyme–inhibitor binding. An important difference in experimental design between our work and that of Nishikimi et al. is the reaction temperature. Their use of 15 °C for kinetic experiments is in the middle of

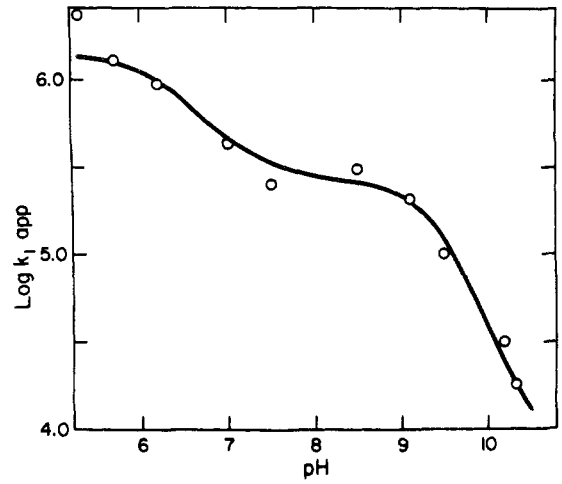


FIGURE 5: The relationship between the log $k_{1,app}$ vs. pH at 19 °C. The solid line is calculated using the parameters of mechanism II, as listed in Table II, and eq 4.

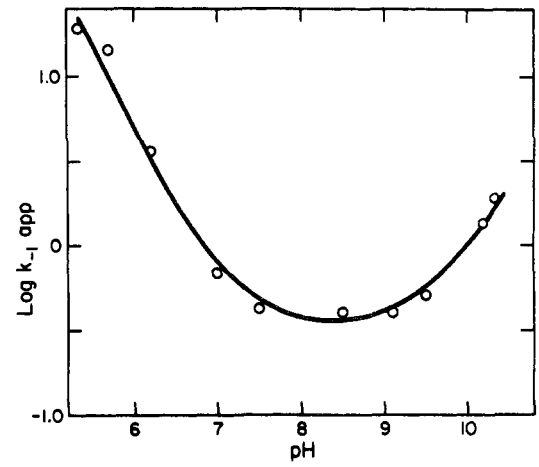


FIGURE 6: The relationship between the log $k_{-1,app}$ vs. pH at 19 °C. The solid line is calculated using the parameters of mechanism II, as listed in Table II, and using eq 3.

the temperature range (10–20 °C) where DAAO undergoes sharp changes in sedimentation coefficient, ultraviolet difference spectra, tryptophan fluorescence, visible spectra, catalytic properties, and light-scattering properties (Massey et al., 1965; Antonini et al., 1966). The complexity of the enzyme at this temperature may be at the root of the complex kinetic pattern reported by Nishikimi et al. From the present study, it is apparent that no such complexity is observed at 19 °C.

The pK for the ionization of benzoic acid is 4.2 at 25 °C (Handbook of Chemistry and Physics). Since this study was performed above pH 5.3, the only mechanism considered is the binding of the benzoate anion to DAAO. Consideration of Figure 2 by the method of Dixon (1953) indicates a single protonic dissociation in the acidic pH range and a double protonic dissociation in the alkaline pH range could approximate the data. These points are features of the first mechanism we will consider.

$$\begin{array}{ccccccc} EH_3 & \overset{K_1}{\rightleftharpoons} & EH_2 & \overset{K_2}{\rightleftharpoons} & EH & \overset{K_3}{\rightleftharpoons} & E \\ & & + & & & & \\ & & Bz & & & & \\ & & \Updownarrow K_B & & & & \\ & & EH_2 \cdot Bz & & & & \end{array} \qquad (I)$$

The representation of mechanism I contains three simplifying features: omitting hydrogen ions, omitting the net charge of the enzyme species, and designating the DAAO-benzoate complex as the uncharged EH_2·Bz complex. One simple test of mechanism I is the value of $k_{-1,app}$ at different pH values. According to mechanism I, $k_{-1,app}$ should be independent of pH. Since experimental determination of $k_{-1,app}$ gives values which vary from 0.4 s^{-1} at pH 8.5 to 19.3 s^{-1} at pH 5.3 (Figure 6 and Table I), mechanism I must be invalid.

The complex nature of the relationship of log $k_{-1,app}$ and pH indicates at least three DAAO-benzoate complexes exist which are in protonic equilibrium with each other. The one-unit

$$\begin{array}{ccccccc} EH_3 & \overset{K_1}{\rightleftharpoons} & EH_2 & \overset{K_2}{\rightleftharpoons} & EH & \overset{K_3}{\rightleftharpoons} & E \\ \Updownarrow K_4 & & \Updownarrow K_5 & & \Updownarrow K_6 & & \\ EH_3Bz & \underset{K_{1,c}}{\rightleftharpoons} & EH_2Bz & \underset{K_{2,c}}{\rightleftharpoons} & EHBz & & \end{array} \qquad (II)$$

slope of the acidic and alkaline portions of Figure 6 implies a single proton is involved at each ionization. Model II takes these points into consideration.

In order to accommodate the apparent double protonic dissociation in the $-\log K_d$ data at alkaline pH values, as well as the single protonic dissociation at acid pH, both K_4 and K_6 must be significantly larger than K_5. A value of about 10 for K_4/K_5 and K_6/K_5 would fulfill this requirement.

For mechanism II

$$k_{-1,app} = \frac{k_{-4}\frac{[H^+]}{K_{1,c}} + k_{-5} + k_{-6}\frac{K_{2,c}}{[H^+]}}{1 + [H^+]/K_{1,c} + K_{2,c}/[H^+]} \qquad (3)$$

Equation 3 was used to fit the data in Figure 6. The data in the alkaline pH range yields $k_{-5} = 0.30\ s^{-1}$, $k_{-6} \geq 5.0\ s^{-1}$ and $K_{2,c} \leq 10^{-10.7}$ M. The data in the acidic pH range were best simulated with $k_{-5} = 0.30\ s^{-1}$, $k_{-4} \geq 55\ s^{-1}$, and $K_{1,c} \geq 10^{-5}$ M.

Mechanism II implies that the values for $k_{1,app}$ should also be pH dependent. In fact, Figure 5 shows these data and illustrates the rather complex nature of this relationship. Theoretically, the rate equation for the second-order reaction for association in mechanism II is given by eq 4

$$k_{1,app} = \frac{k_4\frac{[H^+]}{K_1} + k_5 + k_6\frac{K_2}{[H^+]}}{1 + [H^+]/K_1 + K_2/[H^+] + K_2K_3/[H^+]^2} \qquad (4)$$

The rather good fit of the data to eq 4, using the values of the kinetic and equilibrium constants listed in Table II, is shown in Figure 5.

Returning now to the experiments on the equilibrium dissociation constant for DAAO and benzoate, the relationship between K_d and pH is given by eq 5.

$$K_d = K_5\frac{(1 + [H^+]/K_1 + K_2/[H^+] + K_2K_3/[H^+]^2)}{(1 + [H^+]/K_{1,c} + K_{2,c}/[H^+])} \qquad (5)$$

Figure 2 contains a plot of $-\log K_d$ vs. pH, with the solid line calculated for mechanism II by applying the equilibrium constants from Table II to eq 5.

In summary, the kinetic and equilibrium experiments indicate the presence of three enzyme groups with pK values of 6.35, 9.22, and 9.60 which are important for benzoate binding. According to mechanism II, EH_3, EH_2, and EH bind benzoate. As indicated earlier, the binding of benzoate to EH and EH_3

TABLE II: Rate and Equilibrium Constants for Mechanism II.

K_1	4.47×10^{-7} M
K_2	2.51×10^{-10} M
K_3	6.03×10^{-10} M
K_4	3.30×10^{-5} M
K_5	1.48×10^{-6} M
K_6	1.86×10^{-5} M
$K_{1,c}$	1×10^{-5} M
$K_{2,c}$	2×10^{-11} M
k_4	1.6×10^{6} M^{-1} s^{-1}
k_{-4}	55 s^{-1}
k_5	2.50×10^{5} M^{-1} s^{-1}
k_{-5}	0.30 s^{-1}
k_6	1.70×10^{5} M^{-1} s^{-1}
k_{-6}	5.0 s^{-1}

must be sufficiently weak to yield the apparent two-proton ionization in the alkaline pH range and the apparent one-proton ionization at acid pH. One can see from Table II that K_4/K_5 and K_6/K_5 are 22 and 12.6, respectively.

The identity of the three enzyme species at alkaline pH has not been made. Previous work (Massey and Ganther, 1965) indicated that the N_3-imino group of the flavin has a pK value at about 9.4 ($FADH \rightleftharpoons FAD^- + H^+$) and that this ionization causes a marked increase in the enzyme extinction coefficient at 360 nm. This property might allow us to assign one of the pK values in mechanism II to this ionization. Mechanism II can be rewritten to illustrate the alternative order of flavin-associated ionization and enzyme-associated ionizations where EH_2FADH is equivalent to the EH_3 species of mechanism II and $EFAD^-$ to E.

$$EH_2FADH \underset{K_1}{\rightleftharpoons} EHFADH \underset{K_2}{\rightleftharpoons} EFADH \underset{K_3}{\rightleftharpoons} EFAD^- \quad \text{(IIA)}$$

$$EH_2FADH \underset{K_1}{\rightleftharpoons} EHFADH \underset{K_2}{\rightleftharpoons} EHFAD^- \underset{K_3}{\rightleftharpoons} EFAD^- \quad \text{(IIB)}$$

For simplification of the analysis of the above models, all enzyme species with the protonated form of the flavin, E_{FH} (i.e., EH_2FADH, EHFADH, EFADH), are assumed to have the same extinction coefficient at 360 nm, while the deprotonated form of the flavin, E_{F^-} ($EHFAD^-$, $EFAD^-$), is also assumed to have the same extinction coefficient at 360 nm, although different from that for the protonated flavin species. Thus, we have only five variables: three dissociation constants and two extinction coefficients. For these analyses, only the extinction coefficients were allowed to vary, the group-ionization constants were assumed to be those contained in Table II. The equations for the absorbancy changes with pH for these mechanisms are given by eq 6A and 6B.

$$\epsilon_{360} = \frac{\epsilon_{FH}(1 + [H^+]/K_1 + K_2/[H^+]) + \epsilon_F - (K_2K_3/[H^+]^2)}{(1 + [H^+]/K_1 + K_2/[H^+] + K_2K_3/[H^+]^2)} \quad \text{(6A)}$$

$$\epsilon_{360} = \frac{\epsilon_{FH}(1 + [H^+]/K_1) + \epsilon_{F^-}(K_2/[H^+] + K_2K_3/[H^+]^2)}{(1 + [H^+]/K_1 + K_2/[H^+] + K_2K_3/[H^+]^2)} \quad \text{(6B)}$$

Figure 7 shows the results of experiments to measure the extinction coefficient at 360 nm between pH 5.7 and 10.74. The solid line represents the theoretical curve obtained by applying a value of 8400 M^{-1} cm^{-1} to the FADH enzyme forms and 10 200 M^{-1} cm^{-1} to the FAD^-enzyme forms according to eq 6B. No set of extinction coefficients could be used with 6A to give a close fit to the data. Thus, we can tentatively identify the ionization constant of $10^{-9.60}$ M as that associated with the enzyme-bound flavin.

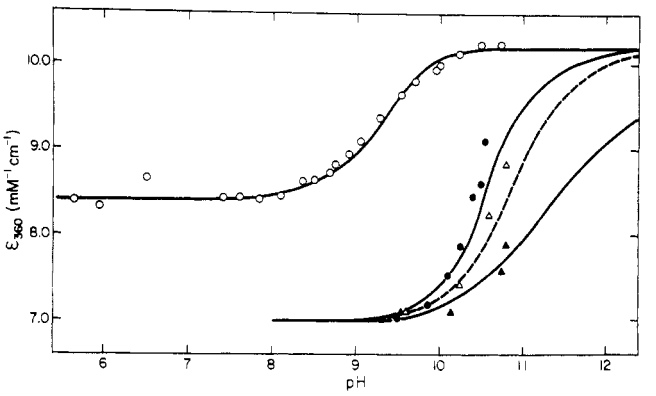


FIGURE 7: Dependence of extinction coefficient at 360 nm on pH. (○) [Bz] = 0. The solid line is a theoretical one, using ϵ_{FH} = 8400 M^{-1} cm^{-1}, ϵ_{F^-} = 10 200 M^{-1} cm^{-1}, and the parameters in Table II, and eq 6b. (●) [Bz] = 2.5×10^{-4} M; (△) [Bz] = 1×10^{-3} M; (▲), [Bz] = 1×10^{-2} M. The lines are theoretical ones for these benzoate concentrations, using ϵ_{FH} = 8400 M^{-1} cm^{-1}, ϵ_{FHBz} = 7000 M^{-1} cm^{-1}, ϵ_{F^-Bz} = 8200 M^{-1} cm^{-1}, ϵ_{F^-} = 10 200 M^{-1} cm^{-1}, and the parameters in Table II and eq 7.

These results led us to a reconsideration of the effects of benzoate on the apparent pK of ionization of the N(3) proton of the flavin coenzyme. Previous studies had shown that, whereas the pK of this ionization was 10.4 with the free coenzyme, it decreased to 9.4 with the holoenzyme of DAAO (Massey and Ganther, 1965). When *excess* benzoate was present, the observed pK for the enzyme-bound flavin was >10.9. However, from the present studies, it is clear that this cannot be an intrinsic pK of the flavin in the enzyme-benzoate complex, since the alkaline enzyme species cannot bind benzoate. That is, the observed pK for the E_{FH} to E_{F^-} transition should be dependent on the *concentration* of benzoate. This prediction was confirmed experimentally (Figure 7).

Model IIB, expanded to include enzyme-benzoate species, was used to analyze the experimental data of extinction coefficients in the presence of benzoate.

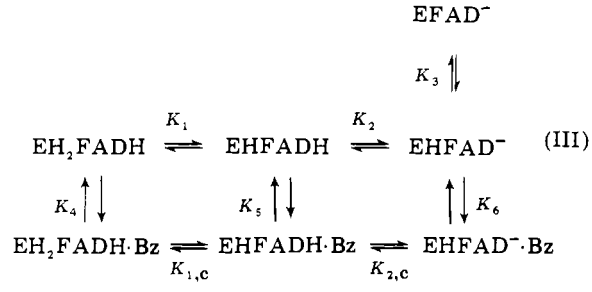
Eq 7 was used to calculate theoretical data with the following simplifications:

$$\epsilon_{360} = \frac{\epsilon_{FH} + \epsilon_{FHBz}([Bz]/K_4) + \epsilon_{F^-Bz}([Bz]K_{2,c}/K_4[H^+]) + \epsilon_{F^-}(K_2/[H^+] + K_2K_3/[H^+]^2)}{1 + K_2/[H^+] + K_2K_3/[H^+]^2 + [Bz]/K_4 + [Bz]K_{2,c}/K_4[H^+]} \quad (7)$$

The molar absorptivities of EHFADH, $EHFAD^-$, and $EFAD^-$ were not varied and were assigned the values obtained in Figure 7, i.e., 8400, 10 200, and 10 200 M^{-1} cm^{-1}, respectively; the values for K_1, K_2, K_3, K_4, K_5, K_6, $K_{1,c}$, and $K_{2,c}$ were not varied and were assigned the values given in Table II; the extinction coefficient of EHFADH·Bz was not varied and was assigned a value of 7000 M^{-1} cm^{-1}. The final assumption seems justified, since at pH 9.5 and 10^{-3} and 10^{-2} M benzoate the mole fraction of total enzyme in the form EHFADH·Bz, according to eq 7, is 0.93 and 0.94, respectively. Thus, the extinction coefficient at this pH can be predominately assigned

to EHFADH·Bz. As can be seen, the only remaining variable is the molar absorptivity of EHFAD$^-$·Bz. Although the simplest considerations might indicate that this value should be close to E_{F^-}, the observation that benzoate lowers the extinction coefficient of E_{FH} from 8400 to 7000 M^{-1} cm^{-1} made it likely that a similar effect would be found for the $\epsilon_{FAD\cdot Bz}$ value. In fact, the best-fit extinction coefficient for EFAD$^-$·Bz is 8200 M^{-1} cm^{-1} (see Figure 7). If this value were set at the ϵ_{FAD} value of 10 200 M^{-1} cm^{-1}, the calculated absorptivity at pH 10.5, 10^{-2} M Bz, would be 8300 M^{-1} cm^{-1} in distinction from the experimentally observed value of 7500 M^{-1} cm^{-1}. Under the conditions of the experiments depicted in Figure 7, the deviations between observed and calculated absorbancies, using $\epsilon_{FAD^-\cdot Bz}$ as 8200 M^{-1} cm^{-1}, do not exceed 0.011 absorbance unit and represent a small proportional error (always <7%). Irreversible denaturation of the enzyme occurs at the extreme alkaline pH values, making extinction measurements imprecise above pH 10.5.

Model III is the simplest one explored which accounts satisfactorily for the interaction of benzoate with D-amino acid oxidase over the whole pH range in which the enzyme is stable.



Four kinds of experimental measurements, apparent dissociation constants, on and off rate constants, and absorbance changes, are all consistent with this model and a single set of kinetic and thermodynamic parameters, listed in Table II. It is obvious, however, that this model could be expanded, ad infinitum, and equally good fits of the experimental data could be obtained. The effect of such expansions is to produce uncertainties in the derived thermodynamic and kinetic constants. For example, the inclusion of the EFADH enzyme species in an expansion of Model III leads to uncertainties in the values of K_2, K_3 and $K_{2,c}$. Until experiments can be devised to measure the importance of this or other expansions of the model, the derived constants shown in Table II can be accepted as tentative ones, compatible with the simplest thermodynamic and kinetic treatment of the data.

The pH behavior of benzoate binding to D-amino acid oxidase (Figure 2) indicates that there are two ionizable groups on the protein as well as the flavin ionization which have to be in the correct state for benzoate binding to occur. Considering that it is the negatively charged benzoate anion which binds to the protein, it would seem that the most likely model to fit these data would be an acidic residue with a pK of 6.3 and a basic group with a pK of 9.2. Histidine is a natural suggestion for the acidic residue, since it has a pK in solution of 6.0. In fact, a histidine residue has been proposed to be part of the active site of D-amino acid oxidase (Thome-Beau et al., 1971), and to function possibly in the withdrawal of the proton from the α-carbon atom of the substrate during catalysis (Walsh et al., 1973a). While perturbations of the ionization constant of lysine residues to pK values as low as 5.9 have been reported (Schmidt and Westheimer, 1971), the available data suggest most consistently that the base involved in proton abstraction from the substrate is an imidazole residue; particularly informative in this respect are the results of intramolecular ^{3}H-transfer studies between β-chloroalanine and pyruvate which strongly implicate a one-proton base (Walsh et al. 1973a).

Due to the many amino acid residues which have pK values in the region of 9.2, speculation about the nature of this group is difficult. However, recent work (T. Nishino, V. Massey, B. Curti, S. Ronchi, unpublished) on reaction of D-amino acid oxidase with dansyl chloride at neutral pH values shows that the enzyme is inactivated with a low degree of modification (<3 dansyl residues per mole of enzyme-bound flavin). This inactivation is prevented by benzoate; work is in progress to determine the site of modification. Studies by Yagi and coworkers (Kotaki et al., 1968) implicate a guanidino residue at the active site of D-amino acid oxidase. These workers found that reaction with glyoxal led to progressive loss of activity as arginine residues were modified, with complete inactivation occurring on reaction of 18 residues. Benzoate was found to protect against inactivation, with one less arginine modified. The resulting enzyme was approximately half as active as native enzyme, and was shown to bind 1 equiv of benzoate per molecule of enzyme-bound FAD. Hence, a guanidino group may possibly be responsible for the pK 9.2 value found to influence benzoate binding in the present study. The role of this group in catalysis is presumably in ionic binding with the carboxyl of the substrate in the formation of the enzyme-substrate complex.

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Bovine Enterokinase. Purification, Specificity, and Some Molecular Properties†

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ABSTRACT: Enterokinase has been isolated from the contents of bovine duodena and purified 1200-fold in 41% yield. The isolation procedure employed DEAE chromatography, affinity chromatography on immobilized *p*-aminobenzamidine, and gel filtration. The resultant pure enzyme exhibits a single band on sodium dodecyl sulfate gel electrophoresis corresponding to a molecular weight of 145 000. Two chains in the molecule, connected by disulfide linkages, have molecular weights of 57 000 and 82 000, respectively. The purified enzyme exhibits a restricted specificity which is directed toward the polyanionic amino acid sequence in the activation peptide of the zymogen substrate. Of the zymogens of the serine proteases tested, including several of those involved in blood coagulation, only native and guanidinated trypsinogen are activated by enterokinase, whereas acetylated trypsinogen is not. Partial heat denaturation of bovine enterokinase causes a differential response toward the activation of trypsinogen and the hydrolysis of benzoyl-L-arginine ethyl ester (BzArgOEt), further suggesting that secondary sites are important in the binding of trypsinogen. A sensitive assay for enterokinase (nanomole range) was developed using tritiated BzArgOEt.

Enterokinase (enteropeptidase, EC 3.4.4.8) occupies a key position in the utilization of dietary proteins. The enzyme initiates intraluminar digestion of proteins by the proteolytic conversion of trypsinogen to trypsin, which in turn activates the other pancreatic zymogens (Kunitz, 1939a,b; Hadorn et al., 1969). The proteolytic attack of enterokinase is directed exclusively toward the Lys_6-Ile_7 peptide bond of trypsinogen leaving all other lysine and arginine bonds in the molecule unaffected (Maroux et al., 1971). The resultant cleavage produces the simultaneous release of active trypsin and of the amino-terminal hexapeptide Val-$(Asp)_4$-Lys (Rovery et al., 1953; Davie and Neurath, 1955). This unique specificity exhibited by enterokinase is of interest as it relates to both the molecular basis of substrate recognition and the control of the digestive process.

Previous work, using synthetic peptide substrates, suggests that the polyaspartyl sequence in the activation peptide is important for optimal interaction of enterokinase and protein substrates (Maroux et al., 1971). This cluster of aspartyl residues is common to all trypsinogens thus far sequenced with two exceptions, both from the African lungfish (Reeck and Neurath, 1972; de Haën et al., 1975). In the investigation reported here, particular attention has been given to the significance of this unique sequence for the specificity of enterokinase. Since much of the critical work on pancreatic enzymes and zymogens has been done on bovine material, the present investigation has been directed toward the isolation of bovine enterokinase and the examination of some of its properties. We have also compared the bovine enzyme to that of porcine origin, purified and characterized by Desnuelle and co-workers (Maroux et al., 1971; Baratti et al., 1973).

Experimental Section

Materials

Bovine trypsinogen (once crystallized) and chymotrypsinogen A were obtained from Worthington Biochemical Corp. Bovine procarboxypeptidase B and plasminogen were purified in our laboratory by D. Grahn and Dr. M. A. Kerr, respectively. Dr. C. de Haën provided the lungfish proelastase B and lungfish trypsinogen B. Prothrombin and blood coagulation factor X were generous gifts from Dr. Earl W. Davie.

1-Guanyl-3,5-dimethylpyrazole nitrate was prepared by Dr. N. C. Robinson using the method of Bannard et al. (1958). Labeled benzoylarginine ethyl ester[1] (BzArgOEt) was synthesized in our laboratory by E. Fodor using a modification of

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[1] Abbreviations used are: BzArgOEt, benzoyl-L-arginine ethyl ester; TosArgOMe, N^{α}-tosyl-L-arginine methyl ester; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Mes, 2-(*N*-morpholino)ethanesulfonic acid; iPr_2P, diisopropylphosphoryl; iPr_2PF, diisopropyl phosphofluoridate; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl.