An Approach to Inhibition Kinetics. Measurement of Enzyme–Substrate Complexes by Electronic Energy Transfer†

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ABSTRACT: The rapid formation and breakdown of enzyme–substrate (E·S) complexes between N-dansylated peptide substrates and carboxypeptidase A can be followed by stopped-flow fluorescence. The maximal fluorescence and the decay of the signal can be related to Michaelis–Menten parameters. The maximal fluorescence, F_{max}, is directly proportional to the steady state concentration of the E·S complex, and the area under the curve, A, is inversely proportional to the hydrolysis rate k_{cat}. Measurement of F_{max} and values of A at different substrate concentrations allow determination of the kinetic parameters K_D and k_{cat}. The mode of action of an inhibitor can be identified directly by the manner in which it affects substrate binding and/or hydrolysis. Competitive inhibitors reduce the initial concentration of the E·S complex while noncompetitive inhibitors reduce its rate of breakdown. Mixed inhibition can be resolved into its competitive and noncompetitive components at a single substrate concentration. This approach has enabled us to delineate the mixed inhibition observed with a number of inhibitors of carboxypeptidase A. The influence of pH on the resolved inhibition modes shows this mixed inhibition to be due to multiple binding of the inhibitor. The pH dependence of the inhibition constants can be interpreted in terms of the protonation scheme proposed for peptide hydrolysis.

Enzyme-catalyzed reactions are usually characterized by the kinetic parameters K_m and V_{max} which can be related mathematically to the steady state concentration of the enzyme–substrate (E·S) complex and its rate of breakdown (Dixon and Webb, 1964). Like other factors which influence the velocity of enzyme-catalyzed reactions, reversible inhibitors affect the apparent K_m, V_{max}, or both. Hence, the assignment of inhibition modes is of necessity indirect. However, if the E·S complex could be observed, the manner in which an inhibitor affects substrate binding and hydrolysis could be recognized directly.

The use of N-dansylated peptide substrates of carboxypeptidase A permits precisely this approach (Latt *et al.*, 1970a,b; Latt, 1971). Energy transfer between tryptophanyl residues of the enzyme and the dansyl group of the bound substrate allows observations of details in the formation and breakdown of the E·S complex. The mode of action of an inhibitor can then be identified by the manner in which it affects substrate binding and/or its hydrolysis.

The data are consistent with those obtained by more classical methods. In addition, mixed inhibition can be resolved into its components using but a single concentration of substrate. This approach has greatly facilitated the interpretation of the complex inhibition patterns seen for a number of inhibitors of carboxypeptidase A. Preliminary communication of this work has appeared (Latt *et al.*, 1970a; Auld and Latt, 1971).

Materials and Methods

Carboxypeptidase A (Anson)[1] was purchased from Worthington Biochemical Corporation as an aqueous, crystal suspension. The crystals were washed with deionized, distilled water and then dissolved in 3 M NaCl to give stock solutions (10^{-3} M). These solutions were then diluted as needed in the appropriate buffer and used in stopped-flow studies of inhibitor action. Fractionation of carboxypeptidase A (Anson) into its multiple alleomorphic forms was performed by DEAE-cellulose chromatography in the presence of 0.05 M β-phenylpropionate (Petra and Neurath, 1969).

Carboxypeptidase A (Cox) was isolated from acetone powders of beef pancreas by the method of Cox *et al.* (1964). Stock solutions were prepared and stored as described elsewhere (Auld and Vallee, 1970a). This enzyme was used in steady state studies and in stopped-flow determination of the Michaelis–Menten parameters of Dns-Gly-L-Trp.[2]

Carboxypeptidase concentrations were measured at 278 nm using a molar absorptivity of 6.42×10^4 M^{-1} cm^{-1} (Simpson *et al.*, 1963).

Peptide Substrates. The syntheses of the peptides employed have been described (Auld and Vallee, 1970a; Latt *et al.*, 1972a). The concentrations of the dansyl peptides were calculated from absorbance measurements at 330 nm in 1 M NaCl–0.05 M Tris, pH 7.5, using an ϵ of 4.5×10^3 M^{-1} cm^{-1}.

Peptidase Activity. Steady state assays using peptides at concentrations greater than 2×10^{-5} M were performed at 25° in 0.05 M Mes, Hepes, or Tris–1 M NaCl, pH 7.5, using a Technicon AutoAnalyzer, by either of two automated ninhydrin methods (Auld and Vallee, 1970a,b).

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[1] Carboxypeptidase referred to in the text is carboxypeptidase A (Anson) unless otherwise specified (Anson, 1937).

[2] Abbreviations used are: blocking groups of peptides, Dns or dansyl, 5-dimethylaminonaphthalene-1-sulfonyl, and Cbz, carbobenzoxy; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpeperazine-*N*-2′-ethanesulfonic acid; ammediol, 2-amino-2-methyl-1,3-propanediol.

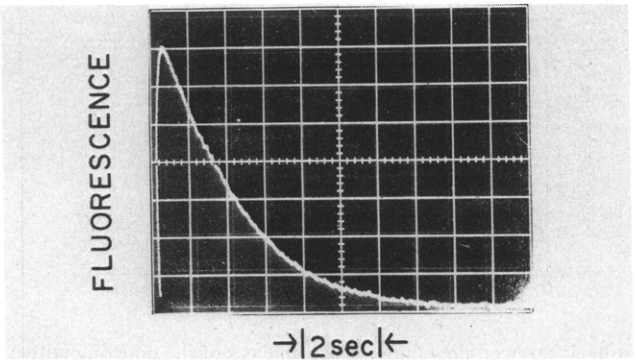

FIGURE 1: Stopped-flow fluorescence observation of the formation and breakdown of E·S complexes of carboxypeptidase A. Equal volume solutions of Dns-Gly-L-Phe and carboxypeptidase A (Anson, 1937) both 2.5×10^{-4} M in 1 M NaCl–0.05 M Tris, pH 7.5, were mixed at 25° and the dansyl fluorescence observed as a function of time. Excitation was at 285 nm and the dansyl emission, above 430 nm, was observed.

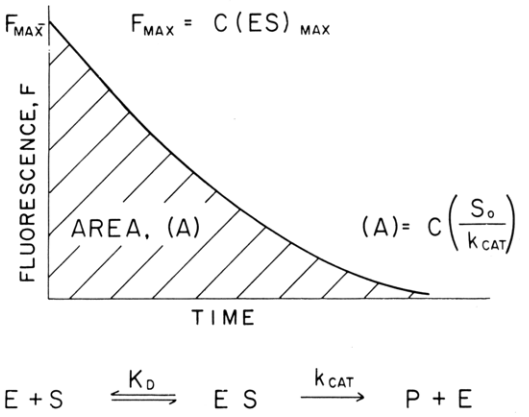

FIGURE 2: Relationship of the maximal fluorescence and decay in signal to Michaelis–Menten parameters. The form of a stopped-flow trace such as is obtained in Figure 1 is schematized. The maximal dansyl fluorescence, F_{max}, is proportional to the concentration of the E·S complex present while the area under the curve, A, is inversely proportional to the rate constant k_{cat} for the rate-determining step.

Stopped-Flow Fluorescence. Assays utilized a Durrum-Gibson instrument equipped with Kel-F connections. All assays were performed at 25° in 1 M NaCl–0.05 M Mes, Hepes, Tris, or ammediol. Enzyme tryptophanyl residues were excited at 285 nm and dansyl emission was measured using a cutoff filter excluding light below 430 nm. Tryptophan emission was measured using a band pass filter peaking at 360 nm. The Durrum fluorescence attachment no. 13018 was modified by using an EMI 9526B photomultiplier tube mounted for end-on observation with a resultant fivefold increase in sensitivity. Most experiments examining inhibitor effects were performed by mixing a solution of enzyme alone with one of substrate plus inhibitor. However, when inhibitor was added to enzyme before mixing with substrate, equivalent results were obtained.

Determination of Stopped-Flow Parameters. Oscilloscope traces of the time course of fluorescence were photographed on Polaroid Type 107 black and white film. The photographs were then traced on Keuffel and Esser Co. Albanese Guide Line 10-5663 millimeter paper. Values of curve height and integrated curve area were obtained from these traces. Corrections for changes in substrate tryptophan fluorescence of Dns-Gly-L-Trp, generally amounting to less than 10%, were based on fluorescence signals following hydrolysis and the known relative contributions of species present. Calculations utilizing the ratio of curve height to area and those using the phenylalanyl series of substrates do not require these small corrections.

When the constant, K_D, was the parameter to be determined by the stopped-flow studies, trial values of it based on steady state K_m values (Latt *et al.*, 1972a) were introduced and then recursively modified. Determinations of K_D at two enzyme concentrations were identical, while the concentration of free substrate, $[S_F]$, varied from 50 to 90% of the total substrate, $[S_T]$.

Results

The rapid formation and breakdown of carboxypeptidase A complexes with N-dansylated peptide substrates can be observed directly by stopped-flow fluorescence (Latt *et al.*, 1970a,b, 1972a). Energy transfer between enzyme tryptophanyl residues and the dansyl group of the bound substrates permits visualization of these E·S complexes. The oscilloscope tracing shown in Figure 1 demonstrates the rapid enhancement in dansyl fluorescence following mixing of equal molar amounts, 2.5×10^{-4} M, of Dns-Gly-L-Phe and carboxypeptidase A. The increase in dansyl fluorescence during the "dead time" of the instrument proceeds to the identical maximal fluorescent values which are observed during the time period apparent from the tracing in Figure 1, demonstrating extremely rapid equilibration of enzyme and substrate to form the E·S complex. The decrease in the signal is a considerably slower process and reflects a diminution in enzyme-bound substrate, as hydrolysis reduces the substrate concentration. A complementary pattern is observed when quenching of enzyme tryptophan fluorescence by the dansyl group of bound substrate is measured.

The maximal fluorescence, F_{max}, is directly proportional to the maximum concentration of the enzyme–substrate complex, $[E \cdot S]_{max}$ (Figure 2). The proportionality factor, C, is a composite of instrumental features, fluorescence quantum yields, and internal filter effects. Proportionality is maintained throughout the time course of the reaction since these factors do not change appreciably as the substrate is hydrolyzed.

The area under the oscilloscope tracing, A, is related to the concentration of the E·S complex by eq 1.

$$A = C\int_0^\infty [E \cdot S]dt \qquad (1)$$

The quantities of F_{max} and A are related to the equilibrium dissociation constant, K_D, and the rate constant for the rate-determining step, k_{cat}[3] (Figure 2), as shown in eq 2–4, in a manner similar to that suggested by Chance for absorbance changes in peroxidase (1943). Since the total substrate added, S_0, is converted completely to products, P

$$S_0 = \int_0^\infty \frac{dP}{dt}\,dt = k_{cat}\int_0^\infty [E \cdot S]dt = \frac{k_{cat}}{F_{max}} A[E \cdot S]_{max} \qquad (2)$$

[3] The rapid equilibration of carboxypeptidase and substrate to form the E·S complex (Figure 1) and previous kinetic data for blocked oligopeptide substrates (Auld and Vallee, 1970a,b) indicate that the action of carboxypeptidase is entirely in accord with classical Michaelis–Menten kinetics, where K_D is the dissociation constant of the E·S complex (Dixon and Webb, 1964).

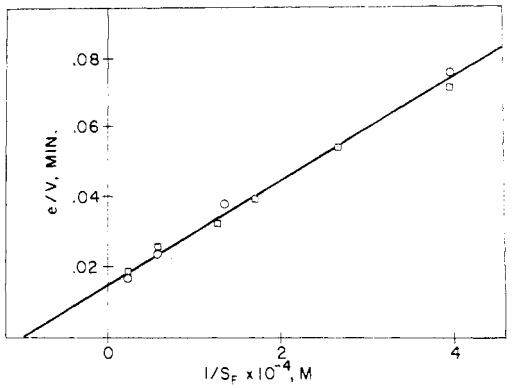

FIGURE 3: Determination of the kinetic parameters, k_{cat} and K_D, for Dns-Gly-L-Trp hydrolysis by stopped-flow fluorescence. Oscilloscope tracing of dansyl fluorescence was obtained at 1.25×10^{-4} M (⊙) or 5.0×10^{-5} M carboxypeptidase (⊡) and Dns-Gly-L-Trp concentrations from 2.5×10^{-5} M to 5.0×10^{-4} M in 1 M NaCl–0.05 M Tris, pH 7.5, at 25°. The rate, V, is obtained from the ratio of F_{max} $[S]_0/A$ for each substrate concentration. The kinetic parameters k_{cat} and K_D are obtained as shown in eq 3.

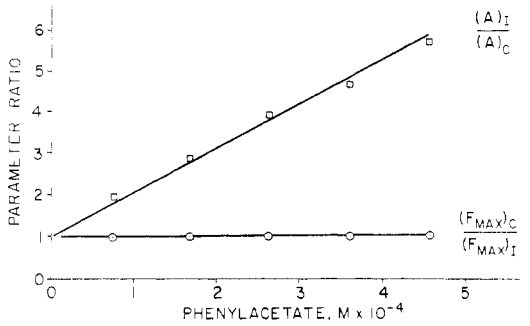

FIGURE 5: Stopped-flow fluorescence analysis of the noncompetitive inhibition of Dns-Gly-L-Trp hydrolysis by phenylacetate. The conditions of the assay were 1.0 M NaCl–0.05 M Mes, pH 5.5, 25°, with enzyme and substrate concentrations of 5.0×10^{-5} and 7.5×10^{-5} M, respectively. For each assay obtained in the presence of inhibitor, values of F_{max} and area are obtained. For convenience in plotting, these values have been normalized to an assay done in the absence of inhibitor. The noncompetitive inhibition constant is calculated from the slope of the line representing A_I/A_C *vs.* inhibitor concentration (eq 4).

Expressing $[E \cdot S]_{max}$ in terms of the total enzyme concentration, $[E_T]$, and K_D, then

$$\frac{F_{max}[S]_0}{A} = k_{cat}[E_T]\frac{[S_F]}{K_D + [S_F]} \quad (3)$$

A negligible amount of product is formed before attainment of F_{max}, due to the large difference between the time which is required for equilibration of the E·S complex as compared with that during which hydrolysis occurs (*vide supra*). The concentration of free substrate, $[S_F]$, therefore equals $[S]_0 - [E \cdot S]_{max}$. When the fraction of substrate in the form E·S becomes appreciable, $[S_F]$ must be evaluated as described in the Methods section. With improved instrumental sensitivity it should be possible to work at conditions of $[E_T] < [S_T]$ such that $[S_T]$ approximates S_F.

$(F_{max}/A)[S]_0$ is analogous to V, the steady state reaction rate; $k_{cat}[E_T]$ is analogous to V_{max} and K_D to K_m. The reciprocal of eq 3 is therefore equivalent to a Lineweaver–Burk plot (1934) and allows the determination of the parameters k_{cat} and K_D. The values of $k_{cat} = 69$ min^{-1} and $K_D = 1 \times 10^{-4}$ M for Dns-Gly-L-Trp (Figure 3) are in excellent agreement with those derived from ninhydrin analysis of tryptophan production, $k_{cat} = 65$ min^{-1} and $K_M = 1.2 \times 10^{-4}$ M (Latt *et al.*, 1972a).

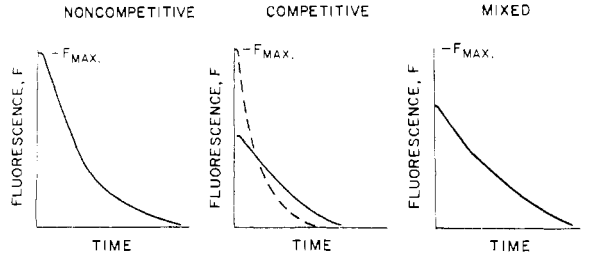

FIGURE 4: Effect of inhibitors on the formation and breakdown of E·S complexes. The uninhibited reaction is represented by the symbol F_{max} on the ordinate and dotted line in the central panel. The solid lines are the traces expected for inhibitors which possess noncompetitive, competitive, and mixed inhibition modes.

Since the area, A, reflects the catalytic rate and F_{max} the binding strength of substrates, modes of inhibition can be deduced directly from the effects of inhibitors on A and F_{max} (Figure 4). Thus, a noncompetitive inhibitor will retard hydrolysis of bound substrate and thereby increase A. However, F_{max} remains unchanged since substrate binding is unaffected.[4] Conversely, a competitive inhibitor will reduce F_{max}, owing to a reduction of substrate binding, but A remains constant since the rate-determining step is unchanged. Mixed inhibition representing both modes would be expected to alter both F_{max} and A. Accordingly such inhibition can be resolved into competitive and noncompetitive components since effects on F_{max} and A can be determined independently.

Therefore, inhibition constants are derived from the dependence of A and F_{max} on inhibitor concentrations *at a single substrate concentration*. The effect of phenylacetate on 8×10^{-5} M Dns-Gly-L-Trp at pH 5.5 is an example of noncompetitive inhibition (Figure 5). For each of the six inhibitor concentrations the stopped-flow assay yields one area and one F_{max} value characteristic of each. These values are normalized to an assay performed in the absence of inhibitor obviating the evaluation of the constant C. The inhibition constant K_I is calculated from the slope of the line A_I/A_C *vs.* [I] as in eq 4,

$$\frac{A_I}{A_C} = 1 + [I]/K_I \quad (4)$$

where the subscripts C and I refer to control and inhibitor values, respectively.

The slope of the line for phenylacetate inhibition yields an inhibition constant of 1×10^{-4} M. This constant is in good agreement with that obtained for phenylacetate inhibition of Dns-Gly-L-Trp, Cbz-Gly-Gly-L-Phe, and Cbz-Gly-Gly-L-Leu when performed by ninhydrin analysis of the rate of production of the amino acid under these conditions.

The stopped-flow fluorescence analysis for L-phenylalanine inhibition of the hydrolysis of Dns-Gly-L-Phe at pH 7.5 is

[4] Classically, uncompetitive inhibition is interpreted as reflecting binding of the inhibitor only to the E·S complex (Dixon and Webb, 1964). Such inhibition should cause an increase both in area and in values of F_{max}.

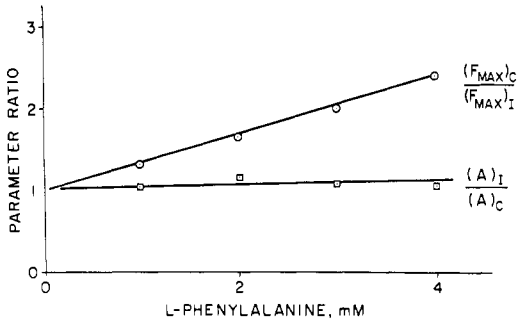

FIGURE 6: Stopped-flow fluorescence analysis of the competitive inhibition of Dns-Gly-L-Phe hydrolysis by L-phenylalanine. The conditions of the assay were 1.0 M NaCl–0.05 M Tris, pH 7.5, 25° with enzyme and substrate concentrations of 5.0×10^{-5} and 7.5×10^{-5} M, respectively. Data were obtained and treated as described in Figure 5. The competitive inhibition constant is obtained from the slope of the line representing $(F_{max})_C/(F_{max})_I$ *vs.* inhibitor concentration (eq 5).

indicative of competitive inhibition (Figure 6). In marked contrast to noncompetitive inhibition, F_{max} decreases with increasing inhibitor concentration while A remains constant, as would be expected for a competitive inhibitor. The inhibition constant, K_I, is derived from the relationship[5]

$$\frac{(F_{max})_C}{(F_{max})_I} = 1 + \frac{[I]/K_I}{1 + [S_F]/K_D} \quad (5)$$

The competitive inhibition constant thereby obtained, 2×10^{-3} M, is in good agreement with results derived from steady state spectrophotometric and fluorometric analysis (Elkins-Kaufman and Neurath, 1949; Whitaker *et al.*, 1966; Latt *et al.*, 1972b).

Phenylacetate noncompetitively inhibits Dns-Gly-L-Trp at pH 5.5, but the stopped-flow fluorescence analyses performed as a function of pH reveal that the type of inhibition observed is markedly dependent on pH. Mixed inhibition occurs at pH 7.5 (Figure 7). Relative to the control there is an increase in A and a slight decrease in F_{max}. However, at pH 9.5 both F_{max} and A are altered markedly.

Mixed inhibition, observed for phenylacetate over most of the pH range, can be resolved into its component noncompetitive and competitive modes at a single substrate concentration. For example, at pH 8.3 with Dns-Gly-L-Trp (8×10^{-5} M) the ratios of both A and F_{max} vary linearly with phenylacetate concentration[6] (Figure 8). At this pH the inhibition constants, calculated from the slopes of these lines, are nearly equal. These results are consistent with the steady state analysis of the inhibition by this agent at pH 8.3. Dixon plots for Cbz-Gly-Gly-L-Leu concentrations at least tenfold below K_m are curved markedly upward as inhibitor concentrations are increased two–threefold above the apparent K_I. If substrate concentrations are chosen at least tenfold above K_m, such curvature disappears, suggesting that multiple inhibitor binding might be involved and that at least one of the modes

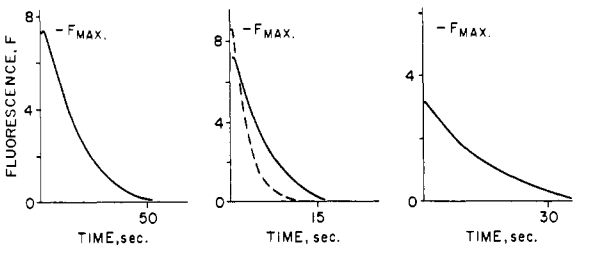

FIGURE 7: Variation in modes of phenylacetate inhibition as a function of pH. The assays were performed at 25° with carboxypeptidase and Dns-Gly-L-Trp both 1.25×10^{-4} M in 1 M NaCl–0.05 M Mes, pH 5.5 (left), –0.05 M Tris, pH 7.5 (center), and –0.05 M ammediol, pH 9.5 (right), with phenylacetate concentrations of 0.1, 2.0, and 10 mM, respectively. The uninhibited reaction represented by the symbol F_{max} on the ordinate and dotted line, is applicable to all three panels.

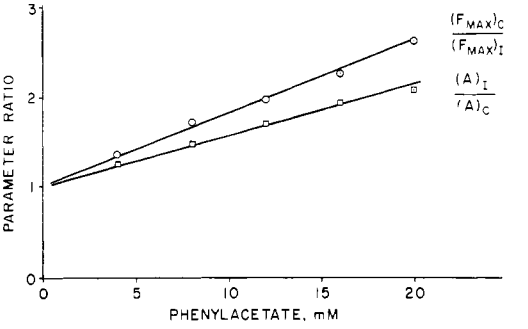

FIGURE 8: Resolution of the mixed inhibition of phenylacetate into its noncompetitive and competitive components. The stopped-flow assays were performed at pH 8.3, 25°, 0.05 M Tris–1 M NaCl. The enzyme and Dns-Gly-L-Trp concentrations were 5.0×10^{-5} and 7.5×10^{-5} M, respectively. The competitive and noncompetitive inhibition constants are calculated from the slopes of these lines (eq 4 and 5).

of inhibition is of a competitive nature (Auld, 1972).[7] Stopped-flow analyses of the effects of β-phenylpropionate, β-iodopropionate, and butyrate demonstrate that they, too, display mixed inhibition, the noncompetitive component being more prominent at pH 7.0 than at pH 8.5.

Further resolution of the mixed inhibition by phenylacetate can be obtained by examining the influence of pH on the inhibition constants of the component modes (Figure 9). The predominant noncompetitive inhibition mode of phenylacetate is strongest at low pH, decreasing 500-fold over the pH range from 6 to 9. The pH dependence of a competitive inhibition mode, apparent at high inhibitor concentrations, is quite different, suggesting that these effects might be due to binding of at least two inhibitor molecules.

Discussion

Reversible inhibitors may affect either the apparent K_m or V_{max} of an enzymatic reaction (Dixon and Webb, 1964). When the mode of action of an inhibitor is defined in the now classical manner, both the substrate and the inhibitor concentrations must be varied in order to assign and quantitate the inhibition mode. However, if an enzyme–substrate complex can be visualized directly, the type of inhibition can be identified using a single substrate concentration (Figure 4).

[5] Equation 5 can be simplified to the same form as eq 4 if $K_D \gg [S_T] > [E_T]$.

$$\frac{(F_{max})_C}{(F_{max})_I} = 1 + [I]/K_I$$

[6] Two modes of phenylacetate inhibition at pH 8.3 are also observed when the purified γ-Val alleomorphic form of carboxypeptidase (Petra and Neurath, 1969) is used as the enzyme.

[7] Unpublished data.

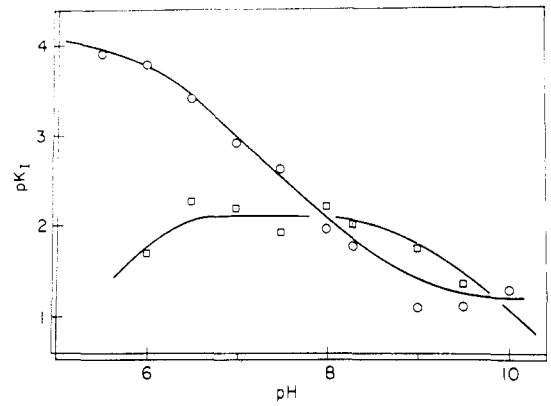

FIGURE 9: The pH dependence of the noncompetitive (⊙) and competitive (⊡) inhibition of Dns-Gly-L-Trp hydrolysis by phenylacetate. The pH-dependent inhibition constants were obtained as shown in Figure 8. The concentrations of Dns-Gly-L-Trp and carboxypeptidase were both 1.25×10^{-4} M. The assays were performed at 25° in 1 M NaCl and 0.05 M Mes (pH 5.5–7.0), 0.05 M Tris (pH 7.5–8.3) and 0.05 M ammediol (pH 9.0–10.0).

In the particular case here studied, carboxypeptidase, the rapid formation and breakdown of E·S complexes can be observed directly by stopped-flow fluorescence. These measurements have utilized a series of N-danylated peptide substrates whose inherent fluorescent properties allow visualization of the E·S complex through measurement of the energy transfer between enzyme tryptophanyl residues and the dansyl group of the bound substrate (Latt *et al.*, 1970a,b). The maximal fluorescence and the decay of the signal can be related to Michaelis–Menten parameters in a manner similar to that suggested by Chance (1943) for absorbance changes in peroxidase. The maximal fluorescence, F_{max}, is directly proportional to the steady state concentration of the enzyme–substrate complex, $[E \cdot S]_{max}$, and the area under the curve, A, is inversely proportional to the hydrolysis rate, k_{cat}. Measurements of F_{max} and A values at different substrate concentrations allow determination of the kinetic parameters K_D and k_{cat} (eq 3). The values of k_{cat} and K_D for the dipeptide Dns-Gly-L-Trp are in excellent agreement with the kinetic parameters k_{cat} and K_M obtained by ninhydrin analyses of tryptophan production. In fact, the stopped-flow studies performed on dansyl peptides are consistent with K_m being a true measure of the binding strength of peptide substrates.

Inhibition constants could be determined by the effect the inhibitors would have on k_{cat} and K_D. However, such constants are more easily derived from the dependence of A and F_{max} on inhibitor concentration (Figures 5 and 6 and eq 4–6). When this is done for noncompetitive and competitive inhibitors of carboxypeptidase A, the inhibition constants obtained are in excellent agreement with those obtained by standard steady state procedures measuring product formation.

The present studies also reveal that the type of inhibition observed for a number of inhibitors such as phenylacetate vary as a function of pH. At pH 5.5 phenylacetate exhibits pure noncompetitive inhibition toward Dns-Gly-L-Trp hydrolysis (Figure 5). However, mixed inhibition is observed at pH 7.5 and 9.5 (Figure 7).[6] The stopped-flow analyses resolve this mixed inhibition into its noncompetitive and competitive components while employing a single substrate concentration. At pH 8.3 the inhibition constants calculated from the variation of the ratios of F_{max} and A as functions of inhibitor concentrations are nearly equal (Figure 8). However, determination of these constants as a function of pH shows that the noncompetitive and competitive inhibition constants differ significantly in their dependence on pH (Figure 9) suggesting that these effects might be due to binding of at least two inhibitor molecules. The marked curvature seen in Dixon plots at substrate concentrations well below K_m is also consistent with multiple binding of such inhibitors (Auld and Vallee, 1970a). Phenylacetate and other inhibitors of similar structure (Latt and Vallee, 1971; Latt, 1971) also perturb the absorption spectrum of cobalt carboxypeptidase in two distinct ways. Moreover, X-ray data indicate two metal dependent binding sites for β-iodophenylpropionate as well as additional binding in the active center region of the crystalline enzyme (Steitz *et al.*, 1967). While all of these studies are consistent with multiple binding of inhibitors such as phenylacetate, the present studies show in addition how such multiple binding can affect the hydrolysis of a peptide substrate.

The pH dependence of the resolved phenylacetate inhibition can be fitted readily to the protonation scheme proposed for peptide hydrolysis (Auld and Vallee, 1970b). This reaction scheme requires minimally three forms of free enzyme species, $EH_2 \rightleftharpoons EH \rightleftharpoons E$, and two forms of enzyme–substrate complexes, one nonproductive, EH_2S, and the other productive, EHS. Noncompetitive inhibition by phenylacetate appears to occur most readily by displacing the equilibrium to EH_2SI. Binding of the inhibitor to EH_2S is at least 100-fold stronger than that to EHS. In marked contrast, the competitive mode of inhibition appears to bind more strongly to the EH form of the enzyme than to the EH_2 or E forms.

Although these inhibition studies of carboxypeptidase E·S complexes have been accomplished by means of fluorescence measurement of energy transfer, the approach is applicable to any technique capable of detecting the formation and breakdown of E·S complexes, *e.g.*, absorption, nuclear magnetic and electron paramagnetic resonance or circular dichroism spectroscopy. The fluorescence method used here is sensitive, and sufficiently rapid for detection of the interconversion of E·S complexes. Although the effectors of substrate hydrolysis employed in this study were inhibitors, the approach is equally suitable to studying the effect of activators on enzyme catalysis. The generality of this fluorescence method for E·S complex measurement should facilitate wide application to mechanistic studies of other enzymes.

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Affinity Labeling of a Mouse IgG2a Myeloma Protein with Binding Affinity for Nitrophenyl Ligands†

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ABSTRACT: A nitrophenyl-binding IgG2a myeloma protein, HPC-3, has been affinity labeled with the reagent [^{3}H]*m*-nitrobenzenediazonium fluoborate under conditions previously used with anti-2,4-dinitrophenyl (Dnp) antibodies. Specific affinity labeling of the active sites of HPC-3 protein was produced, by the criterion that two to three times as much radioactivity was covalently bound to the protein in the absence as in the presence of an excess of the reversibly bound protector, Dnp-aminocaproate. The specific label was predominantly in the form of *m*-nitrobenzeneazotyrosine. About 90% of the affinity label was bound to the H chains of HPC-3 protein, but the 10% of label attached to L chains was also at least partly specific for the active sites. These affinity-labeling results are similar to those obtained with mouse anti-Dnp antibodies, but by several criteria, the active sites of HPC-3 protein and of elicited anti-Dnp antibodies can be distinguished.

Affinity labeling has been applied to the study of the active sites of a variety of anti-hapten antibodies (*cf.* Singer *et al.*, 1967; Wofsy *et al.*, 1967). Detailed structural investigations of the labeled antibodies have been successful to some extent (Thorpe and Singer, 1969; Cebra *et al.*, 1971), although they are made difficult by the gross molecular heterogeneity of most antihapten antibody preparations, even from an individial animal. Myeloma proteins are usually homogeneous immunoglobulins, some of which have been shown to bind certain small molecules with considerable specificity (Eisen *et al.*, 1967, 1970; Potter and Leon, 1968; Cohn *et al.*, 1969). It is clearly of interest to attempt to affinity label these ligand-binding myeloma proteins, and to assess the relevance of the results obtained to those for affinity-labeled elicited anti-hapten antibodies. Metzger and Potter (1968) showed that the mouse IgA[1] myeloma protein MOPC-315, with binding affinity for Dnp[2] ligands, could be affinity labeled with the same specific diazonium reagents used to label elicited anti-Dnp antibodies (Metzger *et al.*, 1963; Good *et al.*, 1967). Further affinity labeling studies with MOPC-315, using other types of reagents, have been reported (Haimovitch *et al.*, 1970). In this paper, we report the results of affinity labeling of a mouse IgG2a myeloma protein, HPC-3 (Warner and Ovary, 1970) with the diazonium reagent [^{3}H]MNBDF. There is specific affinity labeling of the active site of the protein at tyrosine residue(s) only. The specific label is predominantly on the H chain of the protein, but the small amount of label on the L chain appears also to be at least partly specific. The significance of these and other results in assessing the relationship between hapten-binding myeloma proteins and elicited anti-hapten antibodies is discussed.

Materials and Methods

The induction of the plasma cell tumor HPC-3 in inbred NZB mice, and isolation and partial characterization of the pure HPC-3 myeloma protein has been described (Warner and Ovary, 1970). The mouse anti-Dnp antibodies were raised in Swiss-Webster mice by immunization with Dnp-hemocyanin, and the pooled antibodies were isolated, according to published procedures (Thorpe and Singer, 1969).

For most of the experiments reported in this paper, the HPC-3 protein was reacted with the affinity-labeling reagent [^{3}H]MNBDF under the same conditions used in earlier experiments with rabbit and mouse anti-DNP antibodies (Good *et al.*, 1967; Thorpe and Singer, 1969), with or without a 13-fold or 100-fold excess of the specific protector, *N*-Dnp-ε-aminocaproate. For spectral analyses of the products of the affinity-labeling reaction, nonradioactive MNBDF was used under the same conditions. For all the labeling experiments with HPC-3 protein, parallel experiments were carried out with a pooled preparation of pure mouse anti-Dnp antibodies.

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[1] Nomenclature for immunoglobnlins corresponds to that recommended by the World Health Organization (*Bull. W.H.O. 30*, 447 (1964)).

[2] Abbreviations used are: Dnp, 2,4-dinitrophenyl; MNBDF, *m*-nitrobenzenediazonium fluoroborate; DnpNS, 2-(2,4-dinitrophenylazo)-1-naphthol-3,6-disulfonate.