Multiple Inhibitor Binding to Arsanilazotyrosine-248 Carboxypeptidase A†

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ABSTRACT: Selective modification of tyrosine-248 of carboxypeptidase A with diazotized arsanilic acid introduces a chromophoric probe into the enzyme's active center which forms an intramolecular chelate with the active site zinc atom of the enzyme in solution. This single modification actually results in two spectral probes: azoTyr-248 itself and the azoTyr-248·Zn coordination complex. The binding of two different molecules of the inhibitor β-phenylpropionate to two distinct sites was observed, and binding constants were calculated using circular dichroic changes of both probes (Johansen, J. T., Klyosov, A. A., & Vallee, B. L. (1976) *Biochemistry 15,* 296). Thus, each spectral probe can monitor binding of ligands. In principle, circular dichroic titrations should be able to recognize and quantitate the effect of one ligand on the binding of another directly. Toward that end we have now performed equilibrium titrations with pairs of ligands to determine such mutual effects employing several ligand combinations including a pseudosubstrate, glycyl-L-tyrosine, inhibitors, e.g., L-phenylalanine, β-phenylpropionate, and indole 3-acetate, and the activator, cyclohexanol. In this manner we have identified four separate ligand binding sites: site 1, where glycyl-L-tyrosine and L-phenylalalanine bind; sites 2 and 3, where one molecule each of either indole 3-acetate or β-phenylpropionate bind; and site 4, where cyclohexanol binds. Further, we have determined to what extent binding at each of these four sites affects that at the three other sites. Ligand association with site 1, 3, or 4 significantly perturbs the affinity of the two other sites, while association with site 2 has only a small effect on binding at site 1 or 4 and no effect on binding at site 3. The ligands serve as a means of identifying subsites in the area of the enzyme active center and, thus, the results are consistent with the multiple overlapping binding site hypothesis previously proposed by us based on kinetic evidence. Further, with these observations the interactions among a number of binding sites can be deduced, thereby providing a rational basis to relate kinetic to thermodynamic observations regarding the organization of the enzyme's active center.

It was proposed several years ago that multiple overlapping binding sites for substrates, products, and inhibitors characterize the active center of carboxypeptidase A in solution. This hypothesis provides a rational basis for complex enzyme kinetics observed (Vallee et al., 1968). We have now utilized the chromophoric azotyrosine-248 residue of azocarboxypeptidase[1] (Johansen et al., 1972) to monitor the interactions of substrates, products and inhibitors with the enzyme. The selective modification of tyrosine-248 places a spectral probe in its active center region. Remarkably, this modified tyrosine has dual potential as a probe (Johansen & Vallee, 1975; Johansen et al., 1976). On the one hand it forms a complex with the active site zinc atom characterized by typical circular dichroic and absorption spectra with intense bands centered at 510 nm and, hence, monitors the juxtaposition and topological relationships of these components of the active center. On the other hand, the uncomplexed azotyrosine-248 alone also has distinctive circular dichroic and absorption spectra, reflecting its intrinsic, local conformation, but completely lacks the 510-nm maximum. On interaction with azocarboxypeptidase, a wide variety of ligands encompassing substrates, products, inhibitors, and activators disrupt the intramolecular azotyrosine·Zn coordination complex abolishing the 510-nm band and variously affecting the spectrum at shorter wavelengths.

We have previously shown that spectral titrations quantitate ligand binding. Indeed, the spectra of both the azotyrosine·Zn coordination complex and of uncomplexed azotyrosine quantitate the successive binding of two molecules of a single ligand (Johansen et al., 1976). The present studies extend this approach to encompass titrations with pairs of different ligands by holding the concentration of one constant and varying the other systematically. The response of the probe system to such ligand-pair titrations reflects the manner in which the binding of the two ligand molecules mutually influence one another. The results verify the multisite nature of the enzyme active center (Vallee et al., 1968) and, further, quantitate the interactions between its multiple ligand binding sites.

Materials and Methods

Carboxypeptidase A_α, prepared by the method of Cox et al. (1964), was obtained as a crystal suspension from Sigma Chemical Co. Tyrosine-248 was modified selectively by means of diazotized arsanilic acid, as previously described (Johansen & Vallee, 1971). The resultant enzyme contained 0.95–1.01 mol of monoarsanilazotyrosine and less than 0.03 mol of arsanilazohistidine per mol of enzyme. Protein concentrations were determined spectrophotometrically at 278 nm (Johansen & Vallee, 1975), using an absorptivity of $\epsilon_{278} = 7.32 \times 10^{-4}$ M^{-1} cm^{-1}.

β-Phenylpropionic acid was purchased from Aldrich Chemical Co. Inc. and purified by recrystallization from water

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[1] Carboxypeptidase A is also referred to as native carboxypeptidase or carboxypeptidase. To simplify presentation azocarboxypeptidase is used interchangeably with monoarsanilazotyrosine-248·zinc carboxypeptidase. Azotyrosine-248 (azoTyr-248) or azotyrosine refer to the azophenol of monoarsanilazotyrosine-248. Abbreviations employed: Gly-L-Tyr, glycyl-L-tyrosine; L-Phe, L-phenylalanine; C-Hex, cyclohexanol; I-3-Ac, indole 3-acetate; β-φ-Prop, β-phenylpropionic acid.

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and subsequent sublimation. Reagent grade cyclohexanol (Fisher Scientific Co.) was purified further by vacuum distillation. Indole 3-acetate (Sigma Chemical Co.) was sublimed prior to use. L-Phenylalanine and glycyl-L-tyrosine were purchased from Fox Chemical Co. and recrystallized from ethanol and water prior to use. All solutions were freed of adventitious metals by extraction with 0.1% dithizone in carbon tetrachloride. Glassware and cuvettes were freed of metals by soaking in nitric acid and thorough rinsing in metal-free distilled water.

Circular dichroic spectra were obtained using a Cary Model 61 recording spectropolarimeter. Spectral titrations were performed by adding ligands to enzyme solutions either singly or in pairs. All solutions were made 0.5 M in NaCl and buffered in 0.05 M Tris-Cl at pH 8.0.

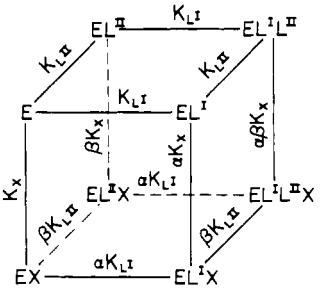
The Tyr-248·Zn complex forms maximally at pH 8.5; hence, that pH would be expected to be optimal to monitor changes in the 510-nm extremum of the circular dichroic spectrum. However, at that pH, the spectral intensity at shorter wavelengths is low. Therefore, to optimize the intensity of the circular dichroic spectrum throughout the visible region, titrations were performed at pH 8.0 where shorter wavelength extrema are more intense and, hence, can also serve to monitor ligand binding (Johansen & Vallee, 1973, 1975). When titrating with a single ligand, successive microliter aliquots of ligand were added to a 1.0-mL volume of enzyme. Spectra from 300 nm to 650 nm were recorded after each addition. When titrating with ligand pairs, the enzyme was incubated with one ligand, hereafter referred to as the "fixed" ligand, and then titrated as described above with a second, the "titrant," ligand.

Analysis of Circular Dichroism Titrations. Titrations with a single ligand were analyzed as described previously by Johansen et al. (1976). One molecule each of β-phenylpropionate and indole 3-acetate binds to both a "primary" and a "secondary" site, a total of two in either instance. The first molecule of either ligand binds at a "primary" site and is quantitated by spectral changes at 550 nm; at this wavelength there are no contributions from extrema below 510 nm. The second molecules of both β-phenylpropionate and indole 3-acetate interacting at an independent and noninteraction "secondary" site are measured at 445 nm and 440 nm, respectively.[2]

In circular dichroism with pairs of ligands, the most complex case encountered dealt with the binding of one ligand to two independent sites plus a second ligand to a third site, as represented in Scheme I.

Enzyme–ligand complexes are depicted at the corners of the cube, and the equilibrium constants defining the reaction leading from one corner to another are placed on the edges. L is a ligand which may bind to two sites, an L^I (primary) site and an L^{II} (secondary) site, while X is a ligand which binds only to a single site, termed an X site. The equilibrium constants for each reaction are expressed as dissociation constants and carry a subscript to indicate which binding site is being considered. α, β, and γ are interaction constants which are equal to the ratio of the dissociation constant defining binding at a particular site—with a specific, second site occupied—to

SCHEME I



that which obtains while that second site is vacant. Thus, where appropriate, dissociation constants are multiplied by one or more interaction constants, i.e., α denoting the interaction between the X and L^I sites, β the interaction between the X and L^{II} sites, and γ the interaction between L^I and L^{II} sites.[2] Ligand binding to the X and the L^I sites disrupts the azotyrosine·Zn complex, while binding at the L^{II} site perturbs the uncomplexed azotyrosine chromophore.

As the circular dichroic spectra for the various ligand complexes vary, so do the different formulations relating the equilibrium parameters to the spectral changes observed at each wavelength. The binding of one or two ligands can be monitored by proper choice of experimental conditions or wavelengths, and binding parameters can be calculated from the spectral titrations. Thus, saturation of the X site or the L^I site with ligand abolishes the ellipticity at 550 nm. In contrast, binding at the L^{II} site does not affect the spectrum at 550 nm but causes substantial changes in the 440–450-nm region. Thus, in the absence of X, K_{LI} and K_{LII} may be obtained directly from such titrations (Johansen et al., 1976). In the presence of saturating quantities of X, spectral changes observed in the 440–450-nm region are the result of binding of L to a secondary site, described by

$$\frac{1}{\Delta\theta} = \frac{1}{[L]}\frac{\beta K_{LII}}{\Delta\theta_M} + \frac{1}{\Delta\theta_M} \qquad (1)$$

Here $\Delta\theta$ is the difference in ellipticity between that of the EX complex and that observed at a given concentration of L; $\Delta\theta_M$ is the analogous difference determined at saturating concentrations of both X and L. Since K_{LII} is known a plot of $1/\Delta\theta$ vs. $1/[L]$ allows determination of β.

The other interaction coefficient, α, is obtained from plots at 550 nm where spectral changes are related to the relevant dissociation and interaction constants as described in eq 2.

$$F = \frac{\Delta\theta}{\Delta\theta_M - \Delta\theta} = \frac{[L]}{K_{LI}}\left(1 + \frac{[X]}{\alpha K_X}P\right) + \frac{[X]}{K_X}P \qquad (2)$$

Here $\Delta\theta$ is the difference in ellipticity between that observed at a given concentration of L and X and that of the enzyme alone. $\Delta\theta_M$ is the maximal ellipticity change observed, i.e., at complete saturation by ligand L and at a given concentration of X and

$$P = \frac{1 + ([L]/\beta K_{LII})}{1 + ([L]/K_{LII})} \qquad (3)$$

A plot of F vs. [L] at various fixed concentrations of X results in a family of lines, one for each concentration of X. When extrapolated, they intersect at a point the coordinates of which are

[2] The linearity of double-reciprocal plots indicates that the two ligand binding sites for a single ligand, I, are independent and noninteracting. If the sites are interacting, the double-reciprocal plot is represented by $1/\Delta\theta = (1/[I])(K_{II}/\Delta\theta_M)((1 + ([I]/K_{II}))/(1 + ([I]/\gamma K_{II})) + (1/\Delta\theta_M)$ even when binding at only one site affects the spectrum. The symbols have the meaning described in the Materials and Methods section and γ represents interaction between the ligand binding sites. When γ is not equal to 1, i.e., when the sites interact, the equation does not represent a straight line. However, when γ does equal one, i.e., the sites do not interact, the equation becomes linear, being $1/\Delta\theta = (1/[I])(K_{II}/\Delta\theta_M) + (1/\Delta\theta_M)$.

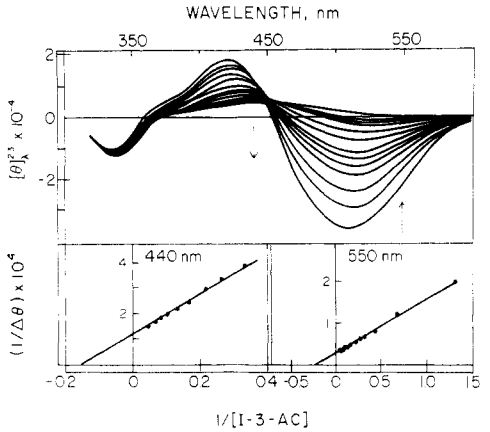


FIGURE 1: (Upper panel) Effect of I-3-Ac on the circular dichroic spectrum of azocarboxypeptidase in 0.05 M Tris, 0.5 M NaCl, pH 8.0, 23 °C. I-3-Ac concentration is varied between 0 and 25 mM. (Lower panel) Double-reciprocal plots ($1/\Delta\theta$ vs. $1/[(I\text{-}3\text{-}Ac])$ on the right at 550 nm and on the left at 440 nm, both calculated from the circular dichroism titrations in the upper panel.

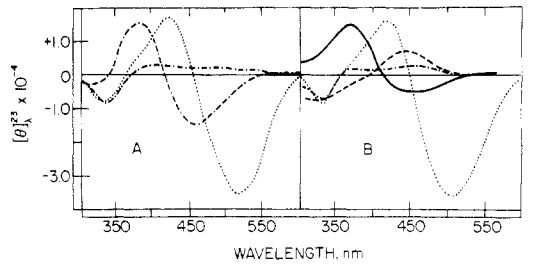


FIGURE 2: (Panel A) Effect of a large excess of β-ϕ-Prop (150 mM) (• -- •) on the circular dichroic spectrum of azocarboxypeptidase preincubated with sufficient I-3-Ac (20 mM) to produce the spectrum of the azocarboxypeptidase·I-3-Ac complex (- • - •). The effect of a large excess of I-3-Ac (130 mM) (- • - •) on the spectrum of azocarboxypeptidase preincubated with sufficient β-ϕ-Prop to produce the spectrum of the azocarboxypeptidase·β-ϕ-Prop complex (• - • -) is also shown. (Panel B) Effect of saturating concentrations of Gly-L-Tyr (----), I-3-Ac (• - • -), and I-3-Ac (30 mM) plus Gly-L-Tyr (20 mM) (—) on the spectrum of azocarboxypeptidase. Solutions used for data in panels A and B were buffered in 0.5 M NaCl, 0.05 M Tris, pH 8.0.

$$-F = \frac{\frac{1}{K_{L^I}}}{\frac{1}{K_{L^{II}}}\frac{(1-\beta)}{\beta} + \frac{1}{\alpha K_{L^I}}} \qquad (4)$$

$$-[L] = \frac{1}{\frac{1}{K_{L^{II}}}\frac{(1-\beta)}{\beta} + \frac{1}{\alpha K_{L^I}}}$$

Since K_{L^I}, $K_{L^{II}}$, and β are known, α may be calculated directly.

When L does not bind at a secondary site (i.e., $K_{L^{II}} = \infty$), the equations simplify so that α is obtained directly from the coordinates of the intersection point as described by

$$-F = \alpha; -[L] = \alpha K_{L^I} \qquad (5)$$

Alternatively after saturating with X, eq 1 can be used to determine α. Plots of such titrations according to eq 1 intersect the $1/[L]$ axis at a value equal to the negative reciprocal of the product, α, and the dissociation constant K_{L^I}. Since K_{L^I} is known, α is calculated easily.

The dissociation and interaction constants governing each step in Scheme I were determined by analyzing spectral changes at one or more wavelengths.

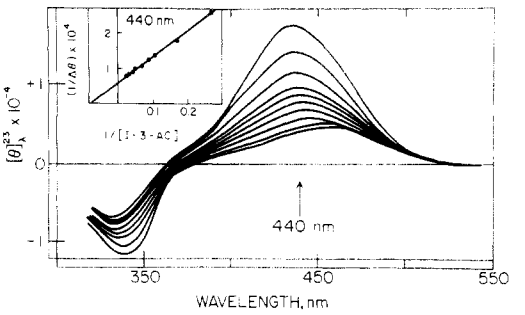


FIGURE 3: Effect of Gly-L-Tyr on the circular dichroic spectrum of azocarboxypeptidase (• • •) partially saturated with the fixed ligand, C-Hex (8.5 mM) (- - -). The titration was performed at 23 °C, pH 8.0, in 0.5 M NaCl, 0.05 M Tris.

TABLE I: Parameters for Ligand Binding to Azocarboxypeptidase Determined by Circular Dichroism Titrations (0.5 M NaCl, 0.05 M Tris, pH 8.0).

Ligand	K_{spec} (mM)
Gly-L-Tyr	2.3 ± 0.6
L-Phe	4.5 ± 0.5
C-Hex	8.6 ± 0.6
I-3-Ac, primary site	3.8 ± 0.4
I-3-Ac, secondary site	5.8 ± 0.3
β-ϕ-Prop, primary site	4.9 ± 0.5
β-ϕ-Prop, secondary site	8.8 ± 0.8

Results

Single Ligand Binding to Azocarboxypeptidase. Upon binding to azocarboxypeptidase, glycyl-L-tyrosine, L-phenylalanine, cyclohexanol, indole 3-acetate, and β-phenylpropionate disrupt the azotyrosine·Zn coordination complex abolishing ellipticity at 550 nm and variously affecting the spectrum at shorter wavelengths. The association of one molecule of each ligand with the enzyme was quantitated at 550 nm. Two molecules each of both β-phenylpropionate and indole 3-acetate associate with independent and noninteracting sites. A β-phenylpropionate or indole 3-acetate molecule binding to its higher affinity or "primary" (L^I) site abolishes the spectrum at 550 nm but has little effect at 445 nm or 440 nm, respectively. In contrast, binding of these agents at a "secondary" (L^{II}) site does result in changes at these wavelengths. On this basis, double-reciprocal plots can determine K_{L^I} and $K_{L^{II}}$ for both agents (Figure 1). Table I summarizes the results. The double-reciprocal plots for indole 3-acetate and β-phenylpropionate titrations are linear both at 550 nm and at 440 or 445 nm; hence, the L^I and L^{II} sites are independent and do not interact.[2]

Spectra of Azocarboxypeptidase–Ligand Complexes. All possible pairs of ligands were examined to determine which ligands could displace one another (i.e., bind to a common site) and which could bind to the enzyme simultaneously, i.e., occupy different sites. This was accomplished by first adding one ligand of a given pair to the enzyme until no further spectral changes were observed and then adding the second ligand in large excess (concentrations typically 15–25 times the dissociation constant). The experiment was then repeated, but reversing the order of addition of the two ligands. If they occupy a common site, then in both cases the final spectrum will be expected to be that characteristic of the ligand present in large

TABLE II: Binding of Ligand Pairs to Azocarboxypeptidase and Resultant Spectra.

Ligand pair	Binding	Final spectrum
Gly-L-Tyr + L-Phe	Mutually exclusive	Ligand added last
Gly-L-Tyr + C-Hex	Simultaneous	Unique
Gly-L-Tyr + I-3-Ac	Simultaneous	Unique
Gly-L-Tyr + β-φ-Prop	Simultaneous	β-φ-Prop
L-Phe + C-Hex	Simultaneous	Unique
L-Phe + I-3-Ac	Simultaneous	I-3-Ac
L-Phe + β-φ-Prop	Simultaneous	β-φ-Prop
C-Hex + I-3-Ac	Simultaneous	Unique
C-Hex + β-φ-Prop	Simultaneous	β-φ-Prop
β-φ-Prop + I-3-Ac	Mutually exclusive	Ligand added last

TABLE III: Interaction Constants as Derived from Ligand Pair Titrations at 550 nm (0.5 M NaCl, 0.05 M Tris, pH 8.0).

Ligand pair	Interaction constant α	Interaction constant β
Gly-L-Tyr + C-Hex	3.5 (3.2)[a]	
L-Phe + C-Hex	6.0 (5.6)	
I-3-Ac + Gly-L-Tyr	1.2	3.0
I-3-Ac + L-Phe	1.0	2.2
I-3-Ac + C-Hex	1.3	5.6
β-φ-Prop + Gly-L-Tyr	1.2	2.4
β-φ-Prop + L-Phe	1.0	3.6

[a] Values in parentheses obtained using eq 1 to analyze titrations performed at 450 nm.

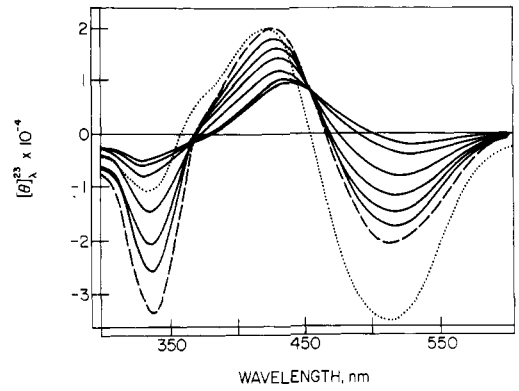


FIGURE 4: Ligand pair titrations of Gly-L-Tyr plus C-Hex performed as in Figure 3 and analyzed according to eq 2. Gly-L-Tyr is the titrant and C-Hex the fixed ligand. The fixed concentration of C-Hex for each titration varied from 0 (bottom line) to 15 mM (top line). All titrations were performed at 23 °C, pH 8.0, in 0.5 mM NaCl, 0.05 M Tris buffer.

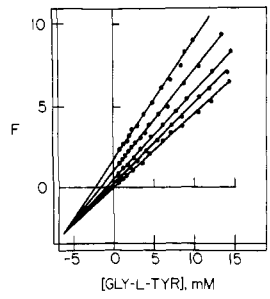


FIGURE 5: Ligand pair titrations of I-3-Ac plus L-Phe analyzed according to eq. 2. I-3-Ac is the titrant and L-phenylalanine the fixed ligand. The concentration of L-Phe, fixed for each titration, varied from 0 (bottom line) to 11 mM (top line). Titrations were performed at 23 °C, pH 8.0, in 0.5 M NaCl, 0.05 M Tris buffer.

excess, as is apparent, e.g., with the pair indole 3-acetate plus β-phenylpropionate (Figure 2A). If, independent of the order of addition, either the final spectrum in the presence of the two ligands differs from that of each ligand alone, as is the case with the pair glycyl-L-tyrosine and indole 3-acetate (Figure 2B), or the final spectrum is that characteristic of only one of the two ligands, again regardless of the order of addition, then binding is simultaneous. This indicates the presence of different sites of interaction for each ligand. The pair indole 3-acetate and L-phenylalanine exemplifies the last case (Figure 3). Here, saturation of the enzyme with L-phenylalanine followed by addition of a large excess of indole 3-acetate yields a spectrum characteristic of indole 3-acetate alone, and on this basis it might be concluded that the binding is competitive. However, the reverse experiment, where the enzyme is first "saturated" with indole 3-acetate followed by addition of a large excess of L-phenylalanine, again results in a spectrum characteristic of indole 3-acetate which cannot be abolished by addition of L-phenylalanine up to its limit of solubility. Therefore, these ligands do not compete; rather the binding of one masks the spectral effects of the other member of the pair. On the basis of these experiments, each ligand pair either binds simultaneously or mutually exclusively (Table II).

Ligands which associate with the enzyme simultaneously might bind either at sites which interact or which are completely independent. In order to characterize interaction between ligand binding sites quantitatively, detailed titrations of azocarboxypeptidase with such pairs of ligands have been performed. Individually, the ligands fall naturally into two groups: The first are those in which only one molecule of the ligand binds to the enzyme, i.e., glycyl-L-tyrosine, L-phenylalanine, cyclohexanol, and the second are those in which two molecules of ligand interact with the enzyme, i.e., β-phenylpropionate and indole 3-acetate (Table I). The first pairs examined comprise ligands of the first group which bind simultaneously, i.e., glycyl-L-tyrosine and L-phenylalanine, both paired with cyclohexanol. They are represented schematically by the front face of the cube in Scheme I. Ligand binding was analyzed using eq 2 based on the disruption of the azotyrosine-Zn coordination complex at 550 nm.

Figure 4 shows a typical example of circular dichroism changes in such ligand-pair titrations where cyclohexanol is the "fixed" ligand and glycyl-L-tyrosine the "titrant". The results of several such titrations with the latter in the presence of different concentrations of cyclohexanol are plotted as described by eq 2 (Figure 5). The resultant pattern characterizes interactions between the binding sites of the ligand pairs both qualitatively and quantitatively. Since the lines intersect, the value of α is finite (eq 5) confirming that both ligands bind to the enzyme simultaneously. The value of α, which quantitates the interaction between their binding sites, is determined by the coordinates of the intersection point as described by eq 5. Table III summarizes the interaction constants determined from such titrants at 550 nm.

The interaction constant, α, was also measured from experiments in which the enzyme was saturated first with one ligand and the resulting enzyme–ligand complex was then titrated with the second ligand. Spectral changes at 450 nm resulting from additions of the second ligand were analyzed according to eq 1. The apparent dissociation constants are products of the intrinsic dissociation constants of the "titrant" ligand and the interaction constant α (Scheme I). These interaction constants (the values in parentheses in Table III) are

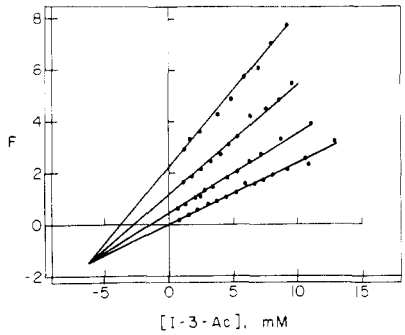


FIGURE 6: Effect of I-3-Ac on the circular dichroism spectrum of the azocarboxypeptidase·L-Phe complex at pH 8.0, 23 °C, in 0.5 M NaCl, 0.05 M Tris buffer. The insert depicts a double-reciprocal plot (eq 1) of spectral changes at 440 nm.

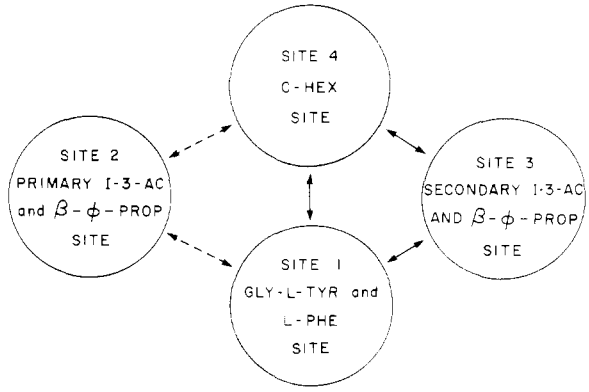


FIGURE 7: Schematic representation of ligand binding sites in the active center of carboxypeptidase A. Circles linked by solid arrows represent interacting sites while circles linked by broken arrows represent *weakly* interacting sites and circles which are not linked by arrows represent independent and noninteracting sites.

in excellent agreement with the values of α obtained from titrations of the azotyrosine·Zn complex.

Two of the ligands examined, β-phenylpropionate and indole 3-acetate, each bind at two independent sites which do not interact (Table I).[2] While limiting spectra (Table II) indicate that either of these ligands binds simultaneously with glycyl-L-tyrosine, L-phenylalanine, or cyclohexanol, they do not indicate how occupancy of each indole 3-acetate or β-phenylpropionate site affects binding of the other three ligands. Spectral titrations characterized the effect of each indole 3-acetate or β-phenylpropionate molecule already bound on the interaction of the second ligand. Equation 2 served to analyze the data at 550 nm when indole 3-acetate or β-phenylpropionate was the titrant ligand (L in eq 2) and glycyl-L-tyrosine, L-phenylalanine, or cyclohexanol was the "fixed" ligand (X in eq 2). Plots at several concentrations of "fixed" ligand extrapolate to a common point of intersection. Figure 6 is a typical plot for the indole 3-acetate plus L-phenylalanine pair. By determination of one interaction constant and of the intrinsic dissociation constants, the other interaction constant was calculated from the value of the intersection point (eq 4). Intrinsic dissociation constants are evaluated readily in the absence of "fixed" ligand, and the interaction constant β is determined independently by analyzing titrations at 440–450 nm according to eq 1. Binding to the secondary indole 3-acetate or β-phenylpropionate site is also quantitated, at 440 nm and 445 nm, respectively, in the presence of fixed ligands, i.e., cyclohexanol, glycyl-L-tyrosine or L-phenylalanine. The straight line double-reciprocal plots, as obtained for example with the indole 3-acetate plus L-phenylalanine pair (Figure 3), allow determination of the value of β from the apparent dissociation constant as described by eq 1; substitution of its value in eq 4 then allows calculation of the value of α. The interaction of both indole 3-acetate or β-phenylpropionate molecules with L-phenylalanine, cyclohexanol, and glycyl-L-tyrosine was evaluated in this manner (Table III).

Titrations with Two Ligands When Each Ligand Binds to Two Independent Sites. Based on the criteria of limiting spectra (Table II) the molecule(s) of β-phenylpropionate which perturb the circular dichroic spectrum of azotyrosine-248 are displaced by the indole 3-acetate molecule(s) affecting the uncomplexed probe. This indicates that at least the secondary indole 3-acetate and β-phenylpropionate sites must be either identical or overlapping. Additionally, ligand-pair titrations of ellipticity at 550 nm did not intersect when plotted according to eq 2 suggesting that the primary sites for indole 3-acetate and β-phenylpropionate are also coincident or at least interact strongly. The limiting spectra and the titrations of the azotyrosine·Zn complex monitored at 550 nm indicate jointly that the two molecules of indole 3-acetate compete with the two molecules of β-phenylpropionate for the same two sites on the azoenzyme.

Discussion

The nature and details of the active center of carboxypeptidase have been probed in numerous ways. Ten years ago, kinetic studies of native and modified carboxypeptidase led us to postulate that its active center comprises overlapping and/or interacting binding domains for substrates, products, activators, and inhibitors (Vallee et al., 1968). More recently kinetic examination of the active center over a wide range of pH has been extended through the study of longer peptide and ester substrates which are devoid of the kinetic anomalies characteristic of dipeptides and depsipeptides employed in the earlier work. The pH dependence of the hydrolysis of these longer substrates and the modes of action, characteristic of a number of inhibitors and activators, have been examined thoroughly (Auld & Vallee, 1970a,b, 1971; Auld & Holmquist, 1974; Auld et al., 1972; Harrison et al., 1975a,b) and the results are consistent with the active site model (Vallee et al., 1968). Further, the chromophoric cobalt (Latt & Vallee, 1971) and azocarboxypeptidases (Johansen & Vallee, 1975; Johansen et al., 1976) have served for spectral studies of psuedosubstrate and inhibitor binding. In both the previous and the present binding studies, however, the time required for experimentation, as well as the chromophoric properties of the modified enzymes themselves, have limited the choice of ligands as well as the conditions under which experiments could be carried out. In particular, the study of substrates has been difficult, since they are destroyed in the course of the very experiments intended to probe their binding. For this reason, thermodynamic studies have primarily relied upon molecules that are not hydrolyzed, and hence, the results of these investigations have been related to the results of kinetic studies by analogy to effects of the molecules on the hydrolytic properties of carboxypeptidase. The present study serves to unify results both from kinetic and thermodynamic approaches while keeping in mind the limits imposed by the above considerations.

We have now employed circular dichroism titrations with several ligands, including a pseudosubstrate, inhibitors, and modifiers of the hydrolytic activity, of carboxypeptidase to delineate the various ligand binding regions of the active center. The results confirm earlier conclusions, are compatible with the "overlapping domain" model which was based on kinetic studies, and delineate four different binding sites in azocarboxypeptidase.

Tyrosine-248 was modified selectively with diazotized arsanilic acid to monitor ligand binding (Johansen et al., 1972). The circular dichroic spectrum of the modified protein, azocarboxypeptidase, has an intense extremum at 510 nm, characteristic of the intramolecular azotyrosine·Zn coordination complex (Johansen and Vallee, 1971), which is abolished by addition of a wide variety of ligands signaling the disruption of the coordination complex (Johansen & Vallee, 1975). The spectrum at shorter wavelengths, which monitors the environment of the azotyrosine residue itself, also changes as a function of the disruption of the coordination complex and the resultant reorientation of the azotyrosine residue. The uncomplexed azotyrosine residue, too, serves as a probe of ligand binding, even in the absence of an azotyrosine·Zn complex, as previously shown (Johansen et al., 1976). Indeed, binding of multiple molecules of a single ligand has been observed previously, using both chromophoric features of this dual probe.[3] We have employed this system of "Janus" like chromophores to monitor simultaneous binding of molecules of different ligands to the active center of azocarboxypeptidase.

Identification of Ligand Binding Sites. Of the five ligands examined two pairs are characterized by mutually exclusive binding: glycyl-L-tyrosine plus L-phenylalanine and β-phenylpropionate plus indole 3-acetate (Table II). Such ligands must associate with the enzyme at sites which are strongly linked energetically and could either overlap partially or be identical. In the case of glycyl-L-tyrosine plus L-phenylalanine, only one site can be discerned, since either one of these two ligands alone solely forms a one-to-one complex with the enzyme (Table I). With β-phenylpropionate plus indole 3-acetate, two sites are identified, since two molecules of either one of these ligands alone bind to the enzyme (Table I), and both molecules of one ligand displace both molecules of the other (Table II). This is consistent with the conclusion that the two indole 3-acetate sites overlap strongly or even coincide with the two β-phenylpropionate sites. Moreover, since glycyl-L-tyrosine binds simultaneously with indole 3-acetate and β-phenylpropionate, as does L-phenylalanine, these two sites differ from the first identified above. Finally, there must be a fourth binding site, for cyclohexanol, since it binds concurrently with all ligands examined.

Interactions between Binding Sites. The organization of the ligand binding sites may be inferred from the interaction constants. Small variations in the values of interaction parameters were found depending on the particular ligands occupying the various sites. This may result from differences either in the steric occupancy of sites or the ability of the specific molecules to affect protein conformation. Values of interaction constants, α or β, equal to one indicate that the sites are independent. When values are between 1 and 2, the sites are considered to be interacting weakly at most; while with values greater than two, sites are considered to interact strongly. Using values of interaction parameters from Table III, the interaction between binding sites is defined by each group of ligands. Ligands binding to site 1 interfere minimally with ligands binding at site 2. The third ligand binding site, however, is affected quite significantly. A pronounced interaction between site 1 and site 4 is also apparent. Finally, binding at site 4 only minimally perturbs ligand association at site 2 but significantly influences such association at site 3. It follows from the lack of curvature in double-reciprocal plots of titrations that sites 2 and 3 are independent.[2] Figure 7 schematically describes the relationships summarized in Table III. Arrows between circles representing each binding site symbolize interaction between the binding areas. The ligands examined perturb the carboxypeptidase catalyzed hydrolysis of both ester and peptide substrates, suggesting that the sites examined must be located in the active center of the enzyme.

Relationship between Ligand Binding Sites and Inhibition Modes of Carboxypeptidase. Interestingly, the identity of ligand binding sites of azocarboxypeptidase determined in this study correlates well with the effects of each of these ligands on the activity of carboxypeptidase in solution. The glycyl-L-tyrosine plus L-phenylalanine pair is comprised of a peptide pseudosubstrate (Auld & Vallee, 1970a) and a competitive inhibitor of peptide hydrolysis (Elkins-Kaufman & Neurath, 1949; Whitaker et al., 1966), respectively. As suggested by their kinetic behavior, both ligands bind to a single site, site 1. Ligands that bind at sites 2 and 3 are characterized as mixed inhibitors of native carboxypeptidase. In the case of β-phenylpropionate, the resultant mixed inhibition has been resolved into competitive and non-competitive modes (Auld et al., 1972), in agreement with the two independent sites for β-phenylpropionate binding identified here. It should be noted that binding of neither of these β-phenylpropionate molecules at sites 2 or 3 precludes binding at site 1, occupied by the competitive inhibitors of native carboxypeptidase's peptidase activity. It appears that occupancy of either one of two independent binding sites can result in competitive inhibition of peptide hydrolysis. Finally, cyclohexanol interacts with the enzyme at yet another site and represents a group of ligands that behave uniquely, activating dipeptide and inhibiting the corresponding ester substrates, which is consistent with the existence of a binding site independent of all others, as here detected. The demonstration of four distinct binding areas, for even such a small selection of ligands, suggests that the enzyme active center is composed of a number of interacting substrate and modifier binding sites.

On the basis of equilibrium studies, an active center area comprised of interacting ligand binding areas is indicated. This suggests that the basis underlying the complex kinetic behavior of carboxypeptidase (Davies et al., 1968b; Vallee et al., 1968) lies in the relatively large active site area of the enzyme. On the basis of kinetic experiments, Abramowitz et al. (1967) suggested previously that there are five recognition sites solely to bind peptide substrates. The present thermodynamic studies now show that binding of substrates or inhibitor ligands at one site affects the binding properties of others situated in the active center consistent with the multiple and overlapping binding-site hypothesis which we proposed previously based on kinetic evidence (Vallee et al., 1968). There is a cluster of interacting sites and a single, group 2, binding site which is largely independent of the others. On the basis of spectral assignments by Johansen & co-workers (1976), site 1 and site 2 might be in the peptide area perhaps being coincident with some of the sites suggested by Abramowitz et al. (1967), while sites 3 and 4 could be in the ester binding region, corresponding with distinct binding loci for these two types of substrates. Though portions of the two substrate areas are completely independent (sites 2 and 3), it is also evident that others interact strongly (the cyclohexanol site, site 4, and site 1). The identification of "subsites" of what may be peptide and ester binding regions as well as the interactions between them provides a topological basis for relating the organization of the enzyme active center in solution to the enzyme's kinetic properties.

[3] While the present probe has a remarkable capacity to visualize the binding of ligands this is, nevertheless, limited by the topographic relationships. Hence, though it detects a large number of vicinal effects, it need not be sensitive to all those outside of its range, much as they may be relevant to catalysis and/or binding.

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Mechanism of Action of Adenosylcobalamin: Hydrogen Transfer in the Inactivation of Diol Dehydratase by Glycerol†

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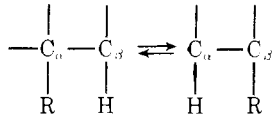
ABSTRACT: We have investigated the kinetic characteristics of the inactivation of the adenosylcobalamin-dependent enzyme propanediol dehydratase by glycerol, (*RS*)-1,1-dideuterioglycerol, (*R*)-1,1-dideuterioglycerol, and perdeuterioglycerol in the presence of 1,2-propanediol and 1,1-dideuterio-1,2-propanediol. The results imply that hydrogen (or deuterium) attached to C-1 of 1,2-propanediol participates in the inactivation process and contributes to the expression of a kinetic isotope effect on the rate of inactivation. The mechanism for this inactivation must involve the cofactor as an intermediate hydrogen carrier, presumably in the form of 5′-deoxyadenosine. Moreover, a mechanism involving a rate-determining transfer of hydrogen from an intermediate containing three equivalent hydrogens quantitatively accounts for all of the results. When diol dehydratase holoenzyme is inactivated by [1-^{3}H]glycerol, 5′-deoxyadenosine which is enriched in tritium by a factor of 2.1 over that in glycerol can be isolated from the reaction mixture.

A number of enzymes require adenosylcobalamin (AdoCbl[1]) as cofactor and catalyze reactions of the general type:



where R can be alkyl, amino, hydroxyl, or carbonyl (Hogenkamp, 1968; Babior, 1975a,b). Propanediol dehydratase is an AboCbl-dependent enzyme which catalyzes the rearrangement of both (*R*)- and (*S*)-1,2-propanediol to propionaldehyde and of ethylene glycol to acetaldehyde.

Until recently (Toraya et al., 1976; Bachovchin et al., 1977), diol dehydratase was thought to be inactive toward glycerol. This behavior distinguished propanediol dehydratase from another very similar AboCbl-dependent enzyme, glycerol dehydratase, which catalyzes the rearrangement of glycerol to β-hydroxypropionaldehyde as well as acting upon 1,2-propanediol and ethylene glycol. In fact, propanediol dehydratase holoenzyme does catalyze the rearrangement of glycerol to β-hydroxypropionaldehyde. However, glycerol also causes rapid and irreversible inactivation of the enzyme, thus explaining why catalytic activity toward glycerol was not detected previously (Lee and Abeles, 1963). Both the inactivation and catalysis reactions exhibit large primary deuterium isotope effects (k_H/k_D = 14 and 8, respectively); thus, hydrogen transfer is an important rate-contributing step in each case. Experiments with various isotopically substituted glycerols demonstrated that two diastereomeric combinations are possible between the enzyme and glycerol; the enzyme–substrate complexes were designated "EG_R" and "EG_S". When glycerol is bound to enzyme in the "EG_R" complex, a hydrogen is abstracted from the pro-R carbon and the catalytic rate actually exceeds that observed with 1,2-propanediol as substrate. When glycerol is bound in the "EG_S" complex, hydrogen is abstracted

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[1] Abbreviations used are: AdoCbl, adenosylcobalamin; NADH, reduced nicotinamide adenine dinucleotide.

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