Circular Dichroism–Inhibitor Titrations of Arsanilazotyrosine-248 Carboxypeptidase A†

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ABSTRACT: Coupling of carboxypeptidase with diazotized arsanilic acid specifically modifies a single tyrosyl residue. Yet, owing to the fact that the resultant azoTyr-248 can form an intramolecular chelate with zinc, two different circular dichroism probes result: azoTyr-248 itself and the azoTyr-248·Zn chelate. Both are environmentally sensitive and, characteristically, each can signal the same or different perturbations, as is apparent from circular dichroic spectra. This dual probe function greatly magnifies the scope of these chromophores in mapping the topography of the active center with respect to sites of interaction of inhibitors (or substrates). Titration of the azoenzyme with a series of synthetic, competitive inhibitors, e.g., L-benzylsuccinate, L-phenyllactate, and L-Phe, and with the pseudosubstrate, Gly-L-Tyr, in turn generates characteristic circular dichroic spectra. Their analysis yields a single binding constant for each of these agents, one molecule of each binding to the active center. Mixed inhibitions, as seen with β-phenylpropionate and phenylacetate, resolved previously into competitive and noncompetitive components, are characterized by different spectral effects. Two molecules of these agents bind to the enzyme, consistent with both thermodynamic and enzymatic studies. The interactions leading to competitive and noncompetitive inhibition, respectively, can be recognized and assigned, based on the manner in which the extrema at 340 and 420 nm, reflecting azoTyr-248, and the negative 510-nm circular dichroism band, typical of its chelate with zinc, are affected and on the pH dependence of spectral and kinetic data. Certain noncompetitive inhibitors and modifiers induce yet other spectral features. Each probe is very sensitive to changes in its particular active center environment, though both can be relatively insensitive to inhibitors interacting at a distance from the active center.

To gain further insight into the mechanism of carboxypeptidase, the interaction of the native and chemically modified enzyme with inhibitors has been examined by kinetics, equilibrium studies, and by x-ray structure analysis (Lipscomb et al., 1970; Vallee et al., 1970; Neurath et al., 1970; Auld and Vallee, 1970; Vallee et al., 1971; Latt et al., 1972). So far, the properties of arsanilazoTyr-248 carboxypeptidase[1] have been found particularly valuable to correlate functional with structural characteristics of the enzyme. The typical circular dichroic spectrum of arsanilazoTyr-248 carboxypeptidase exhibits extrema at 340 nm ($[\theta]^{23}$ −28 000) and 420 nm ($[\theta]^{23}$ +14 000), both similar to such spectra of protonated azophenol chromophores of other azoproteins (Fairclough and Vallee, 1971; Johansen and Vallee, 1975). In addition, at 510 nm both the absorption and circular dichroism spectra with a maximum (ε, 8000) and a large negative extremum ($[\theta]^{23}$ −41 000), respectively, specifically reflect an intramolecular chelate of this residue with the active site zinc atom. The spectral response of this derivative to its physical state, to substrates and inhibitors, pH, and metals exemplifies the capacity of the azochromophore to probe the conformation and immediate environment of the active center (Johansen and Vallee, 1971, 1973, 1975).

In the course of these investigations it has become apparent also that the specific introduction of the azo group into Tyr-248 results in two discrete probes, which distinctively signal different perturbations, on one hand, of the active center azoTyr-248 itself, and, on the other, of the azoTyr-248·Zn chelate. Each can detect the same and/or different interactions and environmental changes, greatly magnifying the scope of these optically active chromophores in revealing the topography of the active center and its interaction with inhibitors and substrates in solution.

Kinetic studies of carboxypeptidase have identified mixed, competitive, and noncompetitive modes of inhibition of the enzyme, and mixed inhibition of peptidase activity has been resolved into competitive and noncompetitive modes (Auld et al., 1972). Such inhibitors affect the absorption and circular dichroic spectra of the cobalt enzyme in characteristic fashion, though spectral manifestations of the interactions or of productive and nonproductive substrate binding modes have not been defined (Latt and Vallee, 1971).

We have now examined the response of the circular dichroic spectrum of the azoenzyme to titration with a series of agents known to inhibit native carboxypeptidase in different modes. Based on their effects on the extrema, characteristic of azoTyr-248 and of its Zn chelate, respectively, they reveal that competitive inhibitors are characterized by one and mixed inhibitors by two binding modes, but assign-

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[1] In order to simplify presentation, zinc carboxypeptidase, zinc arsanilazocarboxypeptidase, the zinc azoenzyme and apocarboxypeptidase, apoazocarboxypeptidase, and apoarsanilazocarboxypeptidase are used interchangeably with zinc monoarsanilazotyrosine-248 carboxypeptidase and apoarsanilazotyrosine-248 carboxypeptidase, respectively, of any enzyme form. Carboxypeptidase A is referred to as native carboxypeptidase. AzoTyr-248 refers to the *azophenol* of monoarsanilazotyrosine-248 and the azophenolate to its ionized species.

ments for noncompetitive inhibitors and modifiers remain uncertain, though they are clearly different. Binding constants for single or multiple azoenzyme sites have been calculated, and the overall results are consistent with the multiple site model for substrate, inhibitor, and product binding proposed previously (Vallee et al., 1968).

Materials and Methods

Carboxypeptidase A_γ, isolated by the method of Anson (1937), and carboxypeptidase A_α, prepared according to the method of Cox et al. (1964), were obtained as crystal suspensions from Worthington Biochemical Corporation and Sigma Chemical Company, respectively. Gly-L-Tyr, indole-3-acetate, and phenylacetate were obtained from Mann Research Laboratories, Inc.; D,L-benzylsuccinic acid was purchased from Burdick and Jackson Laboratories, Inc., and the L isomer was isolated as described by Byers and Wolfenden (1973). β-Phenylpropionic acid (Eastman) was recrystallized from water. L-Lys-L-Tyr-NH_2 and Cbz-Gly were obtained from Cyclo Chemical Corp., and L-phenylalanine was from Pierce Chemical Company. All other chemicals were reagent grade.

Zinc arsanilazoTyr-248 carboxypeptidase was prepared as follows: 25 ml of a 0.02 M solution of diazotized *p*-arsanilic acid was prepared as previously described (Kagan and Vallee, 1969). The pH value of the solution was adjusted to 7.0 with 1 N NaOH followed by dilution with water. The resultant solution was added immediately to 16 ml, 6×10^{-4} M, of a stirred, aqueous crystal suspension of native carboxypeptidase at 0°; then 4.1 ml of 0.11 M $KHCO_3$ was added, and the pH was adjusted to 8.8 with 0.1 N NaOH. This pH was maintained during the initial 10–15 min of reaction. After 3 h of reaction the modified crystals were isolated by centrifugation and washed five times with metal-free water. The modified enzyme was characterized as reported previously (Johansen and Vallee, 1971, 1973; Johansen et al., 1972); apoarsanilazocarboxypeptidase crystals were prepared as described for the native enzyme crystals (Auld and Holmquist, 1974). All solutions were freed of trace metal contamination by extraction with 0.1% dithizone in carbon tetrachloride. Glassware and cuvettes were cleaned by soaking in 1:1 nitric and sulfuric acids, followed by rinsing in metal-free distilled water.

Protein concentration was measured by absorbance at 278 nm, based on a molar absorptivity of 7.32×10^4 M^{-1} cm^{-1} for zinc arsanilazocarboxypeptidase (Johansen and Vallee, 1975).

Absorption spectra were determined with a Cary Model 14R or Model 118C spectrophotometer. A Zeiss PMQ II spectrophotometer was used for absorption measurements at single wavelengths.

Circular dichroism measurements were performed with a Cary Model 61 recording spectropolarimeter using 1-ml quartz cells of 1.0-cm light path at enzyme concentrations ranging from 4 to 7×10^{-5} M. Ellipticity is expressed as molecular ellipticity $[\theta]^{23} = (\theta/10)(M/lc)$ with units of (deg cm^2)/dmol, where θ = observed ellipticity in degrees, M = molecular weight, 34600 for carboxypeptidase, l = path length in cm, and c = concentration in g/ml. Spectral titration with substrates and inhibitors was performed as described previously (Johansen and Vallee, 1975).

The apparent equilibrium constant, K_{app}, was obtained by plotting $1/(A_0 - A)_\lambda$ vs. $1/[I]$, where A_0 is the absorbance of the zinc azoenzyme in the absence of the inhibitor [I] and A is the absorbance in its presence, at wavelength λ.

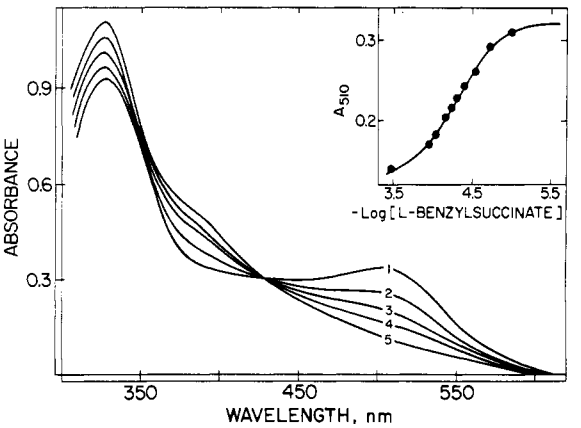

FIGURE 1: Effect of L-benzylsuccinate on the absorption spectrum of zinc azoTyr-248 carboxypeptidase, 5.5×10^{-5} M, in 0.5 M Tris–0.5 M NaCl, pH 8.5, 23°. Numbers indicate the L-benzylsuccinate concentration at which the spectra were recorded: (1) 0; (2) 0.03 mM; (3) 0.06 mM; (4) 0.12 mM; (5) 0.35 mM; all data were corrected for dilution. The insert depicts the titration curve obtained at 510 nm and reflects a dissociation constant of 0.013 mM.

The intercept on the abscissa of the resulting straight line is $-1/K_{app}$. K_{app} was calculated from circular dichroic spectra in the same manner. When spectral titrations exhibit tight isosbestic points, the graphs of $1/(\theta_0 - \theta)_\lambda$ vs. $1/[I]$ are linear when plotted at different wavelengths, but those from spectra which are characterized by shifting points of intersection exhibit curvature due to overlapping spectra of the various species present. However, if two binding modes are present, the K_{app} for each can be calculated at wavelengths where the spectra do not overlap, or at an isosbestic point which reflects one of the binding modes. The program for a Hewlett-Packard 9810A calculator, 9826A plotter, and 9864 digitizer to convert spectral results to double reciprocal plots was developed by Dr. Gerald M. Alter.

Results

The absorption and circular dichroic spectra of arsanilazotyrosine-248 carboxypeptidase are sensitive to substrates and inhibitors which can alter the mutual orientation of or distance between the zinc atom and the azoTyr-248 residue, thereby abolishing the spectra of the intramolecular chelate formed between them (Johansen and Vallee, 1975). Hence, the response of the 510-nm absorption maximum and the corresponding negative extremum of the circular dichroism spectrum of the zinc azoenzyme conveniently measures the affinity of the inhibitor for the enzyme active center. In these instances, the response of the azoTyr-248 extrema at 340 and 420 nm solely reflects the participation of that residue in chelate formation.

Spectral Titrations with Competitive Inhibitors. L-Benzylsuccinate is an effective inhibitor of native carboxypeptidase with an apparent dissociation constant varying from 10^{-8} to 10^{-5} M over the pH range from 6 to 8 (Byers and Wolfenden, 1973). At pH 8.5, where the intramolecular chelate between azoTyr-248 and Zn is maximally formed, L-benzylsuccinate progressively abolishes the 510-nm band of azoTyr-248 carboxypeptidase as its concentration is increased up to 0.3 mM. Concomitantly, the absorbances at both 330 and 380 nm increase to become characteristic of the protonated azophenol (Figure 1). A plot of absorption at 510 nm vs. L-benzylsuccinate concentration fits a titration curve with an apparent dissociation constant of 0.013 mM (Figure 1, insert).

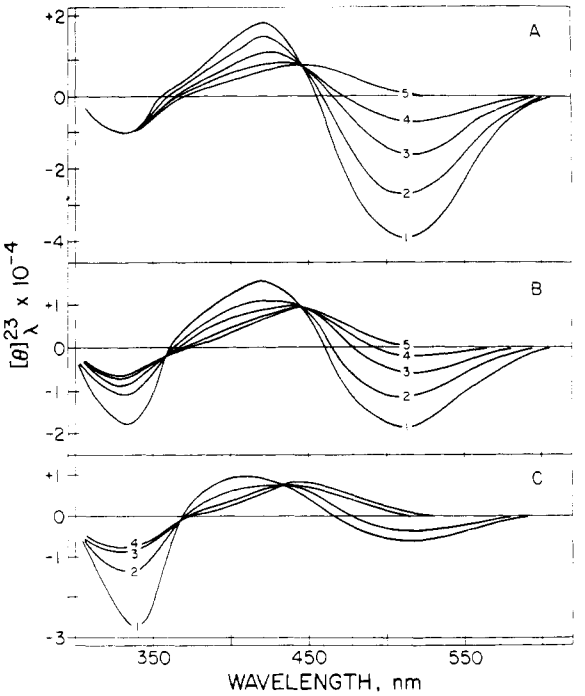

FIGURE 2: Effect of L-benzylsuccinate on the circular dichroism spectrum of zinc azoTyr-248 carboxypeptidase. (A) pH 8.5; (B) pH 7.6; both in 0.05 M Tris-0.5 M NaCl; and (C) pH 6.6 in 0.05 M Mes-0.5 M NaCl, all at 23°. Numbers indicate the L-benzylsuccinate concentration at which the spectra were recorded: (A) (1) 0, (2) 0.03 mM, (3) 0.06 mM, (4) 0.12 mM, (5) 0.3 mM; (B) (1) 0, (2) 0.03 mM, (3) 0.06 mM, (4) 0.08 mM, (5) 0.13 mM; (C) (1) 0, (2) 0.03 mM, (3) 0.06 mM, (4) 0.16 mM; all data were corrected for dilution.

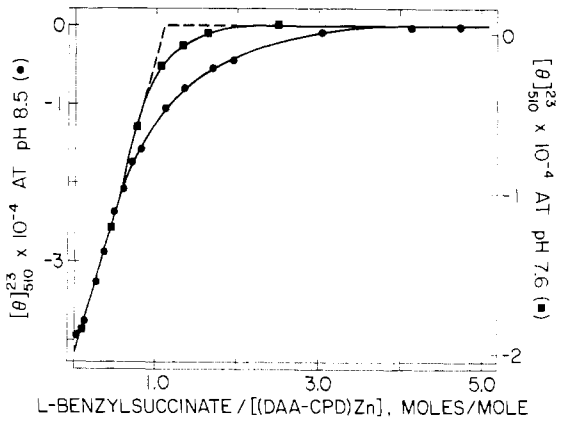

FIGURE 3: Dependence of the circular dichroism spectrum of zinc azoTyr-248 carboxypeptidase on the molar excess of L-benzylsuccinate at pH 8.5 (●) or pH 7.6 (■), both in 0.05 M Tris-0.5 M NaCl, 23°. The ratio of L-benzylsuccinate/azoenzyme and the values of $[\theta]^{23}_{510}$ corresponding to these ratios are taken from Figure 2A and B, respectively.

Circular dichroism titrations with L-benzylsuccinate have been performed at pH 8.5, 7.6, and 6.6 where the azophenolate-zinc chelate is formed maximally, partially, and not at all, respectively (Figure 2). At pH 8.5 the amplitudes of both the negative 510-nm and the positive 420-nm ellipticity bands decrease, the latter shifting to 440 nm, with a single, tight isosbestic point at 450 nm (Figure 2A). In the presence of 0.3 mM L-benzylsuccinate the circular dichroic spectrum now closely resembles that of the apoenzyme at pH 8.5, and further increases in concentration of the agent do not alter the spectrum. At pH 7.6, close to the pK of formation of the intramolecular chelate, the 510-nm band is less intense. At this pH, 0.13 mM L-benzylsuccinate suffices to bring about the same changes, but, in addition, the amplitude of the 340-nm band decreases (Figure 2B). At pH 6.6 only a small amount of the chelate exists and, hence, effects of L-benzylsuccinate are confined largely to changes in ellipticity at 340 and 420 nm, both characteristic of the azophenol. The end points of the titrations are similar at all three pH values.

Figure 3 singles out the effects of L-benzylsuccinate on the 510-nm extremum at pH 7.6 and 8.5. As pH decreases, L-benzylsuccinate binds increasingly firmly to the azoenzyme, and at pH 7.6 only an upper limit of dissociation constant can be estimated. The dissociation constants (Table I) are consistent with those calculated from kinetic data for the native enzyme (Byers and Wolfenden, 1973) suggesting the formation of a stoichiometric, 1:1, azoenzyme-L-benzylsuccinate complex.

Gly-L-Tyr, a pseudosubstrate, abolishes the 510-nm absorption band while increasing the intensity of those at 330 and 380 nm, generating a spectrum characteristic of the protonated azophenol (Johansen and Vallee, 1975). Titrations with L-phenylalanine and L-phenyllactate are qualitatively identical. Figure 4 (upper panel) shows the recordings of circular dichroic titrations of the azoenzyme at pH 8.5 with L-phenyllactate, from 0 to 30 mM. The amplitudes both of the negative and positive extrema at 510 and 420 nm decrease, the latter shifting to 450 nm with a single, tight isosbestic point at 442 nm. With L-phenyllactate, 30 mM, the spectrum closely resembles that of the apoazoenzyme at pH 8.5 (Johansen and Vallee, 1975). The results of titrations at pH 7.6 and 6.6 (not shown) closely resemble those with L-benzylsuccinate (Figure 2) and titrations with Gly-L-Tyr and L-phenylalanine (not shown) are closely similar to those with L-phenyllactate at all pH values studied. Double reciprocal plots of the circular dichroic titrations at 510 and 420 nm are linear with similar dissociation constants, $K_{app\,510}$ = 2.8 mM and $K_{app\,420}$ = 2.5 mM (Figure 4, bottom), virtually the same as that derived from absorbance titration with L-phenyllactate (Table I).

These data suggest a single binding site for L-phenyllactate, Gly-L-Tyr, and L-phenylalanine with reference to the azoprobe and together with kinetic results (Auld and Vallee, 1970; Auld and Latt, 1971) indicate that Gly-L-Tyr and these competitive inhibitors interact with one site of the enzyme in similar fashion.

Mixed Inhibition. Absorption titrations with β-phenylpropionate at pH 8.5 (Figure 5, top) (Johansen and Vallee, 1975) and phenylacetate are virtually superimposable on those with competitive inhibitors. However, the corresponding circular dichroic titrations (Figure 5, middle) differ significantly. While responding to perturbations of the intramolecular chelate, the negative 340- and positive 420-nm extrema characteristic of azoTyr-248 alone also respond to additional inhibitor binding modes.

Concentrations of β-phenylpropionate up to 4 mM decrease the amplitudes of both the negative ellipticity band at 510 nm and the positive one at 420 nm, with an apparent isosbestic point at 445 nm. At higher concentrations the amplitudes of both bands continue to decrease, and the gradual shift of the intersection point to higher wavelengths becomes more apparent. Furthermore, the extremum at 420 nm moves to 380 nm as a new negative band is formed at 450 nm. These spectral titrations are more complex than those in Figures 2 and 4 and suggest at least two modes of β-phenylpropionate binding.

The double reciprocal plot of the change in 510-nm ab-

Table I: Dissociation Constants (K_{app}) for Gly-L-Tyr and Inhibitors of Carboxypeptidase as Determined from Their Effects on the Circular Dichroism and Absorption Spectra of Zinc AzoTyr-248 Carboxypeptidase.[a]

Agent	pH	Spectrum	K_{app}, mM (Wavelength, nm)
Gly-L-Tyr	8.5	Absorption	1.0 (510)
	8.5	CD	1.2 (510)
			1.5 (420)
	7.6	CD	3.3 (510)
			3.4 (420)
	6.6	CD	5.0 (420)
Competitive Inhibitors			
L-Benzylsuccinate	8.5	Absorption	0.013 (510)
	8.5	CD	0.013 (510)
			0.013 (420)
	7.6	CD	<0.01 (510)
			<0.01 (420)
			<0.01 (340)
	6.6	CD	<0.01 (340)
L-Phenyllactate	8.5	Absorption	2.3 (510)
	8.5	CD	2.8 (510)
			2.5 (420)
	7.6	CD	3.5 (510)
L-Phe	8.5	Absorption	1.1 (510)
	8.5	CD	1.5 (510)
	7.6	CD	4.0 (510)
Mixed Inhibitors			
β-Phenylpropionate	8.5	Absorption	7.0 (510)
	8.5	CD	6.4 (550)
			45 (445)
	7.6	CD	2.4 (550)
			9.3 (445)
			9.4 (340)
	6.6	CD	3.5 (445)
			4.0 (340)
Phenylacetate	8.5	Absorption	14.2 (510)
	8.5	CD	16 (510)
			50 (420)
	7.6	CD	9.9 (510)
			24 (420)
			20 (340)
	6.6	CD	17 (340)
Noncompetitive Inhibitors			
Indole-3-acetate	8.5	Absorption	5.0 (510)
	8.5	CD	6.0 (510)
			7.0 (420)
	7.6	CD	3.4 (510)
			4.6 (420)
	6.6	CD	1.0 (420)
L-Lys-L-TyrNH_2	8.5	Absorption	7.0 (510)
	8.5	CD	8.2 (510)
			7.1 (420)
	7.6	CD	41 (510)
Modifiers			
Cyclohexanol	8.5	CD	23 (510)
			20 (340)
Cbz-Gly	8.5	Absorption	200 (510)
	8.5	CD	200 (510)

[a] pH 8.5 and 7.6 (0.05 M Tris–0.5 M NaCl), pH 6.6 (0.05 M Mes–0.5 M NaCl).

sorption yields one K_{app} of 7.1 mM (Figure 5, top, insert). In contrast, that of the circular dichroism titration at 510 nm (not shown) is curved, owing to overlap of the 510- and 450-nm bands, the latter becoming progressively more negative. At 550 nm, however, this overlap is minimal, and the plot is completely linear (Figure 5, right, bottom panel), yielding a K_{app} of 6.4 mM, similar within the error limits of the methods, to that obtained from the absorbance titration.

The binding constant for the second mode of β-phenylpropionate interaction can be obtained from the changes in ellipticity at 445 nm, the apparent isosbestic point for the first binding mode. A double reciprocal plot at this wavelength is linear, yielding a K_{app} of 45 mM (Figure 5, left bottom panel, Table I). Hence, while only one binding mode can be derived from the absorbance titration, the circular dichroism titration resolves two. The spectral changes for the first mode correspond to those observed for the competitive inhibitors (Figures 2 and 4). The distinctive changes on circular dichroism titrations with β-phenylpropionate at pH 7.6 (Figure 6) are consistent with the existence of at least two different binding modes. Up to 3 mM inhibitor, the 510-nm band progressively decreases while that at 420 nm is affected to a much lesser extent and that at 340 nm does not change at all. However, beyond this concentration, effects at lower wavelengths predominate. The apparent isosbestic point, initially at 445 nm, gradually shifts to become an intersection at 495 nm, and a new isosbestic point forms at 400 nm. Simultaneously, the positive maximum at 420 nm inverts and shifts to a negative extremum at 460 nm, while generating a new positive maximum at 380 nm. Importantly, the negative extremum at 340 nm, virtually unaffected by concentrations up to 3 mM, now progressively diminishes, along with the changes just described.

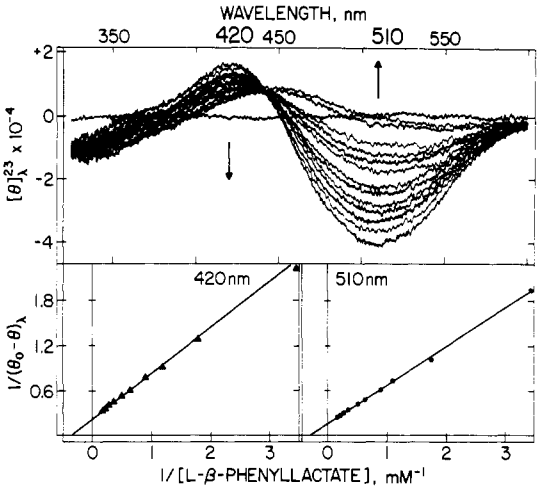

FIGURE 4: (Upper panel) Effect of L-phenyllactate on the circular dichroism spectrum of zinc azoTyr-248 carboxypeptidase in 0.05 M Tris–0.5 M NaCl, pH 8.5, 23°, uncorrected for dilution. L-Phenyllactate concentration is varied from 0 to 30 mM. Arrows indicate the direction of change at the corresponding wavelength set in larger ciphers. (Lower panel) Double reciprocal plots ($1/(\theta_0-\theta)_\lambda$ vs. 1/[L-phenyllactate]), on the right at 510 nm (●), and on the left at 420 nm (▲), both calculated from the spectral circular dichroism titrations in the upper panel.

At pH 6.6 little of the intramolecular azoTyr-248·zinc chelate is formed, and it is largely the azophenol which functions as the probe. Relatively high concentrations of β-phenylpropionate suppress the band at 340 nm, reflecting what would appear to be the noncompetitive binding mode. The final spectrum (not shown) resembles that found at high concentrations of β-phenylpropionate at pH 7.6 (Figure 6).

Thus, at pH 8.5 the negative extremum at 510 most specifically reflects the competitive mode of β-phenylpropionate binding while at pH 7.6 and 6.6, the negative extremum at 340 nm would seem characteristic of the noncompetitive mode consistent with the results of kinetic studies (Auld et al., 1972). Low concentrations affect the circular dichroism

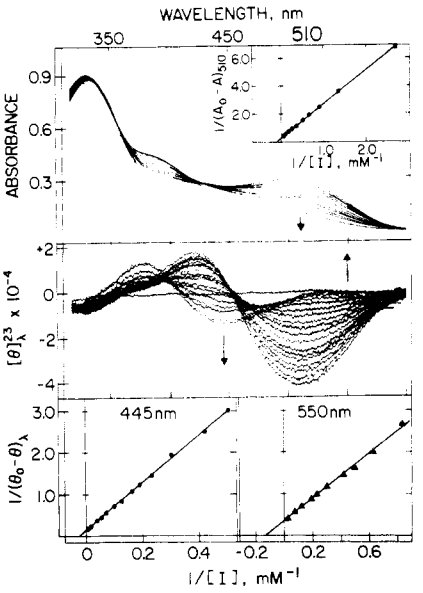

FIGURE 5: (Upper panel) Effect of β-phenylpropionate on the absorption spectrum of zinc azoTyr-248 carboxypeptidase, 4.5×10^{-5} M, in 0.05 M Tris–0.5 M NaCl, pH 8.5, 23°. The concentration of β-phenylpropionate is varied from 0 to 100 mM. The spectra are not corrected for dilution which is 11% for the last spectrum recorded. The insert depicts $1/(A_0 - A)$ vs. 1/[β-phenylpropionate] at 510 nm. (Middle panel) Effect of β-phenylpropionate on the circular dichroism spectrum of zinc azoTyr-248 carboxypeptidase in 0.05 M Tris–0.5 M NaCl, pH 8.5, 23°. β-Phenylpropionate concentration varies from 0 to 150 mM. Spectra are not corrected for dilution, which is 25% for the final spectrum. Throughout, arrows indicate the direction of change at the corresponding wavelength, set in larger ciphers. (Lower panel) Double reciprocal plots $1/(\theta_0 - \theta)_{\lambda}$ vs. 1/[β-phenylpropionate], on the right at 550 nm (▲) and on the left at 445 nm (●), both calculated from the circular dichroism–titrations in the middle panel.

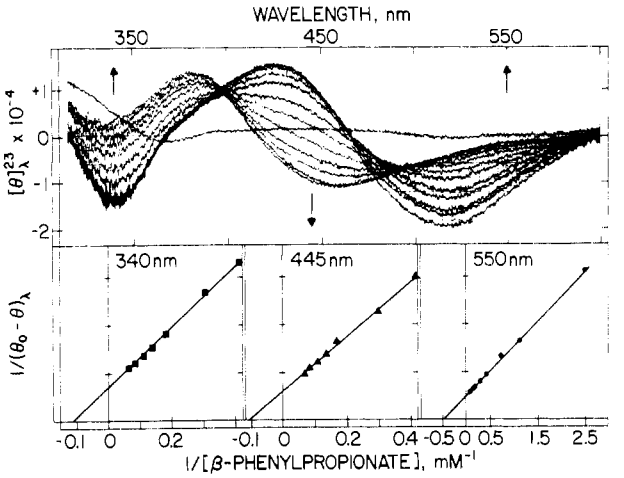

FIGURE 6: (Upper panel) Effect of β-phenylpropionate on the circular dichroism spectrum of zinc azoTyr-248 carboxypeptidase, 0.05 M, in Tris-0.5 M NaCl, pH 7.6, 23°. β-Phenylpropionate concentration was varied from 0 to 91 mM. The spectra are not corrected for dilution, which is 18% for the final spectrum. Arrows as in Figure 5. (Lower panel) Double reciprocal plots of $1/(\theta_0 - \theta)$ vs. 1/[β-phenylpropionate], right panel at 550 nm (●), middle panel at 445 nm (▲), and left panel at 340 nm (■), all calculated from the circular dichroism–titrations in the upper panel.

spectrum of the azoTyr-248·Zn complex and high concentrations that of the azophenol. The first binding mode of β-phenylpropionate does not induce any changes at 445 nm while the second causes changes which result in near identity both of the linear double reciprocal plots at 445 and 340 nm and the K_{app}'s derived from them (Figure 6, lower panel; left and middle, Table I). Thus, the changes in ellipticity at these wavelengths reflect the second binding mode (Table I).

The results for phenylacetate are qualitatively similar, and differ only in the magnitude of the negative band generated at 450 nm by high concentration of the agent as shown in Figure 7 for titrations with 20 and 60 mM reagent at pH 7.6. Phenylacetate, up to 20 mM, decreases the 420- and 510-nm bands, that at 340 nm decreases only thereafter and disappears completely at 100 mM. As for β-phenylpropionate (Figures 5 and 6), two binding modes are apparent (Table I), consistent with the effects on hydrolysis of peptide substrates (Auld et al., 1972).

Noncompetitive Inhibition, Modifiers, and Products. The effects of the noncompetitive inhibitors, indole-3-acetate and L-lysyl-L-tyrosylamide, on the circular dichroic spectrum are very similar to those of the first binding mode of both phenylacetate and β-phenylpropionate, i.e., the 510-nm band is completely suppressed and that at 420 nm is markedly diminished. However, while indole-3-acetate does not affect the 340-nm band, L-lysyl-L-tyrosylamide suppresses it (Figure 7). At present, an explanation for the different effects of the two agents is not apparent, based on their kinetic behavior. Detailed analyses of the circular dichroism titrations now under way may prove this paradox to be apparent only.

Cyclohexanol is a modifier which inhibits the esterase and activates the peptidase activities of native carboxypeptidase (Davies et al., 1968; Vallee, 1969). The circular dichroism titrations with cyclohexanol differ from all others (Figure 8). Concentrations varying from 1.1 to 55 mM abolish the negative 510-nm band, but *increase* the ellipticity at 340 and change that at 420 nm but slightly. There is, however, a single, tight isosbestic point at 420 nm. The changes in ellipticity reflect a single binding site with binding constants of 23 and 20 mM, calculated from the 510- and 340-nm ellipticities, respectively.

The product Cbz-Gly also inhibits the esterase and activates the peptidase activities of native carboxypeptidase (Davies et al., 1968), yet the circular dichroism titrations with this agent differ completely from that with cyclohexanol.[2] Cbz-Gly reduces all three circular dichroism bands at 510, 420, and 340 nm. However, owing to the weak binding and poor solubility of Cbz-Gly, titrations could be performed only up to 100 mM. The changes at 510 nm signal one binding site (Table I).

Discussion

Molecular Basis of AzoTyr-248 and AzoTyr-248·Zn Spectra. Coupling of carboxypeptidase with diazotized arsanilic acid modifies one single tyrosyl residue, azoTyr-248, and the 340- and 420-nm extrema of the circular dichroism spectra reflect its orientation relative to different groups or atoms and other vicinal features of the enzyme. All of the arsanilazoproteins studied by us thus far exhibit bands which have been assigned to azotyrosyl residues at these or closely similar wavelengths (Fairclough and Vallee, 1970). Thus, of itself, this group would be expected to signal changes in its orientation or conformation, constituting an environmental probe of alterations brought about directly or indirectly by, e.g., interaction with inhibitors.

[2] Dr. Gerald M. Alter performed the cyclohexanol experiments which he kindly put at our disposal.

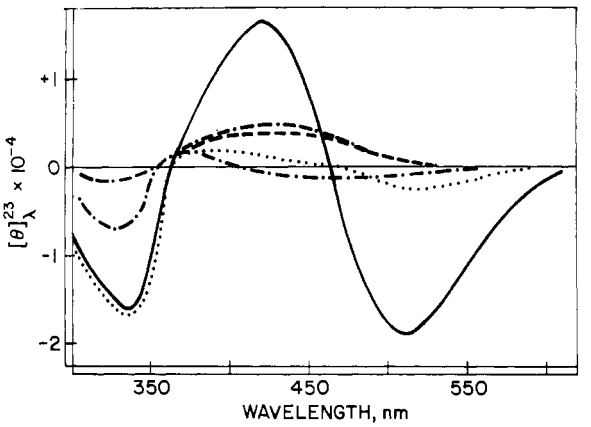

FIGURE 7: Effect of phenylacetate, 20 mM (···) or 60 mM (-·-·-), indole-3-acetate, 10 mM (- - - -), and L-lysyl-L-tyrosylamide, 100 mM (-·-·-), on the circular dichroism of zinc azoTyr-248 carboxypeptidase in 0.5 M Tris–0.5 M NaCl, pH 7.6, 23° (—).

Apart from this general environmental sensitivity of azotyrosyl groups, only the arsanilazocarboxypeptidase family of proteins has been found to exhibit an additional and selective sensitivity of azoTyr-248 to the vicinity of the zinc atom of these enzymes in general and of carboxypeptidase A in particular, since this modified residue forms an intramolecular chelate with a distinctive circular dichroism band at or near 510 nm (Fairclough and Vallee, 1970, 1971; Johansen and Vallee, 1975).

The AzoTyr-248 Probe Differentiated from the AzoTyr-248·Zn Chelate Probe. Coupling of the azo group to Tyr-248 results in two distinct species, each with characteristic properties that can probe their environments, respectively: (1) azoTyr-248 itself and (2) the azoTyr-248·Zn chelate. Hence, given a particular environmental perturbant, each probe may sense the same or different vicinal alterations. Thus, a substrate or inhibitor, e.g., might interact with the azophenol to disrupt its chelate with zinc, to alter its position in three-dimensional space but without interfering with the chelate, or to alter the conformation of azoTyr-248 under conditions where the chelate is not even formed, such as at low or high pH. Since both azoTyr-248 and azoTyr-248·Zn are theoretically optically active, circular dichroic spectra would be expected to detect the interaction of inhibitors—or substrate—when binding to form E·I or E·S complexes in the same or in different modes, or both. Thus, such circular dichroism spectra of azoTyr-248 carboxypeptidase inhibitor (substrate) complexes might qualitatively identify these sites of interaction with respect to azoTyr-248 alone and/or with the chelate and, further, quantitate the resultant stoichiometries.

Specificity and Scope of the Two Azoprobes. The two probes detailed constitute a constellation of sensors which specifically monitor events affecting their environments in the active center of carboxypeptidase. *Pari passu* the achievement of this objective also incurs the possibility that the spectral properties of such probes, based specifically on the interaction and topography of but two components of the active center, need not be sensitive to interactions affecting other neighboring catalytically or structurally important residues, or to events occurring at a distance. These limitations must be borne in mind, of course, when considering numerical assignments of inhibitor stoichiometries to competitive or noncompetitive modes and, ultimately, of substrates to productive or nonproductive sites of interactions.

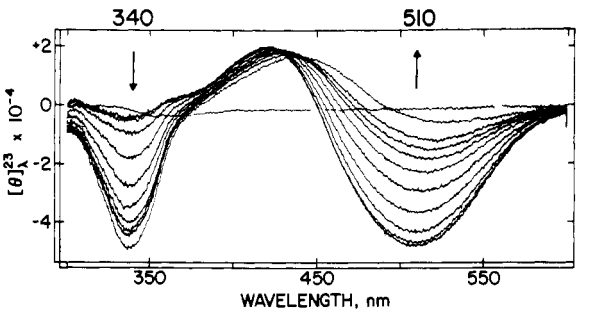

FIGURE 8: Effect of cyclohexanol on the circular dichroism of zinc azoTyr-248 carboxypeptidase in 0.05 M Tris–0.5 M NaCl, pH 8.5, 23°. Cyclohexanol concentration varies from 0 to 55 mM. Arrows as in Figure 5. (See footnote 2)

The system clearly defines spectral responses common to small competitive and mixed inhibitors of the enzyme, respectively. The spectral characteristics correlate well with the kinetic behavior of these two groups of agents. Such inhibition kinetics have long served to explore the mechanism of action of carboxypeptidase A (Snoke and Neurath, 1949; Smith, 1951; Vallee et al., 1968; Auld and Vallee, 1970; Auld et al., 1972). More recently, various equilibrium approaches have served to detail the interaction of inhibitors with functionally essential groups or atoms of the enzyme (Rupley and Neurath, 1960; Coleman and Vallee, 1964; Navon et al., 1968; Lipscomb et al., 1968; Latt and Vallee, 1971; Byers and Wolfenden, 1973; Auld and Holmquist, 1974). The mode of inhibition of native carboxypeptidase catalyzed hydrolysis of peptides is known to be competitive for the inhibitors L-benzylsuccinate, L-phenylalanine, L-phenyllactate, mixed for β-phenylpropionate, L-phenylacetate, and noncompetitive for indoleacetate and L-lysyl-L-tyrosylamide (Auld et al., 1972; Davies et al., 1968; Auld and Holmquist, 1974). Both cyclohexanol and Cbz-Gly are known to increase peptidase while decreasing esterase activities. These latter two were examined as examples of a group of modifiers (Davies et al., 1968; Vallee, 1969).

General Features of Arsanilazocarboxypeptidase–Inhibitor Spectra. All of the inhibitors studied here abolish the 510-nm absorption band of the azoenzyme (Figure 1) by disrupting the azoTyr-248·Zn chelate through alteration of the mutual orientation, proximity, or topographic relationships of the azoTyr-248 and the zinc atom.

In contrast, the effects of the inhibitors on the circular dichroic spectra differ and can be discussed in three categories. The first includes the competitive inhibitors L-benzylsuccinate (Figure 2), L-phenylalanine, and L-β-phenyllactate (Figure 4) and the pseudosubstrate, Gly-L-Tyr (Johansen and Vallee, 1975). The mixed inhibitors β-phenylpropionate (Figures 5 and 6) and phenylacetate (Figure 7) represent the second group. Their inhibitory effects on peptide hydrolysis can be resolved into discrete competitive and noncompetitive components (Auld et al., 1972). The third group is heterogeneous, encompassing the noncompetitive inhibitors indole-3-acetate and L-lysyl-L-tyrosylamide (Figure 7) as well as the modifiers, cyclohexanol (Figure 8) and Cbz-Gly. The effect of each of these is distinctive and differs both from that of any agent in this category and from those of the members of the other two groups.

Comparisons of the present spectral data with inhibition kinetics must of necessity be inferential, since the detailed inhibition kinetics of arsanilazocarboxypeptidase have not been reported as yet, and only those of the native enzyme

are currently available. However, since the kinetic characteristics of the native and azoenzyme species seem virtually the same (Auld and Holmquist, 1973), qualitative comparisons can be made.

Competitive Inhibitors. These competitive inhibitors abolish the 510-nm negative extremum and shift the 420-nm positive extremum of the circular dichroism band of azoTyr-248 to 440 nm, signaling a change in its environment. In the presence of saturating concentrations (Figures 2 and 4), the circular dichroic spectrum becomes virtually identical with that of the apoenzyme (Johansen and Vallee, 1975).

A single molecule of the monoanionic species of L-benzylsuccinate is tightly bound to the active site (Byers and Wolfenden, 1973), as here confirmed by circular dichroism titrations (Figure 3). When Cbz-Gly-Gly-L-Phe is the substrate, this potent competitive inhibitor has a $K_{I_{app}} = 6.3 \times 10^{-8}$ M at pH 6.6, 5×10^{-7} M at pH 7.6, and 6.3×10^{-6} M at pH 8.5, i.e., binding more strongly at lower pH (Byers and Wolfenden, 1973). The kinetic parameters for phenyllactate (McClure and Neurath, 1966; Hall et al., 1969), L-phenylalanine (D. S. Auld, in preparation), and Gly-L-Tyr (Izumiya and Uchio, 1959; Auld and Vallee, 1970) are affected by pH to similar extents (Table I).

In all instances the circular dichroism titrations performed at three pH values indicate that one molecule of these agents binds to one molecule of the enzyme (Figures 2 and 4), consistent with kinetic data that demonstrate them to be competitive inhibitors.

Mixed Inhibitors. β-Phenylpropionate and phenylacetate represent the second group and—dependent on conditions—can inhibit carboxypeptidase catalyzed hydrolysis of peptides both noncompetitively and competitively (Auld et al., 1972). The circular dichroic spectra of the azoenzyme resulting from titrations with these agents differ markedly from those of the first group. While β-phenylpropionate and phenylacetate also abolish the 510-nm ellipticity band, they differ in their effects on the spectrum below 500 nm. Up to 4 mM, β-phenylpropionate decreases the amplitude both of the 420- and 510-nm bands resulting in an apparent isosbestic point at 445 nm, but on increasing concentration from 10 to 50 mM, two new bands appear, centered at 380 and 450 nm, respectively; concomitantly a gradual shift to higher wavelengths in the apparent isosbestic point becomes apparent.

These spectra seem to signal two different binding modes of this inhibitor. The binding of the first β-phenylpropionate molecule apparently disrupts the intramolecular chelate, displacing azoTyr-248 outward, away from the zinc atom (Johansen and Vallee, 1975); the second molecule of β-phenylpropionate binds and perturbs the environment of the protonated azoTyr-248 residue, thereby affecting the circular dichroism bands at 420 and 340 nm. The noncompetitive mode is the only one prominently apparent at pH 6.6 where only azoTyr-248 can function as a probe, making it likely that this second binding mode is responsible for noncompetitive inhibition (see below). Phenylacetate induces very similar changes and likely acts in a similar manner.

Among all inhibitors of carboxypeptidase, β-phenylpropionate has been studied most widely. The large amount of information, obtained through different approaches, can serve to examine the deduction that the present circular dichroism titrations resolve two binding modes of this agent (Figure 6; Table I). Under conditions where [^{14}C]-β-phenylpropionate binds to the zinc enzyme, it does not bind to the apoenzyme (Coleman and Vallee, 1964). Further, its dissociation constant is a function of the particular metal atom at the active site, increasing in the order $Zn^{2+} < Cd^{2+} < Co^{2+}$ (Coleman and Vallee, 1964). Jointly, such data indicate that one of the modes requires a metal atom for binding. The interaction of β-phenylpropionate with cobalt carboxypeptidase perturbs the circular dichroism spectrum in at least two distinct ways. When enzyme and inhibitor are nearly stoichiometric, it induces one ellipticity band at 543 nm but, at much higher β-phenylpropionate concentrations, a second is induced at 580 nm (Latt and Vallee, 1971). Multiple interactions are also consistent with the observation that β-phenylpropionate protects two tyrosyl residues against a number of acylating agents which cause a loss of peptidase and an increase in esterase activities (Simpson et al., 1963; Riordan and Vallee, 1963, 1964). Its analogue, *p*-iodo-β-phenylpropionate, 0.05 M, binds to four enzyme sites, but only two of these were considered primary, i.e., those for the two molecules binding in the domain in which Gly-L-Tyr interacts. At least one of these two *p*-iodo-β-phenylpropionate molecules binds to zinc, though similar binding for the second could not be excluded (Steitz et al., 1967).

β-Phenylpropionate induces polymerization. One binding mode has a dissociation constant, ~0.1 mM, and corresponds to binding at the zinc atom, and a second, ~10 mM, i.e., much weaker, probably interacts with a hydrophobic residue, likely tyrosine. Both zinc removal and acetylation markedly diminish polymerization (Bethune, 1965).

In the presence of this agent, peptide hydrolysis is characterized by multiple inhibitory modes. Below pH 8 noncompetitive inhibition predominates, but at pH 8 and above, inhibition is mixed. Stopped-flow fluorescence analysis resolves this mixed inhibition into competitive and noncompetitive components (Auld et al., 1972; D. S. Auld, personal communication).

The results of circular dichroism titrations of zinc azoTyr-248 carboxypeptidase with β-phenylpropionate are in agreement with conclusions from previous thermodynamic and kinetic data. The β-phenylpropionate binding mode which suppresses the 420- and 340-nm bands is pH-dependent, and the corresponding binding constant is higher than that of the competitive mode (Table I). The competitive binding mode of β-phenylpropionate seems responsible for the disruption of the intramolecular coordination complex, judging from stopped-flow fluorescence data (Auld et al., 1972). The pH-dependent binding likely corresponds to noncompetitive binding in the vicinity of or to the protonated azoTyr-248 residue, analogous to phenylacetate.

Noncompetitive Inhibitors and Modifiers. The enzymatic and spectral responses of this mixed category of agents do not lend themselves to similarly direct interpretations. One each of these molecules primarily affect the 510- and 420-nm bands, in the case of indole-3-acetate similar to the first binding mode of β-phenylpropionate. Major differences relate primarily to their effects on the 340-nm band, which either remains unchanged (indole-3-acetate) or is variously perturbed (Cbz-Gly, cyclohexanol, and L-lysyl-L-tyrosylamide). Though overall, perturbations of the azoTyr-248 spectrum by these agents are similar, it has proven difficult to assign specific significance to the minor differences in details. However, such individual and different spectral consequences are not unexpected, considering the number of possible modes of interaction with the enzyme of peptide and

ester substrates, products, and modifiers (Vallee et al., 1968). We consider it likely that their interactions involve additional residues, other than azoTyr-248 and its zinc chelate. Complete mapping of the modes of interaction of these agents may require extensions of the present methods to encompass other residues.

The view that significant interactions likely involve other residues of the active center is reinforced by recent data demonstrating that nitration of Tyr-198 abolishes peptidase activity, once the enzyme has been coupled first at Tyr-248 with diazonium-1*H*-tetrazole (L. Cueni and J. F. Riordan, in preparation). Inhibitors might well interact with this or other side chains of the molecule in a manner which might not be detected by these spectra.

Both the general and specific features of all circular dichroism titrations performed are consistent with our mechanistic views (Johansen and Vallee, 1975). In the solutions of the azoenzyme at pH 8.5, azoTyr-248 forms a chelate with Zn, and all agents here studied disrupt it, as demonstrated by studies of metal-substituted enzymes, competition between different inhibitors and substrates, and magnetic circular dichroism titrations of the cobalt enzyme (A. Klyosov, J. T. Johansen, G. M. Alter, and B. L. Vallee, in preparation). Further, stopped-flow and temperature-jump studies (Harrison et al., 1975) demonstrate a completely analogous set of events at the very moment of catalysis. Close proximity between Tyr-248 and the zinc atom must be an essential feature of the enzyme's mechanism of action.

Acknowledgment

We are greatly indebted to Dr. Gerald M. Alter for the computer program and the cyclohexanol experiments.

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