Resonance Raman Spectroscopy of Arsanilazocarboxypeptidase A: Mode of Inhibitor Binding and Active-Site Topography†

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ABSTRACT: The interaction of inhibitors with the active site of arsanilazocarboxypeptidase A has been investigated by means of resonance Raman spectroscopy. The resonance Raman bands of the active-site azotyrosine-248 residue have been shown previously to be sensitive to its state of ionization and its interactions with nearby groups. In particular, the azophenol form of azotyrosine-248 can adopt two different coexisting conformations that differ with respect to the presence or absence of an intramolecular hydrogen bond between the phenolic proton and a nitrogen atom of the azo group. Each of these conformations exhibits characteristic ν^{NN} and $\nu^{\phi N}$ azo stretching frequencies. The relative concentrations of these two forms, revealed by resonance Raman spectroscopy, are a sensitive probe of the hydrogen bond accepting ability of the local environment. The present study shows that the binding of L-benzylsuccinate, phenylacetate, L-phenyllactate, and β-phenylpropionate markedly perturbs the distribution of the intra- and intermolecularly hydrogen-bonded forms of azotyrosine-248 in water. In contrast, glycyl-L-tyrosine and L-phenylalanine leave this distribution unperturbed. These results, taken jointly with other data on inhibitor binding, serve to identify common binding sites for groups of inhibitors and result in plausible suggestions concerning the interactions between azotyrosine-248 and these inhibitors that lead to binding.

The active center of carboxypeptidase A is known to contain multiple, overlapping loci for the binding of substrates, inhibitors, and other ligands (Vallee et al., 1968; Johansen et al., 1976; Alter & Vallee, 1978). Multiple binding at these sites is thought to be responsible for the complex kinetic behavior of the enzyme toward dipeptide and ester substrates. In addition, the binding of other ligands (referred to as "modifiers") either inhibits or activates the enzyme. The action of many inhibitors has been studied by steady-state and rapid kinetic techniques, and competitive, noncompetitive, and mixed modes of inhibition have been identified (Auld & Vallee, 1970; Auld et al., 1972). In addition, specific proposals based on chemical (Vallee et al., 1968) and X-ray structural (Lipscomb et al., 1970) data have been advanced to account for the multiple productive and nonproductive binding modes for substrates.

The interaction of inhibitors and other ligands with a chemically modified enzyme has been studied to gain further insight regarding the nature of the active center of carboxypeptidase A. Arsanilazocaboxypeptidase A,[1] the derivatve with the singly modified chromophoric arsanilazotyrosine-248 residue in the active site, has proven particularly useful for spectral characterization of ligand binding. The close correspondence of both the catalytic behavior (Johansen & Vallee, 1971, 1973) and kinetic (Harrison et al., 1975) behavior of the modified and native enzymes supports the relevance of these studies of the azoenzyme. The distinctive circular dichroic (CD)[2] and absorption spectra of the azoprobe are quite sensitive to its local environment and reflect the presence of bound ligands. It has proven possible both to detect and to obtain quantitative data for the binding of single ligands and also to study *multiple* inhibitor binding by monitoring different spectral bands of the azoprobe (Johansen et al., 1976; Alter & Vallee, 1978). These studies not only have provided information about the topology of the active site of the enzyme but also have allowed the identification of distinctive binding regions for the various ligands.

We have recently used resonance Raman spectroscopy (rRS)[2] to study selectively the vibrational bands of the azotyrosine-248 residue of azocarboxypeptidase. These investigations, which include a complete vibrational assignment derived from studies of model azophenols, have provided molecular details of the active center of the enzyme under a variety of environmental influences (Scheule et al., 1977, 1979, 1980). Near pH 8.5, the azoprobe forms an intramolecular complex with the active-site zinc atom. The rR spectra are sensitive to this interaction and, indeed, the observed spectral changes have enabled the structure of the complex to be deduced. At lower pH values, where the phenolic oxygen of azoTyr-248 is protonated, an equilibrium exists in aqueous solution between two forms of the azoprobe in which this proton is hydrogen bonded either *intra*molecularly to an azo nitrogen atom or *inter*molecularly to another acceptor on the enzyme or the solvent. These two species have distinct rR bands, and a shift in the relative populations of the two forms upon crystallization of the enzyme has served to define the nature of the conformational change involving this residue (Scheule et al., 1980).

In the present study, rRS has been used in a similar manner to examine the changes in the vibrational spectrum of azo-

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[1] In order to simplify the presentation, "arsanilazotyrosine-248 carboxypeptidase", "arsanilazocarboxypeptidase", "azocarboxypeptidase", "azoenzyme", and "enzyme" are all terms used interchangeably with monoarsanilazotyrosine-248 zinc carboxypeptidase A; unmodified carboxypeptidase is the native enzyme; "arsanilazotyrosine-248", "azotyrosine-248", "azoTyr-248", "azotyrosine", and "azoprobe" are all terms used interchangeably with monoarsanilazotyrosine-248.

[2] Abbreviations used: rR, resonance Raman; rRS, resonance Raman spectroscopy; CD, circular dichroism; benzylsuccinate, 2-benzyl-3-carboxypropionate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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tyrosine-248 brought about by inhibitor binding. The results identify the interactions of different groups of inhibitors with the active center of the enzyme, thereby supplementing the information obtained from other studies; at the same time, they elucidate the molecular details of these interactions.

Materials and Methods

Carboxypeptidase A, prepared by the method of Cox et al. (1964), was obtained as a crystal suspension (Sigma Chemical Co.) and purified by affinity chromatography (Peterson et al., 1976). Arsanilazotyrosine-248 carboxypeptidase A was prepared by treating a suspension of crystals of the native enzyme with diazotized *p*-arsanilic acid as described previously (Johansen & Vallee, 1971). Concentrations of the azoprotein were determined spectrophotometrically at 278 nm (Johansen & Vallee, 1975) by using an extinction coefficient (ϵ_{278}) of 7.32 $\times 10^4$ M^{-1} cm^{-1}.

Gly-L-Tyr and L-Phe were purchased from Fox Chemical Co. and recrystallized from ethanol and water prior to use. DL-Benzylsuccinic acid was purchased from Burdick and Jackson Laboratories, Inc., and the L isomer was isolated as described by Byers & Wolfenden (1973). β-Phenylpropionic acid was obtained from Eastman Organic Chemical Co. and was recrystallized from water. Phenylacetate was purchased from Mann Research Laboratories, Inc. Tris and Mes were from Sigma Chemical Co., and L-phenyllactate was from Aldrich Chemical Co.

The rR spectra were obtained with an instrument described previously (Scheule et al., 1977). The 4880-Å line of a Coherent Radiation Model CR3 argon ion laser was used to provide an unfocused power at the sample of 50 mW for the pH 8.5 solutions and 100 mW for the pH 6.5 solutions. All spectra were recorded at room temperature with an instrumental resolution of 8 cm^{-1}. Band positions were calibrated with the 981-cm^{-1} symmetric stretching band of sulfate ion (Kohlrausch, 1938). Each spectrum was recorded at least 3 times, and there were no differences between successive spectra.

In all samples, sufficient inhibitor was used to ensure formation of greater than 95% enzyme–inhibitor (EI) complex. In some cases, the nonresonance Raman contribution to the spectrum from the inhibitor was subtracted from the rR spectrum of the corresponding EI complex. The rR spectra of EI complexes with the inhibitors indole-3-acetate and L-lysyl-L-tyrosineamide could not be obtained because of fluorescent inteference.

Results

The inhibitors used in the present study are listed in Table I, together with their apparent dissociation constants. Of these, Gly-L-Tyr is hydrolyzed, albeit very slowly, and may be considered a "pseudosubstrate", but it is characterized better as a competitive inhibitor since its predominant mode of binding results in a nonproductive complex. L-Benzylsuccinate, L-phenyllactate, and L-Phe are also competitive inhibitors of peptide hydrolysis. A single molecule of these inhibitors binds to the enzyme. Phenylacetate and β-phenylpropionate are mixed inhibitors that display predominantly noncompetitive modes of inhibition of peptide hydrolysis at low pH (~6), the proportion of competitive inhibition increasing at higher pH (~8) values. Each of these substances has *two* binding sites and, accordingly, two molecules can be bound to the enzyme simultaneously. The mixed inhibition has been separated into competitive and noncompetitive modes (Auld et al., 1972) which, presumably, correspond to these two distinct binding sites. The rR spectra of these inhibitors will be discussed in groups according to their mode of inhibition of peptide hydrolysis.

Table I: Dissociation Constants (K_{app}) for Inhibitor Binding to Arsanilazocarboxypeptidase A

inhibitor	no.[f]	mode of inhibition[a]	pH[b]	K_{app} (mM)[c]	ref
glycyl-L-tyrosine	1	competitive	8.5	1.2 (510)	*d*
			8.0	2.3 (550)	*e*
			6.6	5.0 (420	*d*
L-phenylalanine	1	competitive	8.5	1.5 (510)	*d*
			8.0	4.5 (550)	*e*
			7.6	4.0 (510)	*d*
L-phenyllactate	1	competitive	8.5	2.8 (510)	*d*
			7.6	3.5 (510)	*d*
L-benzylsuccinate	1	competitive	8.5	0.013 (510)	*d*
			7.6	<0.01 (420)	*d*
			6.6	<0.01 (340)	*d*
β-phenylpropionate	2	mixed primary	8.5	6.4 (550)	*d*
			8.0	4.9 (550)	*e*
			7.6	2.4 (550)	*d*
			6.6		
		secondary	8.0	8.8 (440)	*e*
			7.6	9.4 (340)	*d*
			6.6	4.0 (340)	*d*
phenylacetate	2	mixed primary	8.5	16.0 (510)	*d*
			7.6	9.9 (510)	*d*
			6.6		
		secondary	8.5	50 (420)	*d*
			7.6	24 (420)	*d*
			6.6	17 (340)	*d*

[a] Defined toward peptide substrates. [b] Experiments at pH 8.5, 8.0, and 7.6 were carried out in 0.05 M Tris–0.5 M NaCl, while those at pH 6.6 were carried out in 0.05 M Mes–0.5 M NaCl. [c] The number in parentheses is the wavelength (in nanometers) at which the spectral titration was performed. [d] Johansen et al. (1976). [e] Alter & Vallee (1978). [f] Number of binding sites.

Competitive Inhibitors. The effect of L-benzylsuccinate binding on the rR spectrum of azocarboxypeptidase at pH 8.5 is illustrated in Figure 1. At this pH, and in the absence of inhibitor, the rR spectrum of the azoenzyme is characteristic of the metal complex between azotyrosine-248 and the active-site zinc (Figure 1A). In particular, the bands at 1536, 1435, 1339, and 1214 cm^{-1} are characteristic of the metal complex (Scheule et al., 1977, 1979). On addition of 0.3 mM L-benzylsuccinate, the single azo stretching band (ν^{NN}) at 1435 cm^{-1} is replaced by three new azo bands at 1423, 1439, and 1461 cm^{-1} (Figure 1B). The 1423- and 1461-cm^{-1} bands are due to intra- and intermolecularly hydrogen-bonded rotational isomers of the protonated azophenol species (Scheule et al., 1980), respectively, while that at 1439 cm^{-1} is the azo stretching band of ionized azoTyr-248 (Scheule et al., 1977, 1979). At pH 8.5, a significant fraction of the azotyrosine molecules should be protonated; the disproportionately high peak height of the azophenolate species (even though it is present at low concentration) is merely the results of its greater resonance enhancement releative to the azophenol species when excited at 488.0 nm. Thus, the disruption of the metal complex at pH 8.5 by L-benzylsuccinate results in an equilibrium between two azophenol species and the azophenolate anion. All of the inhibitors studied exhibit this general behavior.

At lower pH values, azoTyr-248 is completely protonated, and the spectrum of the enzyme *before* addition of inhibitors is that of the azophenol species (Scheule et al., 1977, 1979). Both of the azo stretching bands near 1430 and 1460 cm^{-1} are evident, the former being the more intense (Figure 2A). The progressive addition of L-benzylsuccinate at pH 6.5 inverts the relative intensities of these two azo stretching bands (Figure

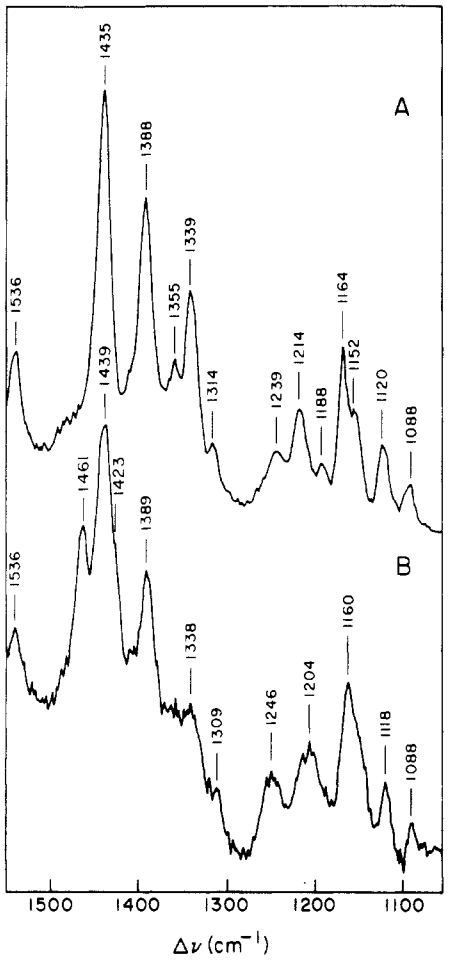

FIGURE 1: Resonance Raman titration of arsanilazocarboxypeptidase A with L-benzylsuccinate at pH 8.5. Enzyme concentration is 0.08 mM in 0.05 M Tris–0.5 M NaCl buffer, and L-benzylsuccinate concentrations are (A) 0.0 and (B) 0.30 mM.

2B,C). The spectrum of the L-benzylsuccinate–enzyme complex at pH 8.5 is a superposition of that at pH 6.5 plus a small amount of the azophenolate species. Some of the subsequent spectra are shown at pH 6.5 only when the effect of the inhibitor on the protonated azophenol form of the probe is seen most clearly without interference from the azophenolate azo stretch.

The competitive inhibitors studied can be grouped according to their effects on the rR spectrum of azoTyr-248. L-Benzylsuccinate and L-phenyllactate both disrupt the azotyrosine–zinc complex at pH 8.5 and also perturb the spectra of the azophenol species at both pH 6.5 (Figure 3A,B) and 8.5. The relative intensities of the azo stretching bands near 1430 and 1460 cm^{-1} reflect the position of the equilibrium between the intra- and intermolecularly hydrogen-bonded forms of the protonated azoTyr-248 residue (Scheule et al., 1980). Thus, these inhibitors shift the azophenol rotamer population to the extent that the 1460-cm^{-1} band becomes predominant. In the absence of inhibitors (Figure 2A), azotyrosine-248 is in an aqueous-like environment; i.e., the rotamer distribution is identical with that of model azophenols, such as monoarsanilazo-*N*-acetyltyrosine, in water (Scheule et al., 1979). The presence of L-benzylsuccinate and L-phenyllactate (Figures 2C and 3A,B), however, shifts this equilibrium in favor of the *inter*molecularly hydrogen-bonded species. In contrast, Gly-L-Tyr and L-Phe disrupt the azotyrosine–zinc interaction but *do not* significantly perturb the aqueous-like environment of the protonated azotyrosine residue (Figure 3C,D).

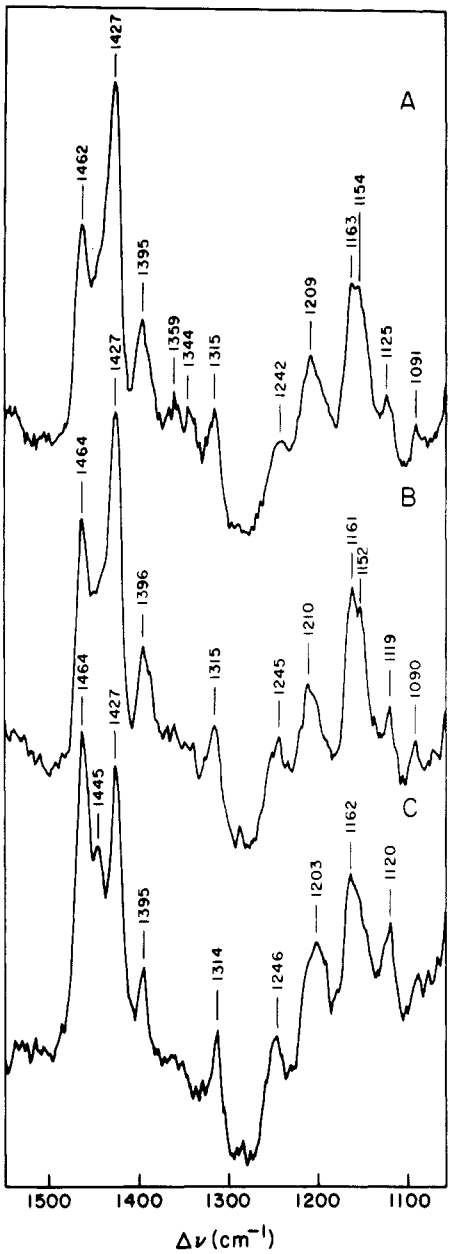

FIGURE 2: Resonance Raman titration of arsanilazocarboxypeptidase A with L-benzylsuccinate at pH 6.5. Enzyme concentration is 0.10 mM in 0.05 M Mes–0.5 M NaCl buffer, and L-benzylsuccinate concentrations are (A) 0.0, (B) 0.03, and (C) 0.3 mM.

Mixed Inhibitors. β-Phenylpropionate and phenylacetate each bind to two different sites on the enzyme (Alter & Vallee, 1978). The binding constants (Table I) do not differ enough to allow the effect of binding at each site on the rR spectra to be determined at any given pH. Hence, all data pertain to the enzyme with *both* sites occuppied. The noncompetitive and competitive modes of inhibition that are thought to correspond to these two sites are pH dependent. The noncompetitive mode dominates the inhibition around pH 6, while the noncompetitive and competitive modes contribute more equally near pH 8.5 (Auld et al., 1972).

The rR spectra of the azoenzyme in the presence of either β-phenylpropionate or phenylacetate are qualitatively very similar. At pH 8.5, the zinc–azotyrosine complex is disrupted and the azophenol rotamer distribution is inverted from its normal, aqueous state (Figure 4A,C). At pH 6.5, where only the azophenol species is present, these inhibitors perturb the rotameric equilibrium in essentially the same manner as at pH 8.5 (Figure 4B,D).

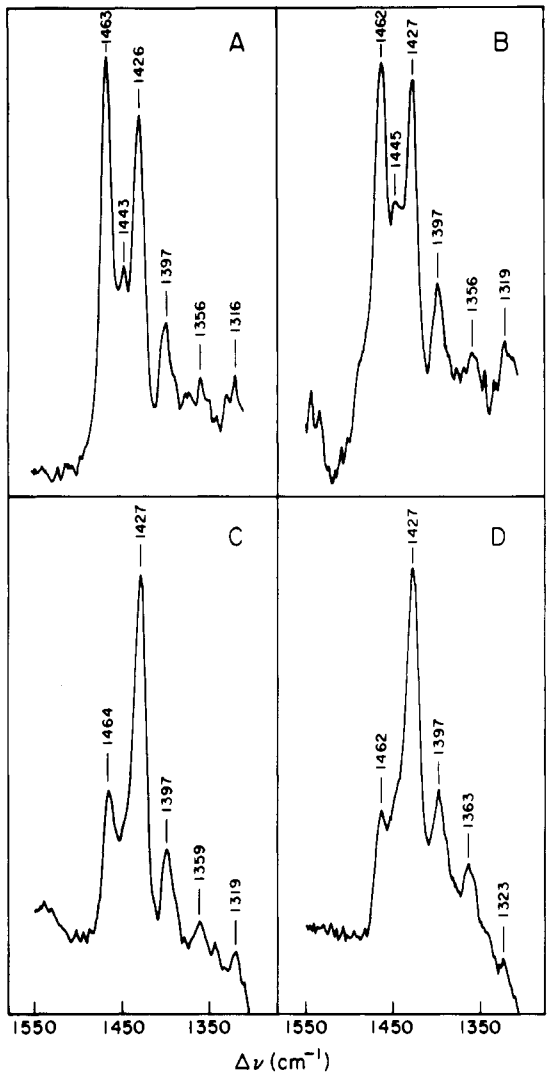

FIGURE 3: Resonance Raman spectra of the azo stretching region of arsanilazocarboxypeptidase A at pH 6.5 in the presence of saturating concentrations of (A) L-benzylsuccinate, (B) L-phenyllactate, (C) Gly-L-Tyr, and (D) L-Phe.

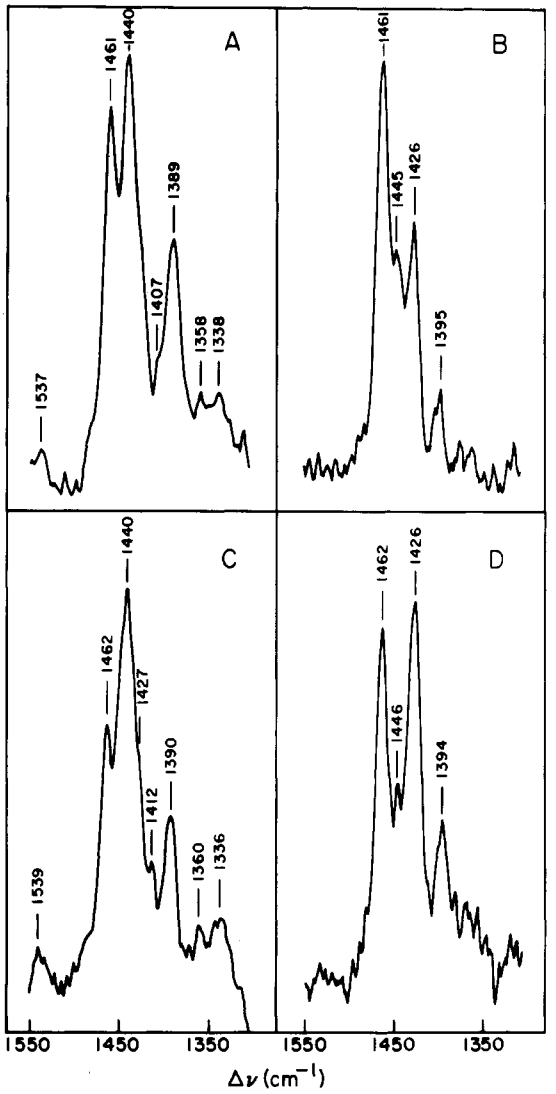

FIGURE 4: Resonance Raman spectra of the azo stretching region of arsanilazocarboxypeptidase A in the presence of saturating concentrations of β-phenylpropionate at (A) pH 8.5 and (B) pH 6.5 and of L-phenylacetate at (C) pH 8.5 and (D) pH 6.5.

Discussion

rRS is a form of vibrational spectroscopy that can provide specific information about the molecular interactions of a chromophoric molecule within the active site of an enzyme. The technique results in a relatively simple spectrum of the region of interest without interference from the rest of the protein. The full utilization of the potential of such a technique, however, requires the prior identification of the molecular motions responsible for the observed vibrational bands. Such a detailed analysis has been completed for the azotyrosine-248 residue of azocarboxypeptidase A (Scheule et al., 1979, 1980). Studies of isotopic substitutions and of model compounds have led to assignments for the rR bands of the azophenol, azophenolate, and zinc complex species of the azotyrosine-248 residue.

By use of these band assignments, the rR spectra have provided the following new detail about the interactions and environment of the azoTyr-248 residue in the enzyme. As a direct consequence of the analysis of its rR spectrum, a structure for the complex of azotyrosine with the active-site zinc atom was proposed (Scheule et al., 1977). In addition, the azophenol form of azoTyr-248 was found to exist in two distinct rotameric states, depending on its environment. Studies of model compounds have confirmed that these two rotamers are the result of an equilibrium involving the tyrosyl hydroxyl group (Scheule et al., 1979, 1980). In aqueous solution, the phenolic proton can be hydrogen bonded either *intra*molecularly to a nitrogen atom of the azo group or *inter*molecularly to a water molecule or to another hydrogen-bond acceptor. These two species exhibit strong azo stretching bands near 1430 and 1460 cm^{-1}, respectively. In the solution-phase azoenzyme, the predominant mode of azoTyr-248 hydrogen bonding is *intramolecular*. Crystallization of the azoenzyme shifts the relative proportion of these species dramatically such that azoTyr-248 is hydrogen bonded predominantly *intermolecularly* to another acceptor group, on either the same or another enzyme molecule (Scheule et al., 1980). Hence, the azo stretching region of the azoenzyme provides direct information about the state of hydrogen bonding of azoTyr-248 and, thus, about interactions within the active site of the enzyme. In the present study, we have used the characteristic rR spectra of the various azotyrosine species to study inhibitor binding to the active site. In particular, the position of the azophenol rotameric equilibrium is used to correlate the behavior and binding sites of various inhibitors.

The active site of carboxypeptidase A is known to consist of multiple overlapping binding sites, first postulated on the basis of kinetic studies (Vallee et al., 1968) and subsequently confirmed by thermodynamic approaches such as circular

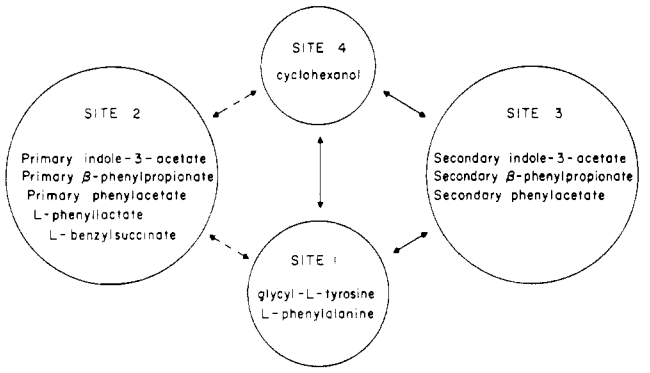

FIGURE 5: Schematic representation of inhibitor binding sites in the active center of carboxypeptidase A. Circles linked by solid arrows represent strongly interacting sites while circles linked by broken arrows represent weakly interacting sites; circles which are not linked represent independent and noninteracting sites.

dichroism titrations (Alter & Vallee, 1978). The number of sites no doubt contributes to the complex kinetics observed for dipeptide substrates (Auld & Vallee, 1970). Recent circular dichroism studies of inhibitor binding to the azoenzyme have mapped out four regions of the active site (Alter & Vallee, 1978). According to this model, Gly-L-Tyr and L-Phe bind at the same site (site 1). At site 2, one molecule of either β-phenylpropionate or indole-3-acetate binds. This is the *primary* binding site for these two inhibitors, i.e., that with highest binding affinity. A second molecule of either of these mixed inhibitors binds to a third, independent site (site 3) which has a weaker binding constant than site 2. Finally, the modifier cyclohexanol binds at a separate fourth site (site 4).

The binding of any two molecules at a given site is mutually exclusive. In addition, some of the four sites presumably overlap; therefore, binding at separate sites may or may not be independent. Specifically, binding at sites 1 and 3, 1 and 4, or 3 and 4 is mutually exclusive. On the other hand, binding at sites 2 and 3 is independent. Finally, binding at sites 1 and 2 or 2 and 4 is weakly dependent, suggesting that both sites *may* be occupied simultaneously, but with the result that the respective binding constants are altered.

Three of the inhibitors studied here—L-benzylsuccinate, phenyllactate, and phenylacetate—can be incorporated into this scheme on the basis of CD inhibitor titrations of the kind that led to the above model (G. M. Alter and B. L. Vallee, unpublished data). Since L-benzylsuccinate can bind simultaneously with Gly-L-Tyr, but competes strongly with the primary indole-3-acetate site, it would seem to bind at site 2. Phenyllactate competes with both the primary indole-3-acetate and primary β-phenylpropionate sites. Hence, it probably also binds at site 2. Phenylacetate resembles both β-phenylpropionate and indole-3-acetate kinetically. It exhibits mixed inhibition that can be resolved into competitive and noncompetitive modes which are pH dependent (Auld et al., 1972). It is therefore likely that two molecules bind—one at site 2 and the other at site 3. The binding scheme for the inhibitors studied is shown in Figure 5. Sites linked by solid arrows are thought to interact strongly while those linked by broken arrows are considered to interact weakly.

The rR data obtained in this study provide new details about the molecular interactions of inhibitors which constitute independent evidence to characterize and classify their binding. They can be separated into two groups based on whether their binding perturbs or fails to affect the azophenol rotameric equilibrium. Thus, Gly-L-Tyr and L-Phe *do not* affect the azophenol rR spectrum while β-phenylpropionate, phenylacetate, phenyllactate, and L-benzylsuccinate *do*. This classification does not necessarily imply that two inhibitors exhibiting similar behavior as judged by rRS bind to the same site, nor does it necessarily mean that two inhibitors exhibiting different behavior do not bind to the same site. Taken jointly with previous findings based on other data, however, possible correlations for the binding and modes of inhibition of different classes of inhibitors emerge.

Two independent hypotheses can be advanced to account for the fact that binding of certain inhibitors perturbs the azophenol rotameric equilibrium, while that of others does not. First, it is possible that the binding of an inhibitor to a certain site causes a conformational change in the enzyme which brings azotyrosine-248 into close proximity to a hydrogen-bond acceptor on the enzyme molecule. This could then promote the formation of a hydrogen bond between the azophenol proton and this acceptor and account for the observed changes in the azo stretching region of the rR spectra. Such conformational changes as a result of inhibitor binding have been observed in rapid kinetic studies with azocarboxypeptidase (Harrison & Vallee, 1978). This hypothesis is attractive since the four inhibitors that perturb the azophenol equilibrium all bind to the same site (site 2).

Alternatively, it is possible that the inhibitor-induced shift in the rotameric equilibrium, to favor the intermolecularly hydrogen-bonded azophenol species, is due to the direct interaction of azoTyr-248 with a hydrogen-bond accepting group on the *inhibitor itself*. Indeed, all of the inhibitors that elicit this behavior have functional groups that could fulfill this role. In this case, the rR spectral changes would provide information regarding the interaction of inhibitors with azotyrosine-248 that likely gives rise to part of the binding energy. With regard to this hypothesis, the specific molecular interaction possible for each inhibitor will be discussed separately.

L-Benzylsuccinate is a potent inhibitor of carboxypeptidase A (Table I). Its tight binding to the active site may be related to the fact that it is a "byproduct analogue", resembling the collected products of peptide hydrolysis (Byers & Wolfenden, 1973). This inhibitor has a single binding site, and one of its two carboxyl groups is thought to interact with the zinc atom (Byers & Wolfenden, 1973). In support of this hypothesis, Figure 1 shows that this inhibitor does displace the azotyrosine residue from the active-site zinc atom at pH 8.5. Moreover, Figures 1 and 2 indicate that the hydroxyl group of the noncomplexed azotyrosine-248 residue is also perturbed by the presence of L-benzylsuccinate. It is therefore possible that one carboxyl group interacts electrostatically with zinc while the second is involved in a hydrogen bond with the phenolic proton of azotyrosine-248. This would not only account for the rR data at pH 6.5 and 8.5 but would also explain the high affinity of L-benzylsuccinate and other dicarboxylic acid substrate product analogues for the active site as compared to inhibitors with a single carboxyl group (Byers & Wolfenden, 1973).

L-Phenyllactate, a product of ester hydrolysis, competitively inhibits both ester hydrolysis and peptide hydrolysis. A single molecule of phenyllactate or L-benzylsuccinate is thought to bind to the same site (Byers & Wolfenden, 1973), in agreement with the binding scheme shown in Figure 5. The rR spectrum of the enzyme–phenyllactate complex (Figure 3B) indicates that the azophenol form of azotyrosine-248 is hydrogen-bonded intermolecularly to another acceptor. Even though it has only one carboxyl group, phenyllactate can also interact simultaneously with both the zinc atom and tyrosine-248 by utilizing its carboxyl and hydroxyl moieties. Alternatively, phenyllactate may bind in such an orientation that its carboxyl group can interact *only* with Tyr-248; this

could explain why it binds so much more weakly than benzylsuccinate to the same site.

Two molecules of β-phenylpropionate are known to bind at the active site, and one molecule is thought to interact with the zinc atom (Coleman & Vallee, 1962, 1964). Inhibition by phenylacetate is similar to that by β-phenylpropionate, with two binding modes that can be resolved and one of which also appears to involve zinc (Auld & Vallee, 1970; Auld & Holmquist, 1974). Since in this study both binding sites are occupied, that corresponding to the interaction with azoTyr-248 cannot be established unambiguously. Inasmuch as the binding at the primary β-phenylpropionate site, the primary phenylacetate site, and the L-benzylsuccinate site is all mutually exclusive, however, it would seem likely that all inhibitors that can bind at site 2 interact with the active-site zinc atom. It is plausible that binding at site 3 involves an interaction between azoTyr-248 and a carboxyl group of the inhibitor. These two binding sites may well account for the noncompetitive and competitive modes of inhibition demonstrated by kinetic studies.

The interaction of Gly-L-Tyr and L-Phe with the enzyme differs significantly from that of these other inhibitors. The X-ray structure of crystalline carboxypeptidase with Gly-L-Tyr bound in the active center indicates that the complex is stabilized by interaction between its terminal carboxyl group and Arg-145, between the amide oxygen and the zinc atom, and between the tyrosyl ring and a hydrophobic pocket of the enzyme (Reeke et al., 1967; Lipscomb et al., 1968). In addition, the phenolic group of Tyr-248 was found to be close to the amide bond of Gly-L-Tyr, and it was suggested that this residue might donate its phenolic proton to the scissile peptide bond of specific substrates. It should be pointed out, however, that these interpretations of the X-ray structure assume that all of the Gly-L-Tyr is bound in the product mode (Lipscomb et al., 1970), a view which does not seem tenable any longer (Johansen & Vallee, 1975).

The rR data (Figure 3C) are consistent with the deduction that the inhibitor binds to the solution-phase azoenzyme in this manner in that, at pH 8.5, Gly-L-Tyr displaces tyrosine-248 from the active-site zinc atom. Since Gly-L-Tyr, however, does not appear to perturb the intramolecular hydrogen bond of azotyrosine-248, these results eliminate the possibility of hydrogen bonding between Gly-L-Tyr and Tyr-248. While this observation need not necessarily contradict the proposed role of tyrosine-248 in catalysis, it does show the absence of the hydrogen-bonding interaction, which, if existent, would have greatly supported this concept. L-Phenylalanine also fails to perturb the rR spectrum of the uncomplexed azotyrosine (see Figure 3D), consistent with the observation that Gly-L-Tyr and L-Phe appear to bind to the same locus (Alter & Vallee, 1978). Finally, the weak interaction between sites 1 and 2 may be due to the fact that inhibitors bound to each site interact with the metal. The fact that binding is not mutually exclusive could be interpreted to denote either that interaction with zinc is not essential for these binding modes or, more likely, that both interactions can occur simultaneously, albeit with each being weakened.

The rR spectra of the arsanilazocarboxypeptidase A–inhibitor complexes studied here clearly reveal marked and specific differences between the binding modes of various inhibitors. At pH 8.5, the breakdown of the zinc–azotyrosine complex was observed for all the inhibitors studied, reaffirming results and conclusions from earlier circular dichroism and absorption spectra (Johansen et al., 1976). At both pH 8.5 and 6.5, certain inhibitors elicit a spectral response from the azotyrosine residue which implicates its hydroxyl moiety in specific hydrogen-bonding interactions. It cannot be stated with certainty whether this difference in behavior is due to conformational changes induced in the enzyme or to specific interactions between azoTyr and the bound inhibitor. Taken jointly, however, with other data available, these results greatly enhance our knowledge of the mode of inhibitor binding and the active-site topology of the enzyme.

The application of the rR technique to the study of arsanilazocarboxypeptidase under various conditions has provided detailed information that would have been difficult to obtain by other means. Thus, the discrete nature of the vibrational bands in the resulting spectra, and the assignment of the molecular motions responsible for them (Scheule et al., 1979), has enabled us, first, to elucidate the nature of the azotyrosine-248–zinc complex in solution (Scheule et al., 1977), and, second, to compare the structure in aqueous solution with the conformational change induced in the enzyme by crystallization (Scheule et al., 1980). In the present work, we have confirmed previous conclusions and extended this approach to study the binding of inhibitors at the active center. The information provided by rRS in each of these studies was much more detailed than that obtained from other spectroscopic techniques (e.g., circular dichroism and absorption) and illustrates the power of rRS when used in conjunction with a suitably modified enzyme (H. E. Van Wart, R. K. Scheule, B. L. Vallee, and H. A. Scheraga, unpublished experiments).

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Potassium- and Calcium-Induced Alterations in Lipid Interactions of Isolated Plasma Membranes from *Blastocladiella emersonii*. Evidence for an Adenosine 5′-Triphosphate Requirement†

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ABSTRACT: The physical–chemical properties of the isolated plasma membranes from zoospores of the chytridiomycete *Blastocladiella emersonii* were investigated, with electron spin resonance (ESR) spectroscopy, using the spin-label 5-nitroxystearate (5-NS). Both isolated plasma membranes and aqueous dispersions of the lipids extracted from the plasma membranes were spin-labeled and analyzed. Plots of the hyperfine splitting parameter ($2T_{\parallel}$) vs. temperature indicated that the middle break point, T_M, initially observed in experiments with spin-labeled zoospores in vivo [Leonards, K. S., & Haug, A. (1980) *Biochim. Biophys. Acta 600*, 805–816], was the result of a lipid–lipid interaction (glycolipid–glycolipid or glycolipid–neutral lipid) rather than a lipid–protein interaction. This interaction was markedly affected by Ca^{2+} ions, which interacted directly with the lipid components, increasing T_M from 11 ± 1 (Ca^{2+} removed by EDTA) to 21 ± 1 °C (10 mM Ca^{2+}) in the lipid dispersions and from 12 ± 1 to 23 ± 1 °C in the plasma membrane preparations. The initial ESR studies on spin-labeled zoospores in vivo had also demonstrated that the addition of K^+ ions could reverse the Ca^{2+} ion effect, downshifting T_M from 22 ± 1 to 10 ± 1 °C. The addition of K^+ ions to the isolated plasma membrane had no affect on T_M, indicating that K^+ ions do not simply replace Ca^{2+} ions but exert their effect indirectly on the membrane. However, after the inclusion of ATP, K^+ ions could reverse the Ca^{2+} ion effect. It was determined that the ATP generated an "energized membrane" state which permitted the K^+ ions to reverse the Ca^{2+} effect. Since K^+ ions have been shown to depolarize the membrane potential in both zoospores and isolated zoospore plasma membrane preparations (generated by ATP), we suggest that the K^+ ion induced reversal of the Ca^{2+} ion effect, and therefore the change in the lipid–lipid interactions responsible for T_M, is a consequence of the K^+ ion induced depolarization of the membrane potential.

Zoospores of the chytridiomycete *Blastocladiella emersonii* have proved to be an excellent model system for studying the role(s) of the plasma membrane in eukaryotic cell differentiation and development. The plasma membrane is intimately involved in the differentiation process. Although zoospore encystment does not seem to require either protein or RNA[1] synthesis (Soll & Sonneborn, 1971; Lovett, 1975), it does involve extensive membrane alterations. The first detectable changes which occur during encystment include the induction of cell adhesiveness (Cantino et al., 1968), alterations in cell surface monitored by FITC-concanavalin A (Jen & Haug, 1979), and the fusion of vesicles derived from the γ particles with the plasma membranes (Truesdell & Cantino, 1970; Myers & Cantino, 1974). In addition, the differentiation process is markedly affected by zoospore's ionic environment, with Ca^{2+} ions inhibiting and K^+ ions inducing encystment (Cantino et al., 1968; Soll & Sonneborn, 1969, 1972).

We have previously demonstrated a correlation between cation- and temperature-induced changes in the physical–chemical properties of the zoospore plasma membrane, in vivo, and the effects of temperature and cations on zoospore differentiation and viability (Leonards & Haug 1979a, 1980a). These studies indicated the presence of three break points (T_L, T_M, and T_H), as observed with ESR spectroscopy, in plots of $2T_{\parallel}$, S, and f vs. temperature. Of the three, T_L and T_H were observed in zoospore total lipid extracts. Further studies on the isolated lipid components of the zoospore indicated that it was the zoospore glycolipids rather than the phospholipids which gave rise to T_L and T_H (Leonards & Haug, 1979b, 1980b).

However, it was the middle break point, T_M, which was affected by the external cation concentration. The cation-induced shifts in T_M were closely correlated with the temperature dependence and physiological effects of cations on zoospore differentiation, suggesting that the physical–chemical properties of the plasma membrane were involved in regulating the initial changes during zoospore encystment (Leonards & Haug, 1979a, 1980a).

The absence of the middle break point in both the total lipid extracts and in the zoospore phospholipid and glycolipid dis-

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[1] Abbreviations used: Mops, 3-(*N*-morpholino)propanesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; AMP-PNP, adenylyl imidodiphosphate; 5-NS or 5-nitroxystearate, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy; TLC, thin-layer chromatography; RNA, ribonucleic acid; ATP, adenosine 5′-triphosphate; cAMP, adenosine cyclic 3′,5′-phosphate.

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