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Resonance Raman Spectroscopy of Arsanilazocarboxypeptidase A: Conformational Equilibria in Solution and Crystal Phases†

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ABSTRACT: Resonance Raman spectroscopy has been employed to explore the environment of the azoTyr-248 residue of arsanilazotyrosine-248 carboxypeptidase A in solution and in crystal phases. In solution near pH 6, azoTyr is protonated and studies of model compounds indicate that azoTyr-248 is in an aqueous environment. At pH 8.5, the spectrum of the arsanilazoenzyme correlates well with those of model azophenols forming complexes with zinc, in accord with earlier data demonstrating an intramolecular complex between azoTyr-248 and the active-site zinc atom of the enzyme. Zinc is bound to the phenolic oxygen and an azo nitrogen atom of azoTyr, with the azo group in the planar trans conformation. At pH 11, this intramolecular complex is dissociated and azoTyr-248 then exists as the ionized azophenolate species. Resonance Raman studies of *o*-hydroxyazobenzene models for azoTyr show that in solution the azophenol forms of these molecules coexist in two different conformations which 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 $\nu^{\phi N}$ bands, with intensities proportional to their concentrations. The relative amounts of these two forms which are in equilibrium depend on the capacity of the local environment to act as hydrogen-bond acceptors. Hence, the relative intensities of both the pair of ν^{NN} and the pair of $\nu^{\phi N}$ bands in the resonance Raman spectrum of azocarboxypeptidase provide specific information about the state of hydrogen bonding of the phenolic proton of azoTyr-248, which has been thought by some to be transferred to the substrate during catalysis. In solution, azoTyr-248 is predominantly hydrogen-bonded *intra*molecularly and exists in an "aqueous-like" environment. However, crystallization apparently induces a conformational change that enables the phenolic proton of Tyr-248 to form an *inter*molecular hydrogen bond to a group of the protein. This interaction may be related to the marked reduction of the enzyme's activity brought about by crystallization.

Arsanilazocarboxypeptidase A,[1] the derivative of carboxypeptidase A, obtained by selective chemical modification of its active-site Tyr-248 residue with diazotized arsanilic acid, has been studied intensively in our laboratories. In particular, absorption and circular dichroism spectra have characterized both its local conformation as a function of its physical state (Johansen & Vallee, 1971, 1973, 1975) and its interaction with inhibitors (Johansen et al., 1976; Alter & Vallee, 1978). Recently, we have further elucidated chemical details pertinent to these molecular events by means of resonance Raman (rR)[2] spectroscopy (Scheule et al., 1977, 1979).

The rR spectra of the azoenzyme and the apoazoenzyme in solution (Scheule et al., 1977) identify the same pH-dependent species detected earlier by absorption spectrometry. In addition, the rR spectra provide structural details of the intramolecular coordination complex between azoTyr-248 and the active-site zinc atom and have similar potential for the conformation of the rest of the enzyme. However, for this purpose more extensive band assignments are required. This has now been achieved through the study of the rR spectra of several azophenols and their isotopically substituted derivatives which serve as models of azotyrosine (Scheule et al., 1979). The resultant data are employed here to identify the vibrational bands in the rR spectra of azocarboxypeptidase, and these assignments are used to interpret the spectral changes in the enzyme induced by various environmental perturbations, including crystallization and changes in pH. Crystallization is known to affect the spectral properties of

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

[2] Abbreviations used: rR, resonance Raman; DAC, monoarsanilazo-*p*-cresol; DAT, monoarsanilazo-*N*-acetyltyrosine; DMS, dimethyl suberimidate.

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azocarboxypeptidase markedly (Johansen & Vallee, 1975) and to reduce its enzymatic activity significantly (Spilburg et al., 1974, 1977). These spectral and kinetic data both indicate conformational differences between the crystal and solution phases of the enzyme (Johansen & Vallee, 1971, 1973, 1975; Riordan & Muszynska, 1974; Spilburg et al., 1977).

A valid mechanism for carboxypeptidase A must be consistent with all relevant information and requires understanding both of the interdependence between structural and functional data and conjoint changes in the two phases. While electronic absorption spectra both of azoTyr-248 and its zinc complex signal the occurrence of conformational changes, they cannot provide chemical details of their precise nature. However, rR spectroscopy does have this capability and is one of the few methods currently available that can provide such information on suitable enzymes in both the solution and crystal phases. These circumstances were the primary reasons for the present study of phase-induced conformational changes of azocarboxypeptidase.

At pH 7.5, both absorption and rR spectroscopy show that crystallization induces conformational changes in azocarboxypeptidase. However, at pH 6, the absorption spectra of crystals and solutions of the enzyme are identical, while their rR spectra differ significantly. Analysis of the rR spectra indicates that the conformational change in the enzyme observed upon crystallization at either pH involves the movement of Tyr-248 into an environment in which its phenolic proton is hydrogen bonded to a group either on the same or a neighboring molecule. Further, preliminary studies indicate that certain inhibitors induce similar conformational changes.

Materials and Methods

Sodium nitrite (^{15}N, 99.5%) and *p*-arsanilic acid (^{15}N, 99%) were obtained from International Chemical and Nuclear Corp. and Isotope Labeling Corp., respectively. Dimethyl-d_6 sulfoxide (99.5 atom % D) was a product of Merck Sharp & Dohme. *p*-Arsanilic acid (Sigma Chemical Co.) was recrystallized from warm water under a nitrogen atmosphere after treatment with activated charcoal and filtration through Celite. Recrystallization was achieved by cooling under nitrogen.

Monoarsanilazo-*N*-acetyltyrosine (DAT)[2] and monoarsanilazo-*p*-cresol (DAC) were synthesized by the method of Sokolovsky & Vallee (1966). The reaction products thus obtained were heterogeneous and the monoarsanilazo derivatives of interest were purified by silica gel chromatography using an eluant of chloroform–methanol–17% ammonia (30:50:20 v/v). In 0.1 N NaOH, DAT exhibits maxima at 326 and 483 nm with extinction coefficients of 14.0×10^3 and 10.5×10^3 M^{-1} cm^{-1}, respectively. As the pH is lowered, an isosbestic point at 416 nm ($\epsilon = 6.29 \times 10^3$ M^{-1} cm^{-1}) is apparent. These spectral parameters all compare favorably with those reported by Tabachnik & Sobotka (1959) for mono(*p*-azobenzenearsonic acid)chloroacetyltyrosine. Elemental analysis (DAT): C, 43.62; H, 4.25; N, 8.98; As, 16.02. Calculated for $C_{17}H_{18}N_3O_7As \cdot H_2O$: C, 43.50; H, 4.34; N, 8.96; As, 15.99. Elemental analysis (DAC): C, 46.32; H, 3.80; N, 8.26; As, 22.26. Calculated for $C_{13}H_{13}N_2O_4As$: C, 46.45; H, 3.90; N, 8.33; As, 22.29.

$^{15}N_1$ or $^{15}N_2$ derivatives[3] of DAT and DAC were synthesized by using [^{15}N]*p*-arsanilic acid or sodium [^{15}N]nitrite, respectively, in the diazotization reaction. The *O*-methyl derivative of DAC (with the phenolic –OH converted to an $-OCH_3$ group) was prepared by reaction of DAC with dimethyl sulfate (Aldrich, 99+%, Gold Label). Dimethyl sulfate and equivalent amounts of base were added in aliquots to a basic aqueous solution of DAC at 0 °C. Neutralization of the reaction mixture gave a product containing ~15% of the unreacted starting material, according to its proton nuclear magnetic resonance spectrum. A subsequent reaction of this product with dimethyl sulfate was used to convert the remaining unreacted DAC to the *O*-methyl form. This second product was found to be greater than 99% methylated by proton nuclear magnetic resonance and rR spectroscopy. The much larger degree of resonance enhancement of the azophenolate ion relative to the *O*-methyl derivative (~20 times) enabled the detection of as little as 1% of the azophenolate species in the rR spectrum of a basic solution of the product.

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 the native enzyme crystals with diazotized *p*-arsanilic acid as described previously (Johansen & Vallee, 1971). The $^{15}N_2$ derivative was prepared by using sodium [^{15}N]nitrite in the diazotization reaction. Azocarboxypeptidase was cross-linked with dimethyl suberimidate hydrochloride (DMS) (Aldrich). Crystalline azoenzyme (5 mg/mL) was suspended in 0.02 M Veronal buffer at pH 8.5, and solid DMS (0.4 mg/mg of enzyme) was added in aliquots over a period of 3 h during which the pH was maintained between 8.0 and 8.5. The crystals were then rinsed exhaustively with metal-free water followed by 0.05 M Tris-HCl at pH 7.5 containing 0.5 M NaCl.

Protein concentration was measured by the absorbance at 278 nm with $\epsilon_{278} = 7.32 \times 10^4$ M^{-1} cm^{-1} (Johansen & Vallee, 1971). The concentrations of DAT and DAC were also determined spectrophotometrically by using $\epsilon_{416} = 6.29 \times 10^3$ M^{-1} cm^{-1}.

rR spectra were obtained as described previously (Scheule et al., 1977). The spectra of colored solids were obtained by using a spinning sample (2% w/w in KBr) pressed between two glass microscope cover slips. All rR spectra were obtained with the 488.0-nm line of a Coherent Radiation Model CR2 argon ion laser. Most rR spectra were obtained with 40–50 mW of unfocused power at the sample. Spectra of the crystalline azoenzyme (and of solid model compounds) were taken with 10–15 mW of unfocused power to minimize thermal effects. Temperature control (±2 °C) of rR samples was achieved by mounting them in a brass block through which water was circulated from a constant temperature bath. Precooled dry nitrogen was blown over the sample tube to prevent condensation at low temperatures. All rR spectra were frequency-calibrated by using the 981-cm^{-1} symmetric stretching band of sulfate ion (Kohlrausch, 1938), which was added as an internal standard.

Results

Characterization of Species of AzoTyr-248. In aqueous solution, zinc azocarboxypeptidase exhibits three pH-dependent species of the azoTyr-248 residue, each displaying characteristic absorption and circular dichroism spectra (Johansen & Vallee, 1975). Near pH 8.5 azoTyr-248 is coordinated to the active-site zinc atom while at lower and higher pH values the complex dissociates to yield the protonated azophenol or azophenolate anion, respectively. In contrast, the spectra of the apoazoenzyme lack the bands attributable to the metal complex. Thus, the metal-free enzyme has only two pH-dependent azoTyr-248 species, i.e., its azophenol and azo-

[3] See Figure 2 for numbering scheme.

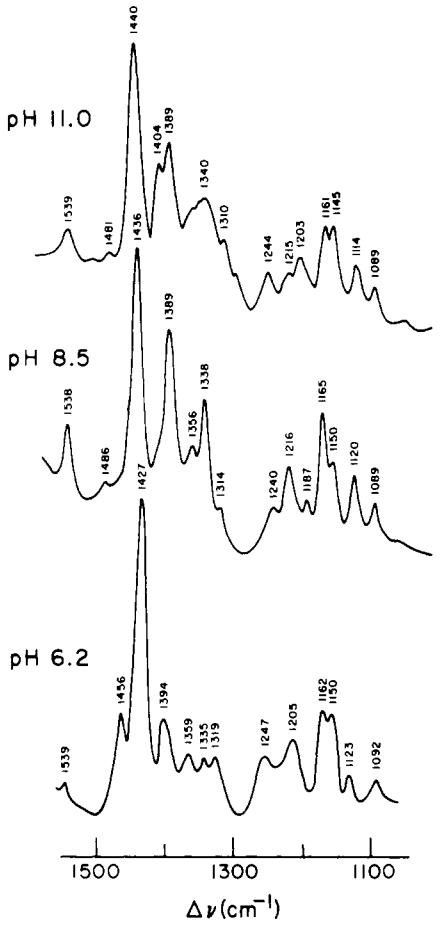

FIGURE 1: Resonance Raman spectra of the three species (see text) of arsanilazotyrosine-248 carboxypeptidase at the pH values indicated. See Table I for band assignments.

Table I: Resonance Raman Spectra of Arsanilazotyrosine-248 Carboxylpeptidase[a]

azophenol, pH 6.2	complex, pH 8.5	azophenolate, pH 11.0	assignment[b]
-	1538 m	1539 w	$\nu\phi O + {}^{2}\nu_{8b}CC$
?	1486 w	1481 w	${}^{2}\nu_{19a}CC$
1456* m 1427* s	1436 s	1440 s	$\nu^{NN} + {}^{2}\nu_{19b}CC$
?	1408 sh	1404 w	${}^{1}\nu_{19b}CC$
1394 m	1389 s	1389 s	${}^{2}\nu_{19b}CC + \nu^{NN}$
1359 w	1356 w	~1355 sh	${}^{2}\beta_{3}CH$
-	1338 s	1340 m, b	${}^{2}\nu_{14}CC + \beta\phi O$
1319 w	1314 w	1310 sh	
1247 w, b	1240 w	1244 w	${}^{2}\nu_{7a}CX$
1205 w, b	1216 m 1187 w	1215 w 1203 w	$\nu\phi N + {}^{2}\nu_{13}CX$ ${}^{2}\nu_{13}CX + \nu\phi N$
1162 m	1165 s	1161 m	${}^{1}\nu_{13}\phi X$
1150* m 1123* w	1150 m	1145 m	${}^{2}\beta_{15}CH + {}^{2}\nu_{1}CC + \nu\phi N$
-	1120 m	1114 w	${}^{2}\nu_{12}CC + {}^{2}\beta_{18b}CH$
1092 w	1089 w	1089 w	${}^{1}\nu_{1}CC$

[a] Only bands in the 1100–1550-cm^{-1} region are listed. Bands with asterisks arise from rotamers (see text). All spectra are of 0.05 mM aqueous samples in 0.5 M NaCl and 2 mM Tris-HCl at 22 °C. A dash denotes t e absence of a band, and ? denotes uncertainty as to its presence. Abbreviations: s, strong; m, medium; w, weak; b, broad; sh, shoulder; v, very. [b] Abbreviations: ν, stretch; β, in-plane bend; X, ring substituent. The subscripts (lower right) identify the normal mode according to the nomenclature of Wilson (1934). The superscripts (upper left) refer to modes arising from the aromatic ring on the left (Ar_1) or right (Ar_2) side of the azo group when the molecule is viewed as shown in Figure 2. Where more than one mode designation is used, the order given reflects their relative contributions to the total potential energy change occurring during the vibration, the largest contribution coming from the mode listed first.

phenolate forms (Johansen & Vallee, 1973, 1975).

rR titrations of the aqueous zinc azoenzyme confirm the existence of three species of azoTyr-248. The rR spectra at pH 6.2, 8.5, and 11.0 (Scheule et al., 1977) are shown in Figure 1 and are summarized together with band assignments in Table I. The rR spectra of the apoazoenzyme at pH 6.2 and 11.0 (not shown) are characteristic of the azophenol and azophenolate species, respectively, the two being superimposed near pH 8.5 where the rR spectrum, characteristic of the metal complex, is clearly nonexistent. Finally, values of pK_{app} resulting from rR titrations of the zinc azo- and apoazoenzymes are in excellent agreement with those obtained by spectrophotometric titration.

Assignment of Vibrational Bands of Arsanilazocarboxypeptidase. The assignment of the vibrational bands of the three azoTyr-248 species of azocarboxypeptidase is based on the rR spectra of a series of azophenols and their ^{15}N and ^{2}H derivatives (Scheule et al., 1979). Both the structures (Figure 2) and rR spectra (Scheule et al., 1979) of the model azophenol DAC and azoTyr-248 correspond closely. Thus, the assignments for the azoenzyme listed in Table I are obtained by direct comparison of the spectra of these substances at various pH values and by reference to the band assignments for DAC. The bands observed are due to skeletal stretching and bending motions of the central C—N=N—C azo group and the para-disubstituted (superscript 1) and asymmetrically trisubstituted (superscript 2) benzene rings. Importantly, in solution the azophenol (pH 6.2) species of azotyrosine and structurally related model azophenols exhibit *two* modes with azo stretching (ν^{NN})[4] and *two* modes with phenyl–nitrogen

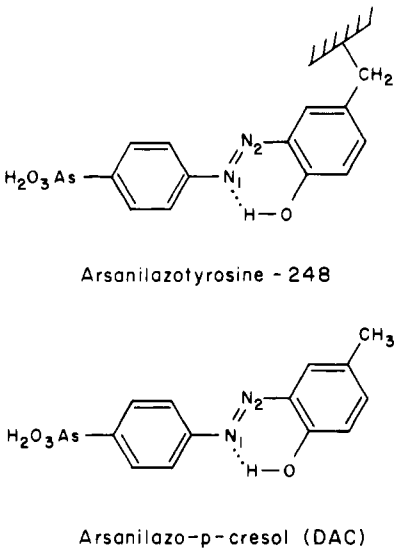

FIGURE 2: Azophenol forms of the azoTyr-248 residue of arsanilazocarboxypeptidase and the model azophenol, arsanilazo-*p*-cresol (DAC).

stretching ($\nu^{\phi N}$)[4] character, indicating the coexistence of two distinct (and previously undefined) conformations.

Rotameric Equilibria of Azotyrosine and Other Azophenols. The pairs of ν^{NN} bands near 1460 and 1430 cm^{-1} and $\nu^{\phi N}$

[4] The ν^{NN} and $\nu^{\phi N}$ modes are actually coupled modes (see Table I), but these designations are retained throughout the text for simplicity.

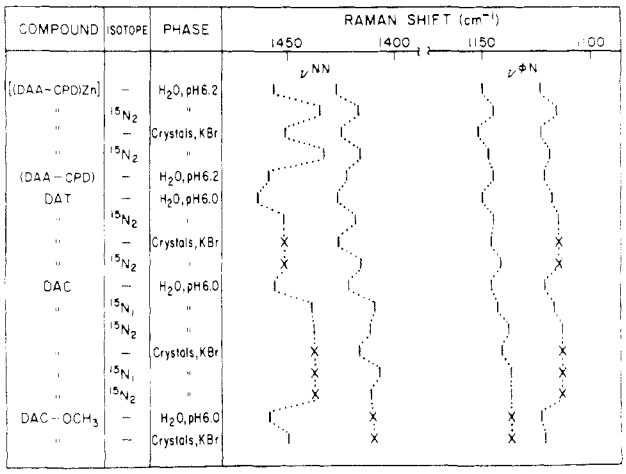

FIGURE 3: Frequency correlation diagram for the azo modes of arsanilazotyrosine-248 carboxypeptidase and several model azophenols. The ν^{NN} and $\nu^{\phi N}$ modes are actually coupled (see Table I), but the respective designations are retained for simplicity. "X" denotes the absence of a band.

bands near 1150 and 1120 cm^{-1} in the solution-phase rR spectra of *o*-hydroxyazobenzenes exhibit characteristics that arise from rotational isomers. The correlation diagram in Figure 3 summarizes their behavior upon ^{15}N-substitution and crystallization. Upon ^{15}N-substitution, each band within a given pair shifts in frequency by the same amount, reaffirming that they both arise from essentially the same mode. For example, $^{15}N_2$-substitution lowers the frequencies of the bands at 1456 and 1427 cm^{-1} of the solution-phase enzyme at pH 6.2 by 11 and 10 cm^{-1}, respectively, and those at 1150 and 1123 cm^{-1} by 5 and 7 cm^{-1}, respectively. The ^{15}N shifts for the model azophenols DAT and DAC are similar.

Whereas the solution-phase spectra of DAC and DAT contain both bands of each pair, those near 1460 and 1120 cm^{-1} disappear on crystallization (Figure 3); the disappearance on crystallization of one band from each pair indicates that these model compounds adopt a single conformation in the solid state, as would be expected. Finally, in the crystalline azoenzyme the relative intensities of the bands within each set vary reversibly with temperature, those near 1430 and 1150 cm^{-1} gaining intensity as the temperature is raised (not shown). Collectively, the above observations are consistent with the coexistence of two rotational isomers of azotyrosine in aqueous solution; one has ν^{NN} and $\nu^{\phi N}$ bands near 1460 and 1120 cm^{-1}, respectively, while in the other they are near 1430 and 1150 cm^{-1} (Table I).

Assuming that maximal π conjugation leads to a nearly planar structure and that the azo group is in the trans conformation [see Scheule et al. (1979) for supporting evidence], four nonequivalent rotational isomers are possible for the azophenol species of azotyrosine, the geometries of which are illustrated in Figure 4. The phenolic proton of the two rotamers on the left is hydrogen bonded to a nitrogen atom of the azo group. These two rotamers differ with respect to which one of the nitrogen atoms of the azo group serves as the hydrogen-bond acceptor. Hydrogen bonding to N_1 or N_2 produces six- or five-membered hydrogen-bonded rings, respectively, interconvertible by rotations of 180° about the phenyl–N_2 bond. For the rotamers shown on the left-hand side of Figure 4, rotation about the phenyl–OH bond produces the rotational isomers shown on the right. Such rotation disrupts the *intra*molecular hydrogen bond and, presumably, would occur only if the phenolic proton could form a stable *inter*molecular hydrogen bond with another hydrogen-bond acceptor.

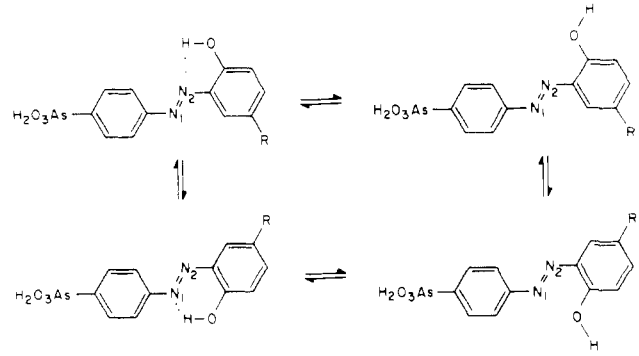

FIGURE 4: Possible rotational isomers for *o*-hydroxyazobenzenes.

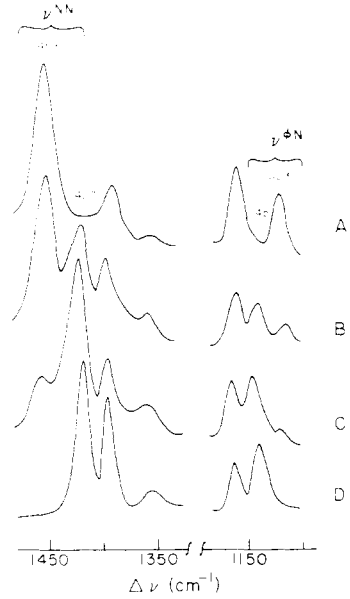

FIGURE 5: The ν^{NN} and $\nu^{\phi N}$ regions of the resonance Raman spectra of *O*-methylmonoarsanilazo-*p*-cresol (DAC–OCH_3) in water (A) and of monoarsanilazo-*p*-cresol (DAC) in dimethyl sulfoxide (B), in water or methanol (C), and in chloroform or a crystalline solid (D). The sample concentrations are 0.05 mM in all solvents. The ν^{NN} and $\nu^{\phi N}$ modes are actually coupled modes (see Table I), but these designations are retained for the sake of simplicity.

The rR spectra in Figure 5 show the solvent dependence of the relative intensities of the two ν^{NN} and two $\nu^{\phi N}$ bands of the model compound DAC. Correlation between the relative intensities of these rR bands and the hydrogen bond accepting ability of the solvent establishes the rotamers of Figure 4 which are responsible for each of the ν^{NN} and $\nu^{\phi N}$ bands. Thus, in chloroform, which cannot accept hydrogen bonds, there are single ν^{NN} and $\nu^{\phi N}$ bands near 1427 and 1145 cm^{-1} (Figure 5D). In water or methanol, good hydrogen-bond acceptors, both ν^{NN} and both $\nu^{\phi N}$ bands are apparent, those near 1427 and 1145 cm^{-1} being the most intense (Figure 5C). Finally, in dimethyl-d_6 sulfoxide (Me_2SO-d_6), an excellent hydrogen-bond acceptor, the ν^{NN} band near 1459 cm^{-1} is the most intense in the spectrum, while the $\nu^{\phi N}$ band at 1120 cm^{-1} further increases in intensity (Figure 5B). Thus, solvents which can compete effectively with the azo group as the acceptor for the hydrogen bond to the phenolic proton elicit the ν^{NN} band near 1459 cm^{-1} and the $\nu^{\phi N}$ band near 1120 cm^{-1}, indicating that these arise from a rotamer whose phenolic proton is hydrogen bonded *inter*molecularly to solvent.

The rR spectrum of DAC–OCH_3 (Figure 5A) supports the above conclusion. Its azo group *cannot* form an intramolecular hydrogen bond, since there is no phenolic proton. Thus, DAC–OCH_3 exhibits *only* the ν^{NN} and $\nu^{\phi N}$ bands near 1459

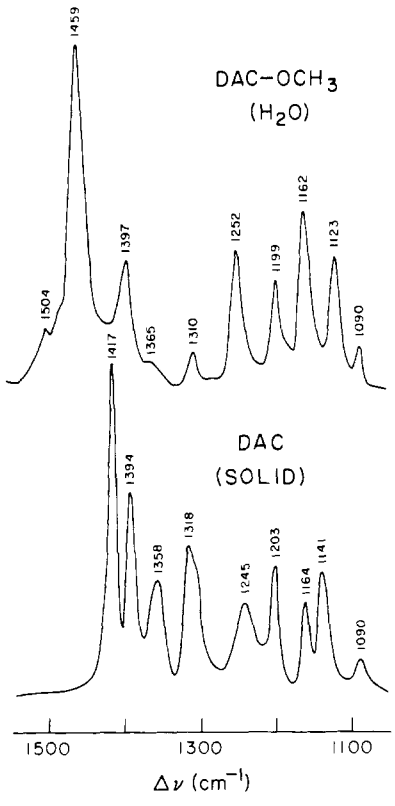

FIGURE 6: Resonance Raman spectra of *O*-methylmonoarsanilazo-*p*-cresol (DAC–OCH_3) in water (sample concentration 0.05 mM) and of monoarsanilazo-*p*-cresol (DAC) crystals. The DAC–OCH_3 spectrum is independent of pH. The frequency shifts induced by crystallization are small. See Table I for band assignments.

and 1120 cm^{-1}, respectively. Hence, we conclude that it is the presence or absence of a hydrogen bond between the azo group and the phenolic proton (rather than rotation about the phenyl–N_2 bond) that leads to the pairs of ν^{NN} and $\nu^{\phi N}$ bands observed. The present data indicate that either rotational isomerism about the phenyl–N_2 bond does not occur or, if it does, it does not perturb the ν^{NN} and $\nu^{\phi N}$ frequencies. Thus, the rR spectra distinguish between the rotamers on the left and right sides of Figure 4 (and both are present) but not between those at the top and bottom.

Figure 6 shows the full rR spectra of DAC–OCH_3 and crystalline DAC, which are models for nonbonded and bonded azo groups, respectively, and hence for the totally *inter*molecularly and *intra*molecularly hydrogen-bonded species of azophenol, respectively. Since these two species exhibit distinct ν^{NN} and $\nu^{\phi N}$ bands, rR spectroscopy provides a means to determine the state of hydrogen bonding of the phenolic proton of azophenols, such as azotyrosine. The appearance of both pairs of ν^{NN} and $\nu^{\phi N}$ bands in the rR spectra of DAC in water shows that an equilibrium exists between these *intra*- and *inter*molecularly hydrogen-bonded species. Moreover, the position of this equilibrium is reflected by the intensities of the 1459- and 1123-cm^{-1} bands (Figure 6, top) relative to those near 1417 and 1141 cm^{-1} (Figure 6, bottom). Since the percentage of the intermolecularly hydrogen-bonded species is determined by the capacity of the microenvironment of the azophenol to compete with the azo group for the hydrogen bond to the phenolic proton, the observed rotamer distribution for azotryosine-248 is a sensitive index of its environment in the azoenzyme, as will be described.

Spectra of Arsanilazocarboxypeptidase in Solution and Crystals. Both the absorption and rR spectra of azocarboxypeptidase have been investigated in solution and crystal phases. Since the enzyme is soluble in 0.5 M NaCl and crystallizes when the salt is removed by dialysis, the crystals were first cross-linked with DMS to prevent solubilization. This allowed the subsequent study of the spectral properties in both phases under the same conditions (i.e., in the presence and absence of 0.5 M NaCl). The absorption and rR spectra of the uncross-linked azoenzyme in the absence of salt and of the cross-linked azoenzyme in the presence and absence of 0.5 M NaCl are indistinguishable.

Figure 7 shows the absorption and rR spectra of the solution- and crystal-phase enzymes at pH 7.5. In solution, the azoenzyme is red with an absorption band centered at 510 nm, the result of the intramolecular complex between the azophenolate form of azoTyr-248 and the active-site zinc atom. Crystallization of the enzyme at this pH, however, disrupts the complex and converts azoTyr-248 to the yellow, protonated azophenol form (Johansen & Vallee, 1971, 1973, 1975). This change in the absorption spectrum of the azoenzyme indicates that crystallization induces a conformational change in the enzyme that blocks complex formation between azoTyr-248 and zinc in the crystalline state.

The rR spectra of the azoenzyme in the solution and the crystal phases affirm the above conclusion. The crystal-phase spectrum contains all of the bands characteristic of a protonated azophenol, while those of the azoenzyme in solution are characteristic of a zinc complex (Scheule et al., 1979). The formation of the intramolecular coordination complex between azoTyr-248 and the active-site zinc atom is accompanied by marked changes in the rR spectrum of the azoenzyme. Thus, there is a significant increase in the intensities of the bands near 1538, 1389, 1338, 1216, and 1165 cm^{-1}. All of these bands are associated with motions of atoms in the C—N=N—C and C—O groupings of the azophenolate ligand (Table I). The bands at 1538 and 1338 cm^{-1} arise from vibrational modes with a high degree of C–O stretching and bending character, respectively. The 1389- and 1216-cm^{-1} bands are associated with N=N and phenyl–N stretching motions, respectively, and the $\nu_{13}{}^{\phi X}$ mode which lies near 1165 cm^{-1} is a ring substituent stretching mode that also involves some phenyl–N stretching character. These very same intensity enhancements have been observed for tridentate model azophenols which are known to complex with zinc (Scheule et al., 1979).

While the rR spectrum of the crystalline azoenzyme clearly is due to an azophenol species, it differs from that of solution-phase model azophenols. The differences lie in the relative intensities of the pair of ν^{NN} bands near 1451 and 1425 cm^{-1} and the pair of $\nu^{\phi N}$ bands near 1148 and 1123 cm^{-1}. Whereas the 1425- and 1148-cm^{-1} bands are dominant in the spectra of the aqueous-phase model azophenols (Figure 5), those at 1451 and 1123 cm^{-1} are the most intense for crystalline azocarboxypeptidase. Hence, the change in the physical state of the enzyme brings about a change in the distribution of these rotamers of the azoTyr-248 residue.

This change is more apparent in Figure 8, which compares the absorption and rR spectra of the azoenzyme in the solution and crystal phases at pH 6.2. While the absorption spectra of the azoenzyme in the two phases are nearly identical, the rR spectra exhibit the same differences in the intensities of the bands near 1451 and 1425 cm^{-1} and near 1148 and 1123 cm^{-1} noted above. Specifically, crystallization of the enzyme favors the intermolecularly hydrogen-bonded conformation of azoTyr-248 with ν^{NN} and $\nu^{\phi N}$ bands at 1451 and 1123 cm^{-1}, respectively, at the expense of the intramolecularly hydro-

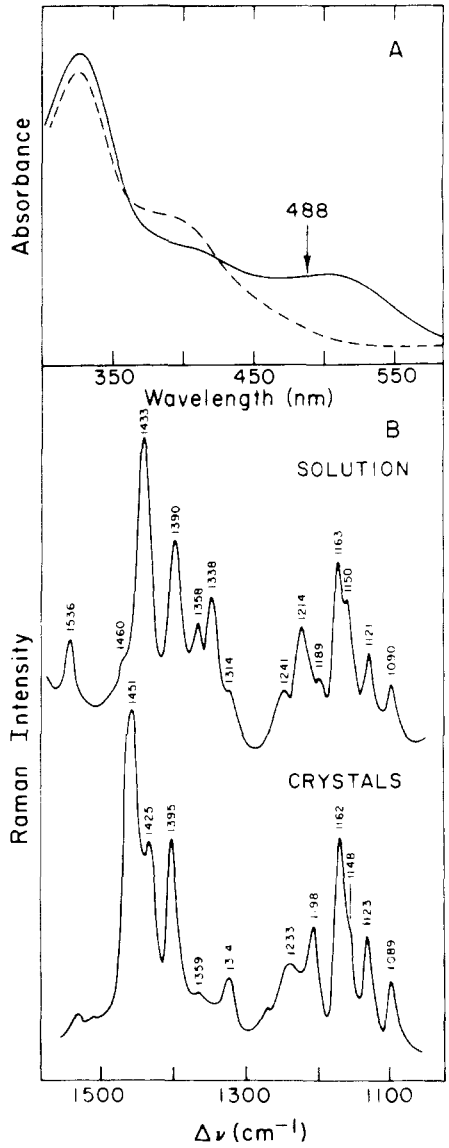

FIGURE 7: (A) Absorption spectra of azoTyr-248 carboxypeptidase dissolved in 2 mM Tris-HCl and 0.5 M NaCl, pH 7.5 (—), and of cross-linked azoTyr-248 carboxypeptidase crystals suspended in 2 mM Tris-HCl and 0.5 M NaCl, pH 7.5 (---). (B) Resonance Raman spectra of the solution- and crystal-phase samples whose absorption spectra are shown in (A). See Table I for band assignments.

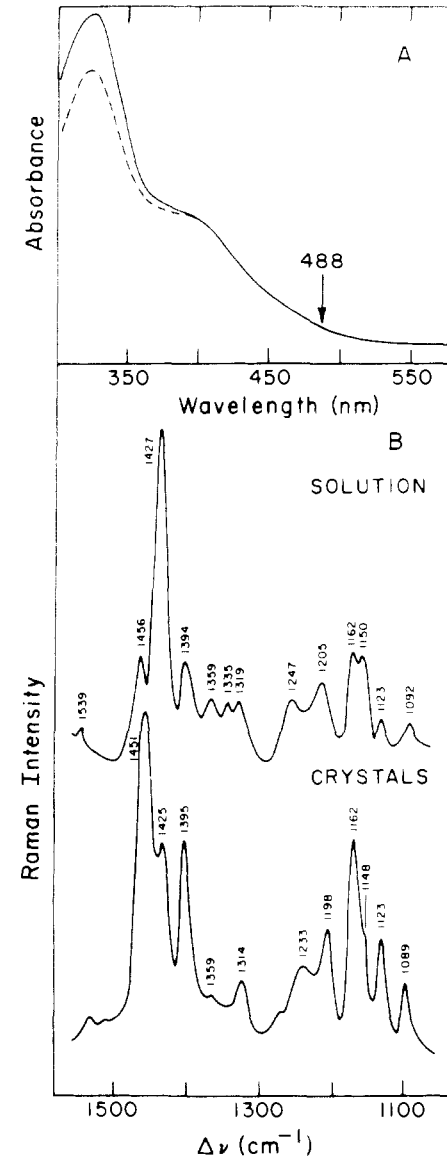

FIGURE 8: (A) Absorption spectra of azoTyr-248 carboxypeptidase A dissolved in 2 mM Tris-HCl and 0.5 M NaCl, pH 6.2 (—), and of cross-linked azoTyr-248 carboxypeptidase crystals suspended in 2 mM Tris-HCl and 0.5 M NaCl, pH 6.2 (---). (B) Resonance Raman spectra of the solution- and crystal-phase samples whose absorption spectra are shown in (A). See Table I for band assignments.

gen-bonded conformation with bands at 1425 and 1148 cm^{-1}.

Discussion

The spectra of a chromophoric, active-site probe, such as arsanilazotyrosine-248, can delineate conformational and structural properties of the active center. Indeed, its characteristic absorption and circular dichroic spectra have revealed important conformational features of carboxypeptidase in crystals and solutions (Johansen & Vallee, 1971, 1973, 1975; Johansen et al., 1976; Alter & Vallee, 1978). The present rR studies of this enzyme provide more detail about the microenvironment of azoTyr-248.

The pH dependence of the rR spectra of azocarboxypeptidase in solution reveals the existence of the three species of azoTyr-248 detected in earlier studies, and rR titrations confirm that the apparent pK values for their interconversion are identical with those observed in visible absorption titrations. To characterize these species further, the rR spectra of a series of model *o*-hydroxyazobenzenes and their isotopically substituted derivatives have been analyzed to identify the normal modes responsible for the vibrational bands observed for all three species of the azoenzyme (Scheule et al., 1979). The results of these studies, summarized in Table I, allow each band to be identified with the motions of specific atoms of the azoprobe. In addition, the effects on the spectra of factors such as proton ionization, tautomeric isomerization, azo cis-trans isomerization, out-of-plane twisting of the azo group, rotameric equilibria, and aggregation have been analyzed, and vibrational spectra of the three species have been described completely.

In general, all three species of azotyrosine and structurally related model azophenols exist in the planar, trans azo conformation. The rR spectra are inconsistent with the existence of any sizable amount of the hydrazone tautomer, and there was no evidence for aggregation under these conditions. On titration from low to high pH, the changes in the rR spectra of the azoenzyme are completely consistent with the conversion of azoTyr-248, first, from the azophenol form to a species which is complexed to the active-site zinc atom and, ultimately, to the free azophenolate ion.

The bands of the azophenolate and of the azophenolato·metal complex species coincide closely. However, the formation of the azoTyr-248·zinc complex selectively perturbs bands associated with motions of the azo group and phenolic oxygen. This observation is consistent with the view that azoTyr-248 complexes to zinc through the N=N and C—O groupings. Furthermore, the frequency observed for the N=N stretching mode is appropriate for that of the trans conformation; the cis azo stretch occurs at higher frequency (Kubler et al., 1960). Finally, the fact that complexation to zinc does not significantly lower the azo stretch establishes that the interaction is through a lone-pair σ orbital rather than a π orbital, since the latter interaction would markedly reduce the azo stretch. Hence, azoTyr-248 is complexed to the active-site zinc atom through lone-pair orbitals on the phenolic oxygen and azo nitrogen, with the azo group in the planar trans conformation [see Figure 5 of Scheule et al. (1977)].

The above discussion focuses on ionization of the phenolic proton and its participation in the formation of a zinc complex as the processes responsible for the spectral changes observed. However, the possible effects of arsonate ionization must be considered also since, in principle, the second ionization of this group ($pK_2 \simeq 8.4$) could affect the visible absorption and rR spectra in the region of interest. Studies with DAC–OCH_3, which permit observation of the effect of arsonate ionization in the absence of that of the phenolic group, demonstrate that arsonate ionization perturbs neither the absorption nor the rR spectrum of DAC–OCH_3 (not shown). These observations confirm previous conclusions (Johansen & Vallee, 1973, 1975) that the ionization of this proton does not account for the spectral changes of the azoenzyme. In addition, this view is consistent with the deduction (Scheule et al., 1979) that the electronic transitions responsible for the observed rR bands are centered on the azo group and phenolic ring and do not involve the arsanilic acid group to any significant extent. This conclusion was based on the observation that the enhanced Raman modes are almost exclusively those arising from vibrations of either the azo group or the phenolic ring.

The vibrational analysis for azotyrosine and other *o*-hydroxyazobenzenes (Scheule et al., 1979) has established that their azophenol species undergo conformational isomerism. These substances can exist in two distinct conformations which 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 $\nu^{\phi N}$ bands, with intensities proportional to their concentrations. The precise position of the equilibrium between these two forms depends on the hydrogen bond accepting ability of the local environment. Hence, the relative intensities of both the pair of ν^{NN} and the pair of $\nu^{\phi N}$ bands in the rR spectrum of arsanilazocarboxypeptidase can be used to specify the state of hydrogen bonding of the phenolic proton of azoTyr-248. Furthermore, the perturbation of this rotameric equilibrium on crystallization of the azoenzyme signals the occurrence of a conformational change which alters its state of hydrogen bonding.

The integration of structural and functional data remains a central problem in mechanistic enzymology. The former are generally obtained by static X-ray analysis of crystals and the latter by kinetic analysis of aqueous solutions. Thus, such structural and functional information is acquired in two different phases and under fundamentally different circumstances. Most often the integration of such data is carried out by assuming that the structural and functional properties of the enzyme in the two phases are the same. While in many cases these assumptions may be valid, they need not always pertain; in instances where they do not, they will likely lead to erroneous mechanistic conclusions. To assess the validity of such assumptions, a comparison of the structural and functional properties of the enzyme and, hence, of the rR spectra in *both* phases is essential.

In solution, the azoenzyme is red with an absorption band centered at 510 nm, the result of the intramolecular complex between the azophenolate form of azoTyr-248 and the active-site zinc atom. However, crystallization of the enzyme at this pH disrupts the complex and converts azoTyr-248 to the yellow, protonated azophenol form. This change in the absorption spectrum of the azoenzyme indicates that crystallization induces a conformational change that prevents complex formation between azoTyr-248 and zinc in the crystalline state (Johansen & Vallee, 1971, 1973).

The rR spectra of the solution- and crystal-phase azoenzymes at pH 7.5 affirm the above conclusion. Extensive vibrational studies of model azophenols and their zinc complexes have shown that at this pH the spectrum of the azoenzyme in solution is that of a zinc complex (Scheule et al., 1977). The crystal-phase spectrum, however, most nearly resembles that of a protonated azophenol. Analysis of the ν^{NN} and $\nu^{\phi N}$ regions of the azophenol spectrum of the crystalline enzyme further reveals a rotamer distribution which clearly differs from that of solution-phase model azophenols manifesting as an inversion in the relative intensities of the ν^{NN} bands near 1456 and 1427 cm^{-1} and the $\nu^{\phi N}$ bands near 1150 and 1123 cm^{-1}. This inversion, brought about by the modification in physical state, reflects a redistribution of the *intra*- and *inter*molecularly hydrogen-bonded species of azoTyr-248 and a change in its environment in the protein.

In aqueous solution, the distribution of rotamers in the azoenzyme (Figure 8) and in the azophenol DAC (Figure 5) is identical, characteristic of a "water-like" environment in both. If the probe were in a hydrophobic environment (Figure 5), i.e., buried in the interior of the enzyme, a spectrum resembling that of DAC in the solid state or in non-hydrogen-bonding solvents (e.g., chloroform) would be expected. However, the rR spectrum of crystalline azocarboxypeptidase (Figures 7 and 8) indicates that the crystal-phase conformation of the azoprobe is neither aqueous-like nor hydrophobic. Instead, azoTyr-248 appears to be in a highly polar environment—one in which the hydrogen bonding of the phenolic hydroxyl group is predominantly *inter*molecular, presumably to a group of the enzyme that can serve as a hydrogen-bond acceptor. The spectrum of the crystalline azoenzyme most closely resembles that of DAC in Me_2SO-d_6 (Figure 5). Figure 9 schematically illustrates the crystal and solution conformations of azoTyr-248.

While the present data cannot as yet identify the hydrogen-bond acceptor of the protein or distinguish between the interaction of azoTyr-248 with an acceptor group of the same or of a different enzyme molecule in the crystal, such differentiations are feasible experimentally and, indeed, are in progress. Moreover, preliminary studies show that the azoenzyme undergoes conformational changes subsequent to the binding of certain inhibitors reminiscent of those brought about by crystallization. Finally, since Tyr-248 has been postulated to be one of the residues essential for enzymatic activity (Lipscomb et al., 1968), the interaction of its phenolic proton with a hydrogen-bond acceptor in the crystal phase could be related directly to the marked reduction in enzymatic activity of carboxypeptidase crystals relative to that of the solution-phase enzyme (Spilburg et al., 1974, 1977). Indeed, the

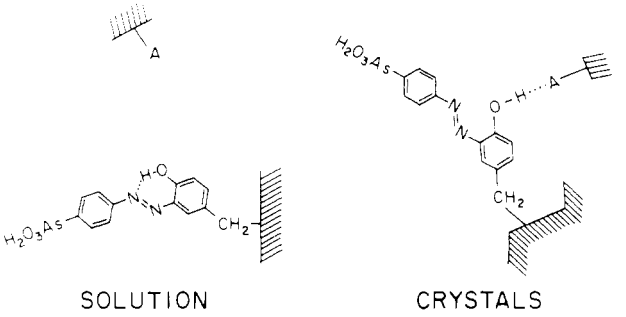

FIGURE 9: Schematic representation of the predominant conformation of the azoTyr-248 residue of azocarboxypeptidase in the solution and crystal phases. The hatched bar represents the protein and the letter "A" an undefined hydrogen-bond acceptor. In the conformation on the left ν^{NN} = 1427 cm^{-1} and $\nu^{\phi N}$ = 1150 cm^{-1}, and in that on the right ν^{NN} = 1456 cm^{-1} and $\nu^{\phi N}$ = 1123 cm^{-1}.

postulated hydrogen bond involves the same proton that is thought to be transferred to the substrate during catalysis.

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Kinetics of Ternary Complex Formation between Dihydrofolate Reductase, Coenzyme, and Inhibitors†

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ABSTRACT: The kinetics of ligand binding to dihydrofolate reductase from *Lactobacillus casei* (MTX/R) to form the ternary enzyme–inhibitor–coenzyme complex have been investigated by the stopped-flow fluorescence technique. The fluorescence changes observed when coenzymes or inhibitors bind to the binary complex of the enzyme with the complementary ligand occur in a single fast phase. Under pseudo-first-order conditions the reaction traces could be fitted with precision to a single-exponential decay, and apparent bimolecular rate constants in the range 2×10^6 to 3×10^7 M^{-1} s^{-1} have been measured assuming a bimolecular–unimolecular model. The kinetic constants obtained suggest that prior binding of an inhibitor to the enzyme may, to a minor extent, interfere with coenzyme binding but the rates of inhibitor binding seem to be unaffected by the presence of a bound coenzyme. Dissociation rate constants appear to be less than 1 s^{-1} which suggests that both coenzymes and inhibitors are tightly bound in the ternary complex. An investigation of the effects of pH on the kinetics of ternary complex formation indicated the involvement of ionizable groups in ligand binding, but this shows some ligand dependence. The rates of ligand binding to form the ternary complex are fairly high, but it is unlikely that these associations are diffusion controlled because their measured activation energies of 7.8–14.5 kcal mol^{-1} are higher than expected from reactions whose rates are limited by diffusion in aqueous solution.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3) catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (H_2folate)[1] to 5,6,7,8-tetrahydrofolate (H_4folate). The enzyme is therefore necessary for maintaining intracellular pools of THF and its derivatives which are essential cofactors in the many important biosynthetic reactions which require the transfer of one-carbon units. In particular, coupled with thymidylate synthetase, the enzyme

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[1] Abbreviations used: H_2folate, 7,8-dihydrofolate; H_4folate, 5,6,7,8-tetrahydrofolate; MTX, methotrexate; TMP, trimethoprim; PYR, pyrimethamine.

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