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Nitrogen-15 Nuclear Magnetic Resonance of Arsanilazotyrosine-248 Carboxypeptidase A and Its Complex with β-Phenylpropionate. Structure and Dynamics in Solution†

William W. Bachovchin,‡ Keiko Kanamori, Bert L. Vallee,* and John D. Roberts*

ABSTRACT: Nitrogen-15 nuclear magnetic resonance has been used to study the structure of arsanilazocarboxypeptidase A and its complex with the inhibitor β-phenylpropionate. Derivatives selectively enriched with ^{15}N were prepared to facilitate observation of the ^{15}N resonances. The results are consistent with the conclusions reached previously from absorption spectroscopic studies and, in addition, provide new information regarding the properties of the azoenzyme and its inhibitor complex. Direct evidence has been obtained for formation of an intramolecular complex between the catalytically essential zinc ion and azoTyr-248, and it has been possible to estimate the degree of complexation. Moreover, the zinc complex involves the distal (N_β) nitrogen of the azo linkage, whereas a model compound, tetrazolyl-*N*-acetyltyrosine, complexes to zinc through the proximal (N_α) nitrogen. The ^{15}N NMR spectra give specific information regarding the intramolecular hydrogen bonding in the azoenzyme. The free azophenol form of the azoenzyme, like that of the model compound arsanilazo-*N*-acetyltyrosine, exists predominantly with the tyrosine phenolic proton intramolecularly hydrogen bonded to N_β of the azo linkage to form a six-membered ring structure. A similar hydrogen bond is also present in the apoazoenzyme and in the azoenzyme–(Gly + L-Tyr) complex, but not in the complex between the azoenzyme and β-phenylpropionate. In the latter complex, there appears to be a new and strong hydrogen bond between the phenolic proton of Tyr-248 and the carboxylate group of enzyme-bound β-phenylpropionate. Thus, azoenzyme-bound β-phenylpropionate, but not azoenzyme-bound Gly + L-Tyr, is apparently able to compete effectively with, and displace, the azo nitrogen as the hydrogen-bond acceptor of the phenolic proton of Tyr-248.

X-ray diffraction studies of carboxypeptidase A have identified only two amino acid residues with side chains close enough to the enzyme-bound pseudosubstrate Gly-L-Tyr to function in catalysis (Lipscomb et al., 1968, 1970). Both of these residues, Glu-270 and Tyr-248, have, in fact, also been demonstrated to be unusually reactive and essential for catalysis through specific chemical modification experiments (Riordan & Hayashida, 1970; Petra & Neurath, 1971; Hass & Neurath, 1971; Nau & Riordan, 1975; Simpson et al., 1963; Riordan & Vallee, 1963; Muszynska & Riordan, 1976). However, in the absence of substrate, X-ray diffraction has localized the Tyr-248 residue at the surface of the molecule, some 12 Å removed from the proposed catalytic site and, moreover, approximately 17 Å from the essential zinc atom (Reeke et al., 1967; Lipscomb et al., 1968). The remarkable movement of Tyr-248 to close to the active site on binding of the pseudosubstrate Gly-L-Tyr has been cited as an example of the induced-fit theory of enzyme–substrate interaction (Koshland, 1958). Whatever the merit of that interpretation, the data clearly underscore the inherent potential for conformational flexibility of globular proteins. Moreover, because understanding of enzyme catalysis is linked to knowledge of structure and because structural change could well be integral to the catalytic act, it further underscores the need for methods that can detect and monitor such conformational variations in solution.

Coupling of carboxypeptidase A with diazotized *p*-arsanilic acid specifically labels Tyr-248 (Figure 1) without significantly affecting its catalytic properties (Johansen & Vallee, 1971, 1973; Johansen et al., 1972). The resulting azoenzyme is intensely chromophoric and has proved to be very useful as a conformational probe.

In aqueous solution, the chromophoric zinc–azoenzyme appears to exist in at least three interconvertible, pH-dependent species involving changes in the azoTyr-248[1] residue. Each of these species exhibits characteristic absorption (Johansen & Vallee, 1973, 1975), circular dichroic (Johansen & Vallee, 1973, 1975), and resonance Raman spectra (Scheule et al., 1977). Furthermore, their separate existence can be inferred

† From the Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, California 91125 (K.K. and J.D.R.), and the Center for Biochemical and Biophysical Sciences, Harvard Medical School, Brigham and Women's Hospital, Boston, Massachusetts 02115 (W.W.B. and B.L.V.). *Received November 17, 1981.* Contribution No. 6543. This work was supported by U.S. Public Health Service Research Grant GM-11072 from the Division of General Medical Sciences (K.K. and J.D.R.), by National Institutes of Health Research Grant GM-15003 (B.L.V.), and by an American Cancer Society Postdoctoral Fellowship (W.W.B.).

‡ Present address: Department of Biochemistry and Pharmacology, Tufts University School of Medicine, Boston, MA 02111.

[1] For simplification of the presentation, "Zn-azo-CPA" and "azoenzyme" are terms used interchangeably with arsanilazotyrosine-248 zinc carboxypeptidase A, "apoazoenzyme" and "apo-azo-CPA" are used interchangeably with apoarsanilazotyrosine-248 carboxypeptidase, and "azoTyr-248" is used interchangeably with arsanilazotyrosine-248.

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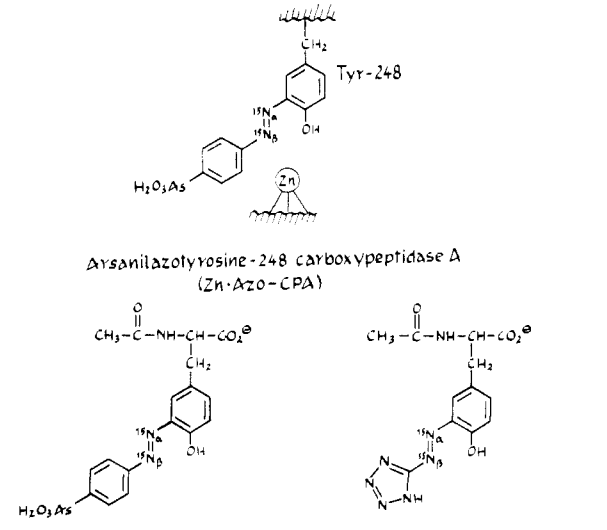


FIGURE 1: Structures of azo-modified carboxypeptidase and of the model compounds DAT and TAT.

from color changes, from yellow to red and, finally, to orange, with increasing pH. The pK_{app} values for these transitions are 7.7 and 9.5, respectively. In contrast, model azotyrosine derivatives in solution undergo only a single transition from yellow to orange with a pK_{app} of 9.5, indicative of ionization of an azophenol to an azophenolate ion (Tabachnick & Sobotka, 1959). The red form of the azoenzyme (λ_{max} 510 nm) is assigned to an intramolecular complex between azoTyr-248 and the catalytically essential zinc ion. The assignment is based on a comparison with TAT,[2] which forms a red zinc complex (λ_{max} 510 nm), and on the fact that the red absorption associated with the azoenzyme is lost when the zinc is removed, when inhibitors or substrates are added, when the pH is reduced, or when the enzyme is denatured (Johansen & Vallee, 1973, 1975; Johansen et al., 1976). This assignment requires a conformation of the enzyme in solution which is different from that allowed by the first X-ray structural analysis. It thus exacerbates the controversy as to whether enzymes have the same conformations in crystals and in solution and, therefore, the degree to which X-ray structures can, in general, be relied upon for assistance in mechanistic interpretations of the catalytic action of enzymes in solution.

The basis of the 510-nm absorption of the azoenzyme has been questioned from several standpoints (Quiocho et al., 1972; White & Legg, 1976), but a plethora of studies employing UV, visible, CD, and, most recently, resonance Raman spectroscopy support assignment of the 510-nm absorption to an intramolecular zinc–azoTyr-248 complex. Furthermore, a reexamination of the X-ray maps of native CPA_α crystals has revealed electron densities which are assignable to Tyr-248 near enough to the zinc ion for complexation, although amounting to only some 15–25% of the total Tyr-248 (Lipscomb, 1973). Thus, while there may be specific points of contention, the current X-ray and spectroscopic data can be reconciled on the basis of multiple conformational states for carboxypeptidase A and an intramolecular azoTyr-248–zinc complex for the modified enzyme.

Because of the importance of the question involved, further investigations of the conformations of the enzyme in solution were very desirable, and for this purpose, a nitrogen-15 NMR study of the azoenzyme appeared to offer the potential for both diagnosis of a zinc–azoTyr-248 complex and for estimating the degree to which is formed. In addition, nitrogen-15 NMR might be able to ascertain which azo nitrogen actually serves as the ligand to zinc, as well as the extent of intramolecular hydrogen bonding between the hydroxyl proton of Tyr-248 and the nitrogen atoms of the azo linkage in each of the various conformations. Furthermore, corresponding studies on TAT and DAT could help to clarify any remaining difficulties associated with these model compounds such as discussed above.

The potential value of nitrogen-15 NMR spectroscopy for application to biochemical problems is becoming increasingly evident (Bachovchin & Roberts, 1978), although the low sensitivity of nitrogen-15 NMR at the natural-abundance level renders many applications difficult. For this reason, we have prepared arsanilazoTyr-248 carboxypeptidase A and the model compounds DAT and TAT specifically isotopically enriched with ^{15}N at one or both nitrogens of the azo linkage. The present work involves application of nitrogen-15 NMR spectroscopy to arsanilazoTyr-248 carboxypeptidase A and its complex with the inhibitor β-phenylpropionate in solution.

Experimental Procedures

Sodium nitrite (^{15}N, 99%) and *p*-arsanilic acid (^{15}N, 95%) were obtained from Isotope Labeling Corp.

Arsanilazo-*N*-acetyltyrosine (DAT) was prepared by treating diazotized arsanilic acid with *N*-acetyltyrosine (Tabachnick & Sobotka, 1959; Scheule et al., 1980). Selective nitrogen-15 isotopic enrichment of N_α or N_β was effected by using sodium [^{15}N]nitrite or *p*-[^{15}N]arsanilic acid, respectively, in the diazotization reaction. The UV–vis spectrum of each preparation of ^{15}N-enriched DAT was carefully examined over the pH range 4.0–11.00. The spectral parameters were found to correspond to those reported previously (Tabachnick & Sobotka, 1959; Scheule et al., 1980).

Tetrazolylazo-*N*-acetyltyrosine (TAT) was prepared by treating diazonium-1*H*-tetrazole with *N*-acetyltyrosine as described by Sokolovsky & Vallee (1966). The $^{15}N_\alpha$ derivative was synthesized by using sodium [^{15}N]nitrite in the preparation of the diazonium-1*H*-tetrazole. UV–vis spectra of the ^{15}N-enriched product were obtained as a function of pH and of added zinc ion; the spectral properties agreed closely with those previously reported.

Carboxypeptidase A (Cox et al., 1964) was obtained as a crystalline suspension from Sigma Chemical Co. and was recrystallized before use. Arsanilazotyrosine-248 carboxypeptidase A was prepared by treatment of carboxypeptidase A crystals with diazotized *p*-arsanilic acid as described by Johansen & Vallee (1971). Nitrogen-15 enrichment at N_α or N_β was achieved with sodium [^{15}N]nitrite or *p*-[^{15}N]-arsanilic acid, respectively, as described above for DAT. UV–vis absorption spectroscopy was used to demonstrate that each preparation of ^{15}N-enriched azoenzyme responded to pH, removal of zinc, inhibitors and substrates, and denaturing agents in the manner expected. All spectral properties were in good agreement with those previously reported.

Peptidase activity was determined spectrophotometrically at 338 nm by using *N*-(2-furanacrylyl)glycyl-L-phenylalanine (2×10^{-4} M in 1.0 M of NaCl and 0.05 M Tris, pH 7.5) as substrate (Breddam et al., 1979). The activity of the modified enzyme measured with this substrate was not discernibly different from that of the unmodified enzyme.

Apoarsanilazotyrosine-248 carboxypeptidase was prepared by suspending crystals of the azoenzyme (5 mg/mL) in 0.01 M of 1,10-phenanthroline and 0.01 M of *N*-(2-morpholino)-ethanesulfonic acid (Mes) buffer, pH 7.0, at 20 °C for 1 h

[2] Abbreviations: TAT, tetrazolylazo-*N*-acetyltyrosine; DAT, arsanilazo-*N*-acetyltyrosine; Tris, tris(hydroxymethyl)aminomethane.

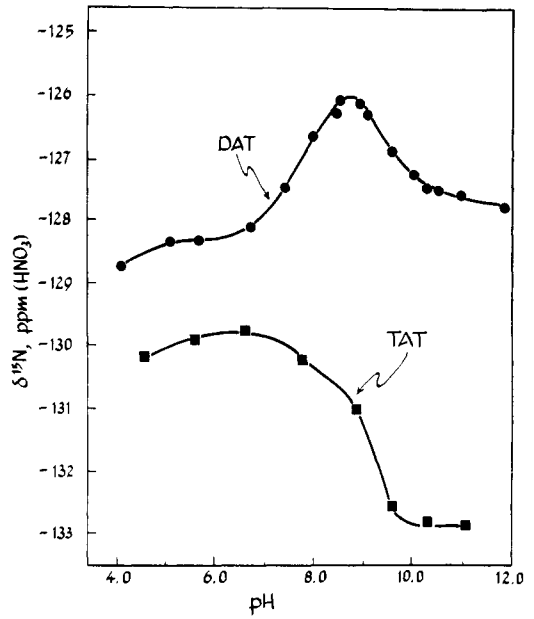


FIGURE 2: ^{15}N chemical shifts of N_α of DAT and TAT as a function of pH: (●) DAT; (■) TAT.

and following this with a 0.5-h washing with 0.01 M Mes buffer, pH 7.0, as previously described (Auld & Holmquist, 1974). The process was repeated 4 times and yielded apoazoenzyme containing less than 0.016 g-atom of zinc/mol of enzyme as determined by atomic absorption spectroscopy.

10-(Phenylazo)-9-phenanthrol was synthesized from 9,10-phenanthraquinone and phenylhydrazine hydrochloride in acetic acid (Bacon & Biggs, 1974). Crystallization from ethanol gave pure 10-(phenylazo)-9-phenanthrol (mp 163–164 °C). The corresponding anion was prepared by addition of 1.5 equiv of sodium hydroxide to a dimethyl sulfoxide solution.

The ^{15}N NMR spectra were obtained with a Bruker WH-180 spectrometer operating at 18.25 MHz. A 1 M solution of $H^{15}NO_3$ in D_2O contained in a 5-mm capillary provided both the external reference signal and field-frequency lock signal. For the model compounds, arsanilazo-*N*-acetyltyrosine (DAT), tetrazolylazo-*N*-acetyltyrosine (TAT), and its zinc complex (TAT·Zn), the spectra were taken of 5 mM solutions, at room temperature. The azoenzyme was dissolved in 1.0 M NaCl, and spectra were obtained on solutions of 1.5–2.0 mM in enzyme. All spectra of the azoenzyme, its inhibitor complexes, and apoenzyme were obtained at 15 °C, except for that of the azoenzyme at pH 7.0 which was obtained at room temperature. On occasion, 0.002 M Tris buffer was used to help stabilize the pH. The sodium chloride was "ultrapure" grade supplied by Alfa Products. Other reagents and solutions were rendered metal-free by dithizone extraction as described previously (Thiers, 1957). The enzyme–inhibitor complexes were made by adding 7.5 mM Gly-L-Tyr or sodium β-phenylpropionate to the azoenzyme solution. The pH of the solution, the activity of the azoenzyme, and the visible absorption spectrum of the NMR sample were determined before and after each NMR measurement. Only data from samples exhibiting no change in the above parameters during acquisition of the ^{15}N NMR spectrum are reported here. Normally, spectra were obtained with a 7000-Hz sweep width, a 90° pulse angle, and an acquisition time of 0.29 s. Proton decoupling was usually not employed.

Enzyme concentrations were determined by absorption at 278 nm, based on a molar absorptivity at 278 nm of 7.32×10^4 M^{-1} cm^{-1} (Johansen & Vallee, 1971). Visible absorption spectra were obtained with a Beckman Acta III and/or with a Varian 219 spectrophotometer.

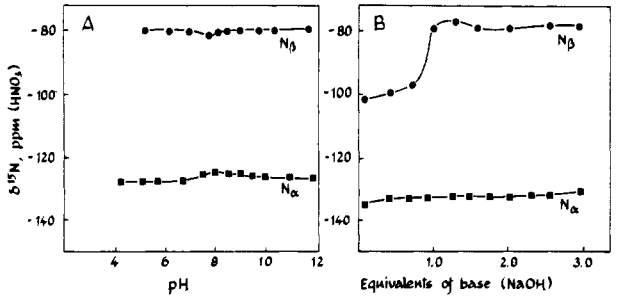
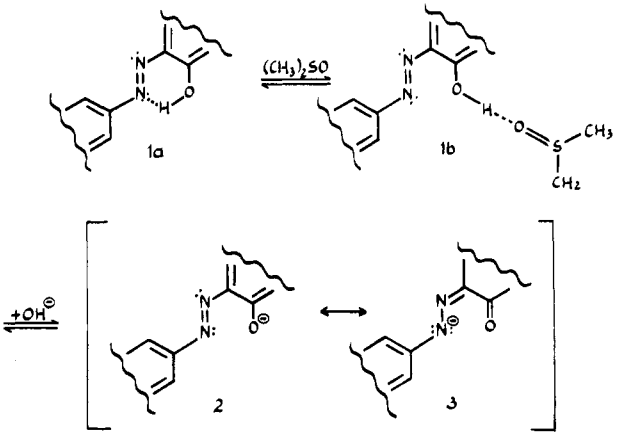


FIGURE 3: ^{15}N chemical shifts of N_α and N_β of DAT as (A) a function of pH in H_2O and (B) a function of added base in 75% $(CH_3)_2SO/H_2O$.

Scheme I



Results

Ionization, Intramolecular Hydrogen Bonding, and Tautomerism in Model Azophenols. Figure 2 shows the changes in ^{15}N chemical shifts of the N_α resonances of DAT and TAT as a function of pH in aqueous solution. The ionization of the phenolic proton corresponds to pK_a's of ~9.5 and ~9.0 for DAT and TAT, respectively (Johansen & Vallee, 1975). In both model azophenols, this ionization is accompanied by a similar, quite small, *downfield* shift in the position of the N_α resonance amounting to about 2 ppm for DAT and 3 ppm for TAT. The pH dependence of the N_α signal of DAT is complicated by a second titrating group with a pK_{app} between 7.0 and 8.0 which can be attributed to the arsonate group. The absence of this complication in the ^{15}N NMR spectra of TAT is consistent with the assignment. The change in the N_α resonance of DAT corresponding to the arsonate ionization is 3 ppm and is opposite in direction to that induced by ionization of the phenolic proton (Figure 2).

Azo nitrogens, in general, exhibit chemical shifts similar to those of azobenzene, which come at ~−133 ppm (Duthaler & Roberts, 1978). On this basis, the ^{15}N chemical shifts for N_α of both DAT and TAT can be considered essentially normal. However, the N_β resonance of DAT occurs at −82 ppm, which is approximately 45 ppm upfield of the resonance of N_α (Figure 3A). This difference is expected if the unshared electron pair on N_β is involved in a hydrogen bond (Schuster & Roberts, 1979a,b; Levy & Lichter, 1979) in the manner known for *o*-hydroxyphenylazo compounds (Burawoy & Thompson, 1953; Hendricks et al., 1936) (Scheme I, structure **1a**).[3] The very small change in shift of N_β on ionization of

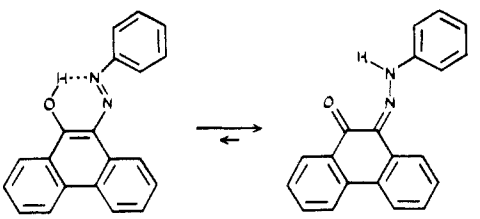
[3] See Figure 1 for structure and numbering scheme.

the phenolic proton is quite surprising and must be the result of nearly equal but opposing effects on the N_β resonance position arising from loss of the hydrogen-bonding interaction to the N_β lone pair and ionization of the phenolic hydroxyl.

So that this hypothesis could be tested, the ^{15}N NMR shifts of DAT were examined in water–dimethyl sulfoxide solutions. Increasing the mole fraction of dimethyl sulfoxide in water caused the N_β resonance to move to lower magnetic fields (see Table I and Figure 3B), as is consistent with competition for the proton of a N···H—O hydrogen bond by a $(CH_3)_2S{=}O{\cdots}HO$ hydrogen bond (Scheme I, structure **1b**). Such changes in hydrogen bonding to =N nitrogens have been shown previously to cause shifts to move toward lower fields (Duthaler & Roberts, 1978; Westerman et al., 1978). The maximal effect is in 100% dimethyl sulfoxide, where the N_β resonance comes at −103.9 ppm, 21.4 ppm downfield from its position in water. Addition of a molar equivalent of base to the dimethyl sulfoxide solution causes ionization of the phenolic hydroxyl group and results in shifting of the N_β resonance back to −78.1 ppm. This sequence clearly shows how scission of the intramolecular hydrogen bond and phenolic ionization exert nearly equal, but opposite, effects on the resonance position of N_β.

In contrast to its effect on N_β, dimethyl sulfoxide has relatively little effect on the resonance position of N_α of either DAT or TAT (Table I; Figures 3 and 4), and this is consistent with substantially less hydrogen bonding to N_α than to N_β in these model compounds. The question remains as to why the ionization of the phenolic hydroxyl in dimethyl sulfoxide solution causes a much greater change in the resonance position of N_β than of N_α and, further, why this effect should result in an upfield shift for N_β.

The principal resonance structures of the ionized form of DAT—structures **2** and **3** in Scheme I—suggest possible answers to these questions. Clearly, contributions corresponding to structure **3** have a greater influence on the shielding of N_β than on N_α because structure **3** corresponds to an increase in the electron density on N_β, thus increasing the shielding of this nitrogen. The ^{15}N NMR shifts of the anion of 10-(phenylazo)-9-phenanthrol (structure **4**) in dimethyl sulfoxide solution



4a 4b

are quite relevant to this question (Table I). The neutral form of this substance is nearly completely the hydrazone (Berrie et al., 1968; structure **4b**). In consequence, the anionic form is expected to have a greater contribution from its resonance structure analogous to structure **3** (Scheme I) than expected for the phenolate form of DAT and should, accordingly, provide a reasonable estimate of the influence of the ^{15}N resonance positions associated with an especially large contribution corresponding to resonance structure **3**. The chemical shift of N_β for the anion of **4** is −33.8 ppm while that of N_α is −110 ppm. The N_β shift clearly supports the idea that contributions of resonance structure **3** should affect the position of the resonance of N_β more than that of N_α and that N_β should be well upfield of azobenzene (−133 ppm).

The ^{15}N chemical shifts of azophenol and ketohydrazone tautomers are generally very different and quite characteristic

Table I: ^{15}N Shifts of Zn-azo-CPA and Complexes Formed with Gly-Tyr and β-Phenylpropionate, Apoazo-CPA, and DAT

pH	Zn-azo-CPA	Zn-azo-CPA + Gly-Tyr	Zn-azo-CPA + β-phenylpropionate	apoazo-CPA	DAT			10-(phenylazo)-9-phenanthrol anion in $(CH_3)_2SO$
					H_2O	$(CH_3)_2SO$	$(CH_3)_2SO/H_2O$ (3:1 v/v)	
				δ ^{15}N for N_β (ppm)[a]				
7.0	−78.0				−82.1			
8.8	−55.6	−88.0	−107.3	−79.1	−82.5	−103.9[b]	−101.4	
9.6	−78.5	−84.4			−82.4			
10.3	−80.8	−80.5	−76.9		−82.6		−78.1[c]	−33.8
				δ ^{15}N for N_α (ppm)[a]				
7.0	−129.8				−127.8			
8.8	−117.9	−125.1	−125.9	−126.2	−126.0		−137.0[b]	
9.6	−122.3	−125.5			−126.8			
10.3	−123.8	−125.9	−125.1		−127.4		−130.1[c]	−110.1

[a] Negative sign indicates the shift is downfield from $H^{15}NO_3$. Because of the substantial line widths, the chemical shifts are considered to be accurate to ±2 ppm or better. [b] Phenol. [c] Phenolate.

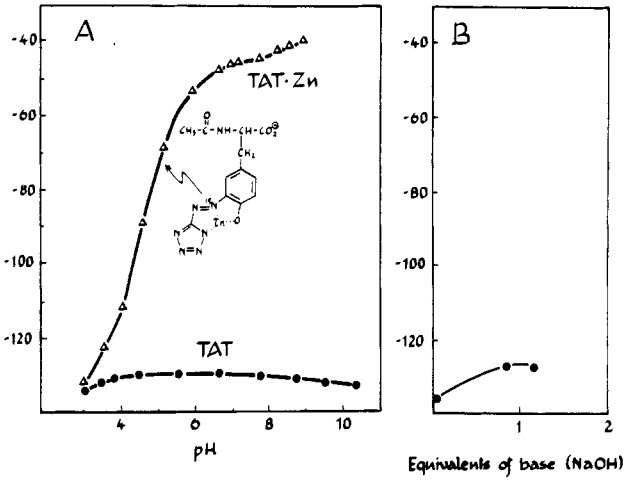


FIGURE 4: ^{15}N chemical shifts of N_α of TAT as (A) a function of pH in H_2O in the presence and absence of Zn^{2+} and (B) a function of added base in 75% $(CH_3)_2SO/H_2O$.

(Witanowski & Webb, 1973). Thus, the N_β resonance of 10-(phenylazo)-9-phenanthrol, which exists predominantly as structure **4b**, comes at +176 ppm compared to about −130 ppm characteristic of azo forms. This fact, combined with resonance Raman, fluorescence, and other data, clearly indicates that DAT and TAT predominantly, if not exclusively, are most stable as the azo tautomers (Gegiou & Fischer, 1971; Scheule et al., 1979, 1980).

In summary, the ^{15}N NMR results are in accord with previous conclusions that DAT and TAT are the azo rather than the hydrazo tautomers, that DAT exists in a six-membered intramolecular hydrogen-bonded ring structure with the phenolic proton hydrogen bonded to N_β, that this hydrogen bond accounts for the upfield position of the N_β resonance, and, finally, that N_α hydrogen-bonded ring structures do not appear to be important for either DAT or TAT.

TAT·Zn Complexation. The N_α shifts of TAT in the presence and absence of zinc as a function of pH are shown in Figure 4A. With zinc, N_α of TAT shows a large upfield complexation shift as the pH is increased. Ionization of the proton from the tetrazole ring ($pK_a \sim 5.0$) is required for formation of the complex.

The upper curve of Figure 4A corresponds to a TAT·Zn ratio of 1:1. The large shielding observed for N_α as the pH is increased levels off at pH 7, as expected if complexation were complete at this pH, and indeed, adding additional zinc causes no further change in the shift of N_α. Above pH 10.0, precipitation of zinc hydroxide occurs. The large upfield shift of ~90 ppm exhibited by N_α as the result of formation of the TAT·Zn complex is in accord with the structure shown in Figure 4A, where TAT acts as a terdentate chelating agent, complexing zinc via N_α, the ionized *o*-hydroxyl group, and one of the nitrogens of the tetrazole ring. This complex has two five-membered chelate rings, which is expected to be more favorable than a terdentate complex involving N_β—an arrangement which would require a six-membered and a four-membered chelate ring (Anderson & Nickless, 1967; Geary et al., 1962).

^{15}N NMR Spectra of Arsanilazocarboxypeptidase A (Zn-azo-CPA). The ^{15}N NMR spectra of arsanilazocarboxypeptidase, isotopically enriched in ^{15}N at both azo nitrogens, at pH 7.0, 8.8, and 9.6 are shown in Figure 5. Only two signals are observed over the pH range investigated, and assignment of these resonances to N_α and N_β are unambiguous as the result of parallel studies of arsanilazocarboxypeptidase A enriched in ^{15}N only at N_α. At pH 7.0, the chemical shifts

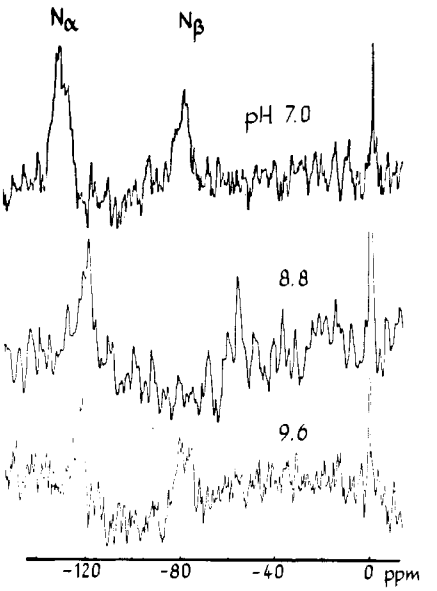


FIGURE 5: ^{15}N spectra of arsanilazoTyr-248 CPA (Zn-azo-CPA) enriched in ^{15}N at both nitrogens at pH 7.0 (43 799 scans), 8.8, (70 010 scans), and 9.6 (52 057 scans).

of N_α at −130 ppm and N_β at −78 ppm (Table I) of Zn-azo-CPA are very close to the corresponding chemical shifts observed for DAT at this pH. The high-field shift of N_β indicates that, at this pH as for DAT, N_β is hydrogen bonded to the Tyr-248 hydroxyl proton.

Formation of a complex between azoTyr-248 and the catalytic zinc ion should result in an upfield shift in the resonance position of the azo nitrogens, and indeed, at pH 8.8, the N_α resonance is 12 ppm and the N_β resonance 26 ppm upfield from their respective positions at pH 7.0. These chemical shifts are not expected for DAT under any circumstances and provide direct evidence of complexation of azoTyr-248 to zinc. The much larger change in shift of the N_β resonance shows that complexation primarily involves the lone pair σ orbital of this nitrogen. The change in shift of the N_β resonance on complexation to zinc (26 ppm) is actually much larger than might first appear because account must be taken of the 21-ppm upfield contribution which arises from the intramolecular hydrogen bond to N_β at pH 7.0 and which is lost on formation of the zinc complex. The overall upfield shift from pH 7.0 to 8.8 is thus on the order of 47 ppm.

On going to pH 9.6 and higher, both azo nitrogen resonances shift back downfield to positions quite similar to those for DAT in this pH range and which are characteristic of the free azophenolate ion.

^{15}N NMR of Azoenzyme Inhibitor Complexes and of Apoazoenzyme. The apoazoenzyme cannot form a zinc complex and does not display the 510-nm optical absorption. Moreover, the enzyme inhibitor β-phenylpropionate and pseudosubstrate Gly-L-Tyr cause the 510-nm absorption to disappear when added to solutions of the red azoenzyme, presumably by binding to the active site and thereby disrupting the intramolecular Zn–azoTyr-248 complex. The Gly-L-Tyr azoenzyme complex was an especially desirable subject for ^{15}N NMR studies because the crystal structure of its complex with the unmodified enzyme (Lipscomb et al., 1970) has provided the basis for much useful information as to the details of the catalytic mechanism. However, although the rate of hydrolyses of Gly-L-Tyr by carboxypeptidase is reported to be sufficiently slow to allow for X-ray diffraction studies of the enzyme–substrate complex, we find that 10 mM solutions of Gly-L-Tyr are completely hydrolyzed to Gly + L-Tyr in a few minutes

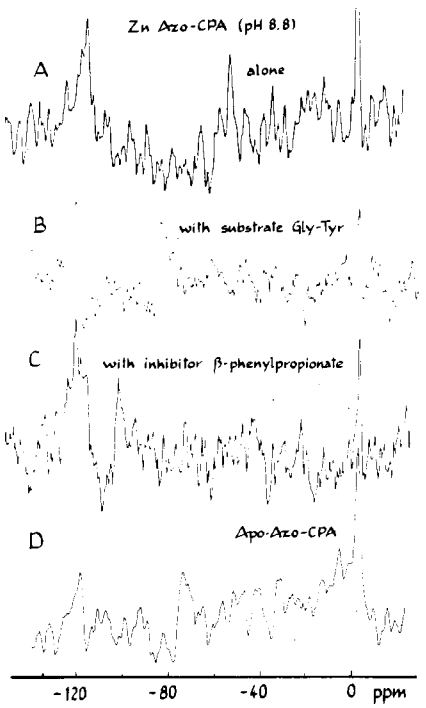


FIGURE 6: ^{15}N spectra at pH 8.8 of (A) arsanilazoTyr-248 CPA (Zn-azo-CPA; 70010 scans), (B) Zn-Azo-CPA with added glycyl-L-tyrosine (245587 scans), (C) Zn-azo-CPA with inhibitor β-phenylpropionate (131054 scans), and (D) apoarsanilazoTyr-248 CPA (apo-azo-CPA; 255327 scans).

by 1 mM enzyme under the conditions required for taking the spectra. Nonetheless, the 510-nm absorption of the azoenzyme does not reappear when hydrolysis is complete, and it seems most likely that L-Tyr is bound to the active site, thus disrupting the zinc–azoTyr-248 complex.

Figure 6 allows comparison of the ^{15}N spectra of solutions formed by addition of β-phenylpropionate and Gly-L-Tyr to Zn-azo-CPA and on removal of zinc from the enzyme. Clearly, removal of zinc or the addition of inhibitor or substrate results in large downfield shifts of the azo nitrogen resonances. There is an important difference between the ^{15}N shifts of the β-phenylpropionate–azoenzyme complex and those of the apoazoenzyme and of the azoenzyme in the presence Gly-L-Tyr complex. In the β-phenylpropionate–azoenzyme complex, the N_β resonance comes at −107.3 ppm, very similar to that of N_β of DAT in dimethyl sulfoxide. In contrast, the N_β resonance is observed at −88 ppm for the (Gly + L-Tyr)–azoenzyme complex and at −79 ppm for the apoazoenzyme. These latter values are reasonably close to the N_β position for the azophenol form of DAT in water (pH 7). From this, we conclude that the phenolic proton is largely hydrogen bonded to N_β in the complex formed from Gly-L-Tyr and the azoenzyme, and also in the apoazoenzyme, whereas in the β-phenylpropionate–azoenzyme complex, this hydrogen bond is absent.

Discussion

The intramolecular complex proposed for the catalytically essential zinc and azoTyr-248 of Zn-azo-CPA which gives the 510-nm optical absorption has been presumed to involve one of the azo nitrogens, and the ^{15}N NMR spectra not only are consistent with formation of such a complex but also further demonstrate that it involves bonding of N_β rather than N_α to the zinc.

Determination of the extent of formation of this complex would be highly desirable but is difficult for lack of a truly appropriate model system. If one assumes that the N_α shifts of the TAT·Zn and the N_β shift of the Zn–azoTyr-248 complexes should be comparable and uses the ~90-ppm upfield shift of N_α of TAT on complexation with zinc to represent the magnitude of the shift expected on coordination of the N_β nitrogen of azoTyr-248 to zinc, then, on this basis, because zinc complexation for Zn-azo-CPA results in about a 50-ppm upfield shift, the extent of complexation would be about 55% at pH 8.8. This value, while very crude, is within the same ballpark as the expected value of 78% of complex formation for pH 8.8 calculated from the pK_{app}'s of 7.7 and 9.5. Unquestionably, the ^{15}N NMR data clearly support the contention that a very important conformation of the azoenzyme in solution at pH 8.8 involves coordination between the catalytically essential zinc ion and β-azo nitrogen of azoTyr-248. The X-ray crystallographic analysis which demonstrates the Tyr-248 of the enzyme to be on the outside of the molecule was made at pH 7.4. Perhaps at pH 8.8, where our results show the azoTyr-248–zinc complex to be formed to the fullest extent, the crystal structure of the native enzyme might so change as to be fully in accord with the spectral data obtained with the azoenzyme.

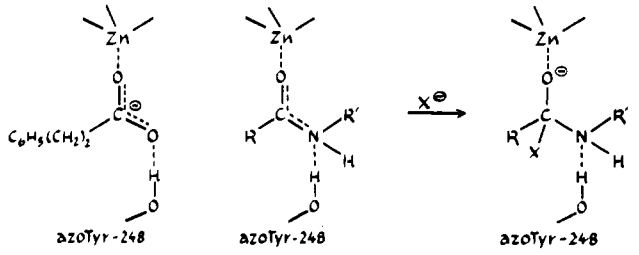
The ^{15}N NMR spectra also help to delineate the importance of intramolecular hydrogen bonding involving the phenolic proton and azo nitrogens for the free azoenzyme and the azoenzyme–inhibitor complexes by determining the presence or absence of the characteristic upfield shifts induced for N_β by hydrogen bonding. Thus, the relatively high-field position of N_β of the azoenzyme at pH 7.0 (Table I) demonstrates that azoTyr-248, at this pH, resembles DAT and exists predominantly as the free azophenol with a hydrogen bond between the phenolic proton and N_β of the azo linkage corresponding to the six-membered intramolecular hydrogen-bonded ring structure. At first glance, the absence of detectable amounts of zinc complex in our samples at pH 7.0 would seem to be at odds with earlier analysis based on the 510-nm optical absorption which indicates 20–30% complex at this pH. The discrepancy is, in fact, only apparent and can be attributed to differences in experimental conditions. The enzyme has a tendency to dimerize as its concentration is increased and/or as the pH is decreased in solutions of high salt content (Bethune, 1965). Thus, some degree of dimerization can be expected for the 1 mM azoenzyme NMR samples at pH 7.0, which should inhibit formation of the zinc complex, and indeed, the NMR samples do not absorb at 510 nm. When the concentrated NMR samples are diluted at pH 7.0 or the pH is increased, then 510-nm absorption is evident.

At pH 8.8, where the phenolic hydroxyl of DAT is not ionized and retains the hydrogen-bonded structure, azoTyr-248 is involved in a zinc complex, and its azo nitrogen chemical shifts are drastically altered. At this pH, addition of Gly-L-Tyr or the inhibitor β-phenylpropionate or removal of zinc results in the disappearance of the 510-nm absorption and appearance of absorptions indicative of azophenol formation. The 510-nm absorption and the extreme high-field position of the ^{15}N resonances of the azoenzyme clearly have the same molecular basis because the azo ^{15}N resonances of the azoenzyme–inhibitor complexes and apoazoenzyme are shifted markedly downfield from the positions characteristic of the zinc complex.

The ^{15}N shifts also reveal an important difference between the hydrogen bonds in the inhibitor complexes. In the (Gly + L-Tyr)–azoenzyme complex, N_β has a chemical shift of −88 ppm, only slightly downfield of the N_β shifts of the azophenol of DAT, the azoenzyme at pH 7.0, or the apoazoenzyme at pH 8.8 (Table I). This indicates that the hydrogen bond between the phenolic proton and N_β of azoTyr-248 is sub-

stantially intact in this azoenzyme–inhibitor complex. The chemical shift of −107 ppm of N_β in the β-phenylpropionate–azoenzyme complex is much farther downfield and is close to the shift of N_β of DAT in 100% dimethyl sulfoxide. Clearly, there is no phenolic hydrogen bond to N_β in this complex.

Carboxypeptidase A is known to have multiple, independent and overlapping binding sites for substrates and inhibitors (Vallee et al., 1968; Alter & Vallee, 1978). Two molecules of β-phenylpropionate bind at or near the active site with similar binding constants; thus, both sites must be fully occupied. Consequently, which molecule of β-phenylpropionate is responsible for causing disruption of the intramolecular hydrogen bond is not easy to determine. However, there is substantial evidence that at least one of these molecules interacts directly with the active site zinc (Coleman & Vallee, 1962, 1964; Navon et al., 1968; Steitz et al., 1967; Rees et al., 1981). A very plausible arrangement for this is shown in structure **5**, wherein the phenolic proton of Tyr-248 hydrogen



5 6a 6b

bonds to one carboxylate oxygen of enzyme-bound β-phenylpropionate, while the second carboxylate oxygen binds to the active site of zinc. This structure places the azoTyr-248 in just the kind of position it should have relative to the catalytic zinc ion (compare structures **5** and **6**) if the role of the phenolic hydrogen is, in fact, to facilitate nucleophilic attack (structure **6b**) on the carbonyl group of the scissile peptide bond by hydrogen bonding to the amide nitrogen. It is important that this interaction is stronger than the very favorable hydrogen bond of the phenolic proton to N_β. The effectiveness of this hydrogen bond to β-phenylpropionate is likely to be related to the effectiveness of β-phenylpropionate as an inhibitor. Thus, it seems likely that the conformational change associated with binding of substrates which places Tyr-248 into the position necessary for its participation in catalysis here promotes hydrogen-bond formation to enzyme-bound β-phenylpropionate.

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Spinach Siroheme Enzymes: Isolation and Characterization of Ferredoxin-Sulfite Reductase and Comparison of Properties with Ferredoxin-Nitrite Reductase†

Rick J. Krueger‡ and Lewis M. Siegel*

ABSTRACT: Sulfite reductase (SiR) has been purified to homogeneity from spinach leaves. Two forms of the enzyme were separated by hydroxylapatite chromatography. One, with subunit M_r 69 000, appears to be proteolytically cleaved to give rise to the other, with subunit M_r 63 000, during the purification procedure. The two species have identical catalytic activities (on a per heme basis) when reduced methylviologen (MV^+) or ferredoxin (Fd_r) is used as electron donor for sulfite reduction, and they exhibit nearly identical optical and EPR spectra. Both enzyme forms exist in 50 mM phosphate buffer (pH 7.7) primarily as dimers at 20 °C. Spinach SiR contains 1 mol of siroheme and one Fe_4S_4 center per subunit. The heme iron is in the high spin Fe^{3+} state in the enzyme as isolated. Near quantitative reduction of the Fe_4S_4 center by dithionite could be achieved if SiR was either converted to the CO complex or treated with 80% dimethyl sulfoxide. Spinach SiR and nitrite reductase (NiR) both catalyze Fd_r- or MV^+-dependent six-electron reductions of SO_3^{2-} and NO_2^-, as well as the two-electron reduction of NH_2OH. V_{max} values are highest with the nitrogenous substrates. However, the K_m of SiR for SO_3^{2-}, and of NiR for NO_2^-, is at least 2 orders of magnitude less than with either of the other substrates. Rates of reduction with Fd_r as electron donor are greater than with MV^+ as donor. No immunological cross-reaction could be detected between spinach SiR and *Escherichia coli* SiR or between spinach SiR and NiR.

A key step in the assimilation of sulfate by plants and microorganisms is the six-electron reduction of SO_3^{2-} to S^{2-}, catalyzed by sulfite reductase (SiR)[1] (Siegel, 1975). This enzyme has been highly purified from *Escherichia coli* (Siegel et al., 1973) and several other microorganisms (Yoshimoto & Sato, 1968; Siegel et al., 1971; Lee et al., 1973a,b). The *E. coli* SiR has been separated into two distinct subunits, each consisting of a single type of polypeptide (Siegel & Davis, 1974). The "flavoprotein" subunit contains FMN and FAD and catalyzes transfer of electrons from NADPH to the "hemoprotein" subunit, which in turn binds sulfite and catalyzes its reduction to sulfide (Rueger & Siegel, 1976). The hemoprotein subunit of *E. coli* SiR exists in solution as a monomeric polypeptide of M_r 56 000; it contains one molecule of siroheme and one Fe_4S_4 center per peptide (Siegel, 1978). Siroheme is an iron dimethylurotetrahydroporphyrin, with the methyl groups attached to adjacent pyrrole rings of the porphyrin nucleus (Murphy et al., 1973; Scott et al., 1978). EPR and Mossbauer studies have indicated that the siroheme and Fe_4S_4 centers exchange spins through a common bridging ligand in the *E. coli* SiR hemoprotein subunit (Christner et al., 1981).

A second enzyme, ferredoxin-nitrite reductase (NiR), which catalyzes the six-electron reduction of NO_2^- to NH_3, has been highly purified from higher plants, algae, and fungi (Hucklesby et al., 1972; Zumft, 1972; Vega et al., 1975; Vega & Kamin, 1977). After some initial evidence suggesting the presence of an Fe_2S_2 center in the enzyme (Vega & Kamin, 1977), recent data of Lancaster et al. (1979) strongly indicate that spinach NiR contains an array of prosthetic groups identical with those of the *E. coli* SiR hemoprotein, i.e., one siroheme and one Fe_4S_4 center on a monomeric polypeptide (in the case of NiR, M_r 61 000).

Asada et al. (1969) have reported isolation of an SiR from spinach which was stated to contain no more than 1 g-atom of Fe/mol of enzyme. The SiR preparation exhibited the absorption spectrum of a hemoprotein, and the sulfite-reducing activity was markedly inhibited by the heme ligands CO and CN^-. The dye MV^+, but not the physiological electron donor ferredoxin, could serve as reductant for stoichiometric reduction of SO_3^{2-} to S^{2-}. The possibility of obtaining an SiR enzyme which could transfer six electrons in a single reaction yet was structurally simpler than the siroheme-Fe_4S_4 protein from *E. coli* prompted our efforts to purify and characterize the spinach SiR. The present paper reports the isolation of SiR from spinach leaves, describes a number of physical, chemical, and catalytic properties of the enzyme, and compares those properties with those of *E. coli* SiR and spinach NiR. We find that spinach SiR is in fact a siroheme-Fe_4S_4 protein

† From the Departments of Biochemistry and Botany, Duke University, and the Veterans Administration Hospital, Durham, North Carolina 27705. *Received December 18, 1981.* This work was supported by Grant AM-13460 from the National Institutes of Health, Grant PCM-7924877 from the National Science Foundation, and Project Grant 7875-01 from the Veterans Administration.

‡ Present address: Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139. This work is part of a dissertation submitted by R.K. to Duke University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[1] Abbreviations: BSA, bovine serum albumin; Fd_r, reduced spinach ferredoxin; Fe_2S_2, binuclear iron-sulfur center; Fe_4S_4, tetranuclear iron-sulfur center; EDTA, ethylenediaminetetraacetic acid; Me_2SO, dimethyl sulfoxide; MV^{2+}, oxidized methylviologen; MV^+, reduced methylviologen; NiR, nitrite reductase; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; SiR, sulfite reductase; 63K SiR, spinach SiR species with subunit molecular weight 63 000; 69K SiR, spinach SiR species with subunit molecular weight 69 000.

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