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# Environmental Sensitivity of Azo Chromophores in Arsanilazocarboxypeptidase*

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ABSTRACT: Coupling of carboxypeptidase A with *p*-azobenzenearsonate generates a visible, optically active absorption spectrum indicative of arsanilazotyrosine, confirmed by amino acid analysis and measurement of protein-bound arsenic which further reveals the modification of lysine. Physicochemical properties of this derivative are virtually the same as those of the native enzyme. The consequences of modifying *O*-acetyl-, *N*-succinyl-, nitro-, and azotetrazolylcarboxypeptidase with *p*-azobenzenearsonate are consistent with the formation of arsanilazotyrosine which reflects in the visible circular dichroic spectrum. Denaturation of the enzyme with guanidine hydrochloride greatly simplifies the circular dichroic spectrum. The binding of a substrate, glycyl-L-tyrosine, or the inhibitor, β-phenylpropionate, and the removal of the catalytically essential zinc atom markedly alter the complex circular dichroic spectrum in a fashion characteristic for each. The data indicate that the arsanilazotyrosyl chromophore of arsanilazocarboxypeptidase is a sensitive probe of the interactions of β-phenylpropionate or glycyl-L-tyrosine with the enzyme and of the topology of the protein molecule.

The application of suitable reagents for the chemical modification of tyrosine has increasingly provided evidence that these residues play an important role in the mechanism of action of a number of enzymes. Certain tyrosyl modifications result in covalent chromophoric derivatives some of which can also serve as probes of enzyme conformation. Thus, the environmentally sensitive absorption spectrum of nitrotyrosine in nitrocarboxypeptidase A was capable of discerning the effects of substrates and inhibitors on the microchemical environment of the active site of this enzyme (Riordan *et al.*, 1967a).

We have now studied *p*-azobenzenearsonate derivatives of carboxypeptidase. This chemical modification can be quantitated by both amino acid and spectral analysis; protein-bound arsenic also serves as an analytical gauge of modification. This reagent was chosen to probe tyrosyl residues of carboxypeptidase A, which have been implicated in catalytic function (Vallee and Riordan, 1968). Consistent with previous studies employing a series of tyrosyl reagents, tyrosyl modification with *p*-azobenzenearsonate is accompanied by a change in the activities of carboxypeptidase. Importantly, the absorption spectrum of arsanilazocarboxypeptidase is optically active and displays multiple extrinsic Cotton effects between 300 and 600 mμ. This property offers novel means to examine the effects of environmental influences on modified tyrosyl residues. Thus, the circular dichroic bands of arsanilazocarboxypeptidase are altered markedly by the binding of substrates or inhibitors and by the removal of the catalytically essential zinc atom. Preliminary accounts of these studies have appeared elsewhere (Vallee and Riordan, 1968; Kagan and Vallee, 1968, 1969).

## Materials and Methods

Carboxypeptidase A prepared by the method of Anson (1937) was obtained as an aqueous crystal suspension from the Worthington Biochemical Corp. The crystals were washed three times with metal-free distilled water and dissolved in 3 M NaCl to give about $10^{-3}$ M stock solutions just before use. *p*-Arsanilic acid was a product of Eastman Organic Chemicals and was used without further purification. All other chemicals were reagent grade. Buffers were extracted with 0.1% dithizone in carbon tetrachloride to prevent contamination by adventitious metal ions (Thiers, 1957).

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Esterase activity was measured routinely with 0.01 M HPLA[1] as substrate in 0.2 M NaCl–0.005 M Tris buffer, pH 7.5, 25°. Hydrolysis of HPLA was determined by pH titration (Snoke *et al.*, 1948) with 0.1 M NaOH of the hydrogen ions released on hydrolysis using a pH-Stat (Radiometer, Copenhagen) and recorder (Ole Dich, Copenhagen). Ester hydrolysis at substrate concentrations below 0.001 M HPLA was followed spectrophotometrically (McClure *et al.*, 1964) using a Cary Model 15 recording spectrophotometer and 3 ml of substrate solution in 0.05 M Tris (pH 7.5) in 1-cm cuvets mounted in a thermostatted cell compartment at 25°.

Peptidase activity was determined at 0° using 0.02 M CGP (Miles Laboratories) as substrate in 0.02 M Veronal–1 M NaCl, pH 7.5 (Coleman and Vallee, 1960). Hydrolysis of CGP was followed by an automated ninhydrin color assay for free phenylalanine released employing a Technicon Autoanalyzer.

Protein concentration of native carboxypeptidase was measured by the absorbance at 278 mμ, based on the molar absorptivity of $6.42 \times 10^{-4}$ $M^{-1}$ $cm^{-1}$ (Simpson *et al.*, 1963). Concentrations of arsanilazocarboxypeptidase were measured after dialysis by the method of Lowry *et al.* (1951), employing native carboxypeptidase as a standard. These values were in good agreement with those obtained by differential refractometry.

Absorption spectra were determined with a Cary Model 15 recording spectrophotometer. A Beckman DU spectrophotometer was used for absorption measurements at single wavelengths. pH was measured with a Radiometer pH meter (Model 25) equipped with a Radiometer GK 2021 electrode.

Amino acid analyses were carried out with a Spinco Model 120B amino acid analyzer according to the procedure of Spackman *et al.* (1958). Proteins were hydrolyzed with 6 N HCl in sealed evacuated tubes at 110° for 22 hr. Tryptophan analyses were performed by the method of Spies and Chambers (1949).

*p*-Azobenzenearsonate was prepared by diazotization of *p*-arsanilic acid (0.5 mmole dissolved in 10 ml of 0.3 M HCl) by the addition of $NaNO_2$ (0.75 mmole dissolved in 7.5 ml of water) at 0°. The solution was adjusted to pH 5 with NaOH after 30 min of reaction and then diluted to 25 ml with water. The concentration of the diazonium salt was established by reacting aliquots of the solution with 50- to 100-fold molar excesses of *N*-chloroacetyl-DL-tyrosine for 30 min at pH 8.8 and room temperature. The concentration of the product of the reaction, monoarsanilazo-*N*-chloroacetyl-DL-tyrosine, was determined by its absorption at 490 mμ in 0.1 N NaOH (Tabachnick and Sobotka, 1959). These titrations indicated that the concentration of the diazonium reagent was 100 ± 2% of the theoretical value of 0.02 M.

Coupling of carboxypeptidase ($2 \times 10^{-4}$ M) was carried out at 0° in 0.33 M $NaHCO_3$–1 M NaCl (pH 8.8) by addition of appropriate aliquots of a freshly prepared 0.02 M solution of *p*-azobenzenearsonate. The reaction was stopped by addition of a fivefold excess of aqueous phenol relative to the initial *p*-azobenzenearsonate. This ratio of phenol to coupling reagent effectively quenched the unreacted *p*-azobenzenearsonate. The modified protein was dialyzed at 4° against several changes of a 100 volume excess of 0.04 M Tris–1 M NaCl (pH 7.7).

Reaction of carboxypeptidase with *N*-acetylimidazole was carried out at room temperature by the addition of a 60-fold molar excess of the solid reagent to the enzyme dissolved in 0.02 M sodium Veronal–1 M NaCl (pH 7.5) (Simpson *et al.*, 1963). The native enzyme was succinylated with a 50-fold molar excess of succinic anhydride, followed by 48-hr dialysis to allow deacylation of tyrosyl residues (Riordan and Vallee, 1964). Nitration of the enzyme was performed with a fourfold molar excess of tetranitromethane (Riordan *et al.*, 1967b) and azo coupling with an eightfold molar excess of diazonium-1H-tetrazole (Sokolovsky and Vallee, 1967). *O*-Acetyltyrosine content was measured by the neutral hydroxamate procedure (Balls and Wood, 1956).

Apoarsanilazocarboxypeptidase was prepared by incubation of the modified metalloenzyme with 1,10-phenanthroline. The zinc–phenanthrolinate complex was then removed by passage of the solution over a metal-free column of Sephadex G-25, as previously described for the native enzyme (Coombs *et al.*, 1964).

The monoarsanilazoderivative of *N*-chloroacetyltyrosine was prepared by the method of Tabachnick and Sobotka (1959). The monoazo-*N*-acetylhistidine and bisazo-α-*N*-acetyllysine derivatives were prepared by a modification of this procedure and were purified by chromatography on DEAE-cellulose but not isolated. Hydrolysis of these products with 6 N HCl at 110° for 22 hr did not regenerate free lysine, histidine, or tyrosine, as evidenced by amino acid analyses of the hydrolysates, consistent with the findings of Gundlach *et al.* (1962). Therefore, modification of these residues in the enzyme was quantitated by the difference in number of residues recovered from the native and modified enzymes, respectively.

Atomic absorption spectrometry was employed to measure zinc (Fuwa and Vallee, 1963) and arsenic (Ando *et al.*, 1969).

Optical rotatory and circular dichroism measurements were performed at 25° with a Cary Model 60 recording spectropolarimeter equipped with a circular dichroism attachment. Slit widths were programmed to give a constant band width of 15 Å between 200 and 650 mμ. Maximum absorbance of samples did not exceed 2.0 optical density units. Optical rotatory dispersion and circular dichroism measurements between 300 and 620 mμ were performed in 1-cm cells at protein concentrations of $8 \times 10^{-5}$ or $4 \times 10^{-5}$ M, depending upon the optical density of the sample. Overlapping rotatory spectra of different dilutions and light paths indicated that there was no concentration dependence nor artifacts due to absorption at or below $8 \times 10^{-5}$ M. Protein concentrations were about $2 \times 10^{-5}$ M in optical rotatory dispersion measurements below 300 mμ, varying the path length as needed from 1 cm to 1 mm. Base-line measurements for circular dichroism and optical rotatory dispersion studies were obtained with dialysates of the protein samples before and after each set of two sample measurements. All samples were in 0.04 M Tris–1 M NaCl (pH 7.7), the standard dialysis buffer. The circular dichroism attachment was calibrated using a 1-mg/ml aqueous solution of *d*-10-camphorsulfonic acid, which gave an observed ellipticity of (+) 0.308° at 290 mμ in a 1-cm cell. Optical rotation is expressed in specific rotation: $[\alpha]_\lambda^{25} = 100\alpha/lc$, where $\alpha$ = observed rotation in degree, $c$ = concentration in g/100 ml, and $l$ = path length in dm. Ellipticity is expressed as molecular ellipticity: $[\theta]_\lambda^{25} = (\theta/10)(M/lc)$ with units of deg $(cm)^2$ per dmole, where $\theta$ = observed ellipticity in degrees, $M$ = protein molecular weight (34,600 for carboxypeptidase A), $l$ = path length

[1] Abbreviations used are: HPLA, hippuryl-*dl*-β-phenyllactate; CGP, *N*-carbobenzoxyglycyl-L-phenylalanine.

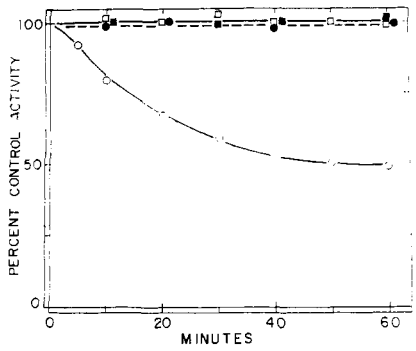


FIGURE 1: Progression of changes in esterase (□—□) and peptidase (○—○) activities on coupling of carboxypeptidase with a 10-fold molar excess of *p*-azobenzenearsonate in the presence (■- - -■, ●- - -●) and absence of 0.1 M β-phenylpropionate.

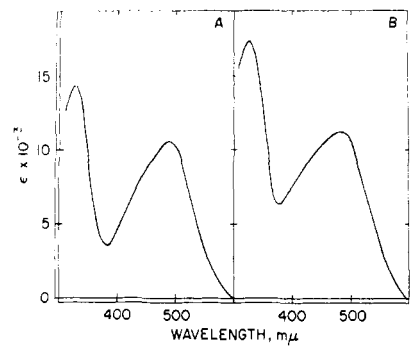


FIGURE 2: Absorption spectra in 0.1 M NaOH. (A) Monoarsanilazo-*N*-chloroacetyltyrosine. (B) Arsanilazocarboxypeptidase prepared with a 10-fold molar excess of reagent.

in centimeters, and $c$ = concentration in grams per milliliter. Molecular ellipticities are not corrected for the refractive index of the solvent (Djerassi, 1960).

## Results

Reaction of carboxypeptidase ($2 \times 10^{-4}$ M) with a 10-fold molar excess of *p*-azobenzenearsonate at pH 8.8, 0°, decreases peptidase activity to 50% of that of the control after 40 min. Esterase activity remains apparently unchanged when assayed under routine conditions. Prior addition of the inhibitor, β-phenylpropionate, prevents the activity changes (Figure 1). These changes in enzymatic activity are seemingly maximal; higher molar excesses (up to 20-fold) have little further effect (Kagan and Vallee, 1969). The specificity and reproducibility of the functional and chemical consequences of this modification critically depend upon pH, reaction time, and effectiveness of the quenching reagent employed to terminate the reaction. Modification of the enzyme ($2 \times 10^{-4}$ M) with a 10-fold molar excess of reagent for 40 min, pH 8.8, 0°, followed by quenching of excess reagent with phenol, was adopted as standard procedure.

The concentration–velocity profile for HPLA hydrolysis by arsanilazocarboxypeptidase is altered markedly while that of the enzyme modified in the presence of β-phenylpropionate is indistinguishable from that of the native enzyme. Initial rate data obtained by the spectrophotometric method of McClure *et al.* (1964) yield a $K_{m\,app}$ for HPLA of $8.2 \times 10^{-5}$ M and a $V_{max}$ of $26.2 \times 10^{3}$ $min^{-1}$ for both the native enzyme and that modified in the presence of β-phenylpropionate. On the other hand, azo coupling of the enzyme in the absence of the protecting agent decreases the maximum rate for HPLA hydrolysis to $15.9 \times 10^{3}$ $min^{-1}$ and marginally increases the $K_{m\,app}$ to $1.1 \times 10^{-4}$ M.

The esterase profiles of the native and modified enzymes intersect in the region of 0.01 M HPLA, the concentration of substrate generally employed for standard assays. Hence, at this concentration of substrate, the alterations in the properties of ester hydrolysis induced by the modification are not apparent.

The absorption spectra at pH 13 of the monoazo derivative of *N*-chloroacetyltyrosine and that of the enzyme modified with a 10-fold excess of reagent, corrected for the absorption of the native enzyme, are compared in Figure 2. Both display absorption maxima at 328 and 490 mμ and an absorption minimum at 380 mμ. The similarity of the protein and *N*-chloroacetyltyrosine spectra indicates the presence of monoarsanilazotyrosine in the enzyme. Employing the absorptivities at 460 and 500 mμ (Tabachnick and Sobotka, 1960), the azotyrosine and azohistidine content of the enzyme can be calculated to account for the modification of 1.1 tyrosyl and less than 0.1 histidyl residue per molecule of enzyme.

The modification of tyrosine is confirmed by amino acid analyses. With the exception of lysine, no other differences were found between the amino acid compositions of the native and modified enzymes (Table I). In particular, the histidine content of the two enzymes were identical. The values for tyrosine obtained by amino acid analysis (Table I) were in close agreement with those obtained from spectral data, *i.e.*, 1.1 at a 10-fold and 1.8 at a 20-fold molar excess. The sums of the tyrosyl and lysyl residues modified, calculated from amino acid analysis and/or spectra, are in good agreement with the amount of arsenic incorporated, as measured by atomic absorption spectroscopy. A one-to-one relationship between arsenic incorporation and total residue modification is ob-

TABLE I: Residue Modification[a] and Arsenic Incorporation[b] on Coupling Carboxypeptidase A with *p*-Azobenzenearsonate.

| | Molar Excess of Reagent | | |
|---|---|---|---|
| Residue | 10× | 20× | 10×, Protected[c] |
| Tyrosine | 1.3 | 1.8 | 0.5 |
| Lysine | 1.0 | 2.3 | 0.9 |
| Sum, Tyr + Lys | 2.3 | 4.1 | 1.4 |
| As (g-atoms/mole) | 2.6 | 4.1 | 1.5 |

[a] The number of amino acid residues modified was determined by comparing the amino acid analyses of native and azo-coupled proteins. All values represent the average of two or three analyses. [b] Arsenic bound to the modified protein was measured after dialysis by atomic absorption spectroscopy. [c] Modification of the enzyme was carried out in the presence of 0.1 M β-phenylpropionate, but, otherwise, under conditions described under Materials and Methods.

served uniformly at all molar excesses up to 20-fold and when the enzyme is modified in the presence or absence of β-phenylpropionate.

With a 10-fold molar excess of reagent, 1.1–1.3 azotyrosyl residues are formed, of which 0.4–0.8 is protected by the presence of β-phenylpropionate. In contrast, this molar excess of reagent modifies the same number of lysyl residues in the presence or absence of β-phenylpropionate (Table I).

Succinic anhydride acylates free tyrosyl and lysyl residues of carboxypeptidase but on dialysis for 48 hr the *O*-succinyltyrosyl residues deacylate spontaneously to leave only *N*-succinyllysyl residues. The peptidase and esterase activities of *N*-succinylcarboxypeptidase differ only slightly from those of the native enzyme (Riordan and Vallee, 1964). On reaction of the *N*-succinyl enzyme with a 10-fold molar excess of *p*-azobenzenearsonate for 40 min, it loses 52% of its peptidase activity while esterase activity decreases only slightly. The product, arsanilazo-*N*-succinylcarboxypeptidase, contains 1.2 azotyrosyl residues and only 0.3 azolysyl residue, as determined by spectral and amino acid analysis (Table II).

TABLE II: Successive Chemical Modifications of Carboxypeptidase A Enzymatic Activities.[a]

| Carboxypeptidase Derivative | Sp Act. (% Control) Esterase | Peptidase |
|---|---|---|
| *N*-Succinyl- | 120 | 108 |
| Arsanilazo-*N*-succinyl- | 110 | 56 |
| *O*-Acetyl- | 515 | 7 |
| Arsanilazo-*O*-acetyl- | 490 | 5 |
| Arsanilazo-*O*-acetyl- + $NH_2OH$ | 112 | 95 |
| Nitro- | 195 | 12 |
| Arsanilazonitro- | 190 | 7 |
| Arsanilazo- | 98 | 53 |
| Nitroarsanilazo- | 107 | 46 |
| Azotetrazolyl- | 190 | 85 |
| Arsanilazotetrazolyl- | 135 | 35 |

[a] *N*-Succinyl-, *O*-acetyl-, nitro-, and azotetrazolylcarboxypeptidase were prepared according to conditions under Materials and Methods. However, in the present experiments the enzyme concentrations were up to twofold greater during the preparation of some of these derivatives than those described in the literature, accounting for the activities of the controls.

The free tyrosyl residues of the native enzyme can be O acetylated selectively with *N*-acetylimidazole increasing esterase and virtually abolishing peptidase activity (Simpson *et al.*, 1963). No further alteration of activity occurs on addition of *p*-azobenzenearsonate to the acetylated enzyme. Treatment of arsanilazo-*O*-acetylcarboxypeptidase with 1 M $NH_2OH$ deacylates the *O*-acetyltyrosyl residues and restores both activities essentially to those characteristic of the native enzyme. Quantitative spectral analysis of the doubly treated enzyme reveals a negligible azotyrosyl content (0.1 residue/molecule) (Table II). Apparently, prior acetylation prevents subsequent azo coupling.

Similarly, the activities of nitrocarboxypeptidase containing 1.2 nitrotyrosyl residues/molecule do not change significantly on reaction with *p*-azobenzenearsonate (Table II) although 0.6 arsanilazotyrosyl residue/molecule is introduced as calculated from the difference spectrum of the azonitro *vs.* the nitro enzyme.

Chemical modification has also been carried out in the reverse sequence. Reaction of arsanilazocarboxypeptidase with a fourfold molar excess of tetanitromethane at pH 8.0 and 23° reduces its peptidase activity by 7% while increasing its esterase activity by 9% (Table II). Consistent with these relatively minor catalytic effects, only 0.2 tyrosyl residue of the arsanilazoenzyme is nitrated.

An eightfold molar excess of diazonium-1H-tetrazole modifies one tyrosyl residue of carboxypeptidase, doubles esterase activity, and decreases peptidase activity only slightly (Sokolovsky and Vallee, 1967). Reaction with *p*-azobenzenearsonate of a derivative containing 1.2 azotetrazolyltyrosyl residues/molecule introduces 0.8 arsanilazotyrosyl residue and decreases the esterase from 190 to 135% and peptidase activity from 85 to 35% of that of the native enzyme (Table II).

Various chemical and physical properties of arsanilazocarboxypeptidase are compared with those of the native enzyme in Table III. Both the modified and native enzymes contain 1 g-atom of zinc/mole. By sedimentation velocity, arsanilazocarboxypeptidase moved as a single, symmetrical peak with an $s_{20,w}$ of 3.34 *vs.* 3.32 S for the native enzyme under the same conditions.

TABLE III: Physicochemical Characteristics of Carboxypeptidase and Arsanilazocarboxypeptidase.

| Property | [(CPD)Zn] | [(AzoCPD)Zn] |
|---|---|---|
| Zn (g-atoms/mole) | 1.0 | 1.0 |
| $s_{20,w}$[a] (S) | 3.32 | 3.34 |
| $[\alpha]^{25}_{235}$ (deg) | −3900 ± 100 | −3900 ± 100 |
| Elution volume (ml)[b] | 132 | 133 (>90%) |
| Sephadex G-75 (ml) | | 100 (<10%) |

[a] Proteins were sedimented at 50,740 rpm at 22°, for 1 hr and 20 min, at a concentration of 4–5 mg/ml in 0.05 M Tris–1 M NaCl (pH 7.5). [b] The gel was equilibrated and eluted at 4° with 0.05 M Tris–1 M NaCl (pH 7.5). The dimensions of the gel column were 70 × 2 cm; 3.12 ml of $1.6 \times 10^{-4}$ M enzyme was applied to the column in each case.

The positions and amplitudes of the negative, intrinsic Cotton effect at 235 mμ of the modified and native enzyme were identical within experimental error.

Arsanilazocarboxypeptidase eluted as a symmetrical peak accounting for 90% of the applied protein on a 70 × 2.2 cm column of Sephadex G-75 in 0.04 M Tris–1 M NaCl, pH 7.5, 4°, in the same position as native carboxypeptidase. A minor peak (less than 10% of the applied protein) was also detected,

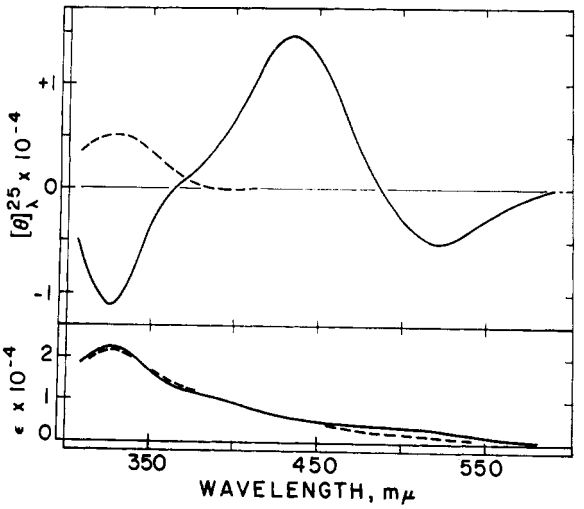


FIGURE 3: Circular dichroic spectra (top) and absorption spectra (bottom) of arsanilazocarboxypeptidase, 0.04 M Tris, 1 M NaCl, pH 7.7, 25°, in the presence (- - -) and absence (—) of 5 M guanidine hydrochloride.

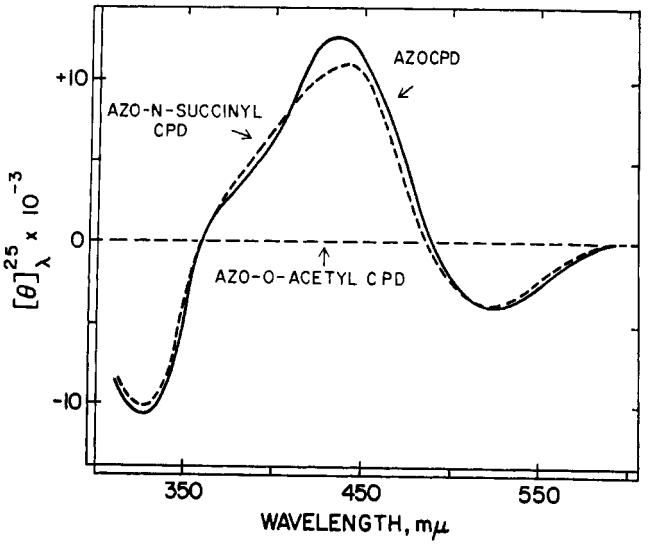


FIGURE 4: Circular dichroic spectra of arsanilazocarboxypeptidase (AzoCPD) (—), arsanilazo-*N*-succinylcarboxypeptidase (Azo-*N*-succinylCPD) (- - -), and arsanilazo-*O*-acetylcarboxypeptidase (Azo-*O*-acetylCPD) (- - -) in 0.04 M Tris, 1 M NaCl, pH 7.7, 25°. Enzyme derivatives were prepared as described in Materials and Methods. The circular dichroism spectrum of arsanilazo-*O*-acetylcarboxypeptidase is virtually identical with that of the base line, *i.e.*, native carboxypeptidase, in this spectral region.

moving slightly ahead of the major component. The spectral and enzymatic characteristics of the major and minor components were virtually identical.

The optical rotatory dispersion of the native and modified proteins are virtually identical in the ultraviolet region of the spectrum, but arsanilazocarboxypeptidase displays multiple extrinsic Cotton effects between 300 and 600 mμ (Figure 3). At pH 7.7, the circular dichroic spectrum of the modified protein exhibits overlapping negative, positive, and negative Cotton effects whose maximal amplitudes occur at 328, 435, and 525 mμ, respectively. At this pH, the absorption spectrum of the modified protein displays a distinct peak at 328 mμ and a broad envelope of overlapping bands with shoulders centered at 395 and 515 mμ (Figure 3). While the position of the troughs of the negative Cotton effects at 328 and 525 mμ occur approximately at the maxima of the corresponding absorption spectrum, the peak of the Cotton effect at 435 mμ seems displaced with respect to a shoulder of the absorption spectrum. This could signify unresolved Cotton effects and/or unresolved absorption bands between 300 and 600 mμ, but the extrinsic Cotton effects may yet be capable of further resolution.

Denaturation of the modified protein by 5 M guanidine hydrochloride simplifies the circular dichroism spectrum. Only a single, small positive Cotton effect centered at 325 mμ remains (Figure 3). Within experimental error, the circular dichroism pattern of the denatured protein is virtually the same as that of monoarsanilazo-*N*-chloroacetyl-L-tyrosine, obtained at the same pH. In contrast, denaturation of the modified enzyme alters its absorption spectrum in the corresponding spectral region only minimally (Figure 3).

Successive chemical modifications were also employed to assign the extrinsic Cotton effects to tyrosyl or lysyl residues. Between 300 and 600 mμ, the circular dichroism spectrum of *N*-succinyl- and *O*-acetylcarboxypeptidase does not differ significantly from that of the native enzyme. Modification of *N*-succinylcarboxypeptidase with a 10-fold molar excess of *p*-azobenzenearsonate, however, generates a circular dichroic spectrum quite similar to that of arsanilazocarboxypeptidase itself (Figure 4). On the other hand, reaction of *p*-azobenzenearsonate with *O*-acetylcarboxypeptidase does not induce any optical activity above 300 mμ and is, therefore, virtually also the same as the circular dichroism spectrum of the native enzyme. Further, the Cotton effects do not appear when the *O*-acetyl groups are removed subsequently by hydroxylamine (Figure 4). These experiments suggest that arsanilazotyrosyl residues of the enzyme account for the extrinsic Cotton effects.

The circular dichroic spectrum of arsanilazocarboxypeptidase varies with the molar excess of reagent employed to modify the native enzyme (Figure 5). The amplitude of the negative band at 525 mμ becomes maximal with a 10-fold excess of reagent, while those of the remaining positive and negative bands continue to change up to a 20-fold molar excess, the maximum amount employed.

Since the extrinsic Cotton effects appear to reflect arsanilazotyrosine, the amplitudes of the three major, optically active bands were correlated with the degree of tyrosyl modification as measured spectrally (Figure 5, insert). The amplitudes of the negative band at 525 mμ is maximal upon modification of one tyrosyl residue. The positive band at 435 mμ and the negative band at 328 mμ also change in proportion to the degree of tyrosyl modification up to one azotyrosyl residue per mole, as is apparent from Figure 5 (insert) which also indicates that additional modification produces relatively lesser change.

Since *p*-azobenzenearsonate alters a tyrosyl residue of carboxypeptidase, the effect of substrate and inhibitors on the optical activity of the modified protein was examined. Increasing concentrations of the inhibitor, β-phenylpropionate, markedly change the circular dichroism spectrum of arsanilazocarboxypeptidase (Figure 6). As the concentration of inhibitor approaches $4.3 \times 10^{-3}$ M, the negative Cotton effect at 525 mμ

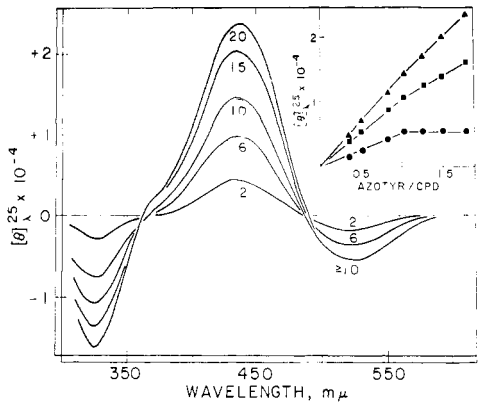


FIGURE 5: Circular dichroic spectra of carboxypeptidase, modified with increasing molar excesses of *p*-azobenzenearsonate. Numbers refer to molar excesses employed. Spectra were recorded in 0.04 M Tris, 1 M NaCl, pH 7.7, 25°. Insert: molecular ellipticity of extrinsic Cotton effects at 328 (■—■), 435 (▲—▲), and 525 mμ (●—●) *vs.* moles of arsanilazotyrosine generated per mole of modified enzyme.

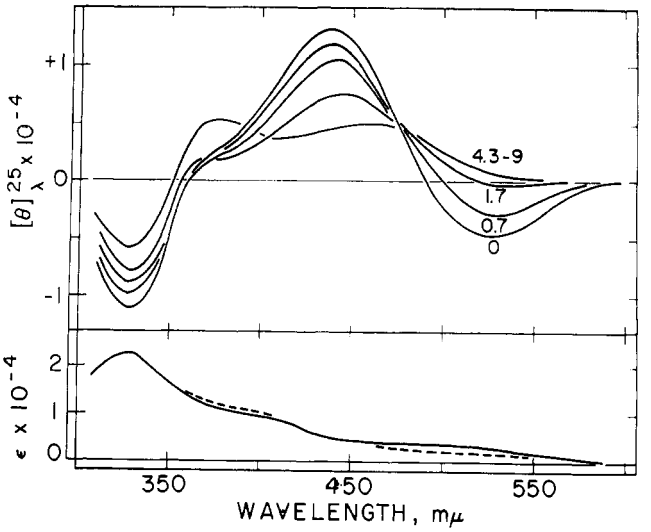


FIGURE 6: The effect of β-phenylpropionate on the circular dichroic spectrum (top) and absorption spectrum (bottom) of arsanilazocarboxypeptidase. Spectra were recorded in 0.04 M Tris, 1 M NaCl, pH 7.7, 25°. The molar concentrations (× $10^3$) of β-phenylpropionate added to the enzyme for the circular dichroism measurements are indicated. The absorption spectra were recorded in the presence (- - -) and absence (—) of 4.3 × $10^{-3}$ M β-phenylpropionate.

disappears and the amplitudes of the positive and negative bands at 435 and 328 mμ decrease. When the concentration of inhibitor is increased to 9 × $10^{-3}$ M, a positive Cotton effect at about 380 mμ is resolved. The intrinsic Cotton effect of arsanilazocarboxypeptidase with a minimum at 235 mμ is not measurably altered at any of these concentrations of inhibitor. In contrast to the effects on the circular dichroism spectrum, the absorption spectrum of arsanilazocarboxypeptidase is not altered extensively by β-phenylpropionate. The shoulder at about 520 mμ disappears while absorption at 360–380 mμ increases slightly (Figure 6).

The peptide substrate, glycyl-L-tyrosine, also perturbs the circular dichroism spectrum, and to a lesser extent, the absorption spectrum of arsanilazocarboxypeptidase (Figure 7). At 3.5 × $10^{-3}$ M the negative ellipticity band at 525 mμ disappears while the amplitude of the negative band at 328 mμ decreases. However, unlike the effect of β-phenylpropionate, the amplitude of the positive band at 435 mμ is unaltered at this concentration of substrate. The absorption spectrum changes include a small increase in the region of the 328-mμ peak, a slight increase at 380–400 mμ and the loss of the shoulder at 490–520 mμ (Figure 7).

These changes do not seem to be due to the products of hydrolysis of the substrate. During the time required to record the circular dichroism spectrum, the concentrations of L-tyrosine and glycine would only reach about 2 × $10^{-4}$ M. Separate experiments have shown that these concentrations do not alter the extrinsic Cotton effects of arsanilazocarboxypeptidase significantly.

Certain details of the circular dichroism spectrum of arsanilazocarboxypeptidase are dependent upon the presence of the catalytically essential zinc atom of the enzyme (Figure 7). The metal atom can be removed by incubation of the enzyme with 1,10-phenanthroline, and the apoenzyme isolated by gel filtration. Arsanilazocarboxypeptidase and apoarsanilazocarboxypeptidase share the negative and positive Cotton effects at 325–330 and 435–440 mμ, but the latter lacks the negative band at 525 mμ. However, addition of 1 g-atom of zinc/mole to the apoenzyme immediately after its elution from the Sephadex column largely restores this band. The changes in the absorption spectrum due to removal of zinc resemble those due to the addition of glycyl-L-tyrosine (Figure 7).

## Discussion

Diazonium salts have been used for affinity labeling of antibodies (Metzger *et al.*, 1964) and for selective modification of enzymes (Gundlach *et al.*, 1962; Burton and Waley, 1967; Sokolovsky and Vallee, 1967), bovine serum albumin (Tabachnick and Sobotka, 1960), and peptides (Borek *et al.*, 1965). *p*-Azobenzenearsonate was selected here because its desirable optical properties facilitate the study of its effect on carboxypeptidase by spectral means. The ease with which incorporation into the protein can be measured independently by virtue of its arsenic content seemed to be a further advantage. Changes in the functional properties of carboxypeptidase A in response to azo coupling with *p*-azobenzenearsonate served as indices of tyrosyl modification. They are reminiscent of those occurring on modification of the enzyme with other functional reagents, though they differ quantitatively.

As in previous instances, the activity changes are due to the covalent modification of amino acid residues. Gross structural or conformational alterations do not seem to be a major consequence of diazo coupling; the physical parameters of the modified enzyme resemble those of native carboxypeptidase closely (Table III). Further, the product contains 1 g-atom of zinc/mole. All of the protein-bound arsenic can be accounted for by covalent modification of lysine and tyrosine, eliminating noncovalently bound reagents as the cause of the observed changes.

The absorption spectrum of arsanilazocarboxypeptidase is consistent with the formation of monosubstituted azotyrosine (Figure 2) (Tabachnick and Sobotka, 1959) but it does not clearly reveal the presence of azolysine. Bisazo derivatives.

*i.e.*, pentazenes, have been thought to be major products of the reaction of diazonium reagents with primary amines, including lysine analogs (Howard and Wild, 1957). In alkali the molar absorptivity of the bisazo derivative of ε-aminocaproic acid at 378 mμ is 30,800 (Tabachnick and Sobotka, 1959). Hence, the presence of bisazolysyl residues should be detectable readily by spectral analysis, but none was found in arsanilazocarboxypeptidase (Figure 2). While this could be accounted for by spontaneous decomposition of pentazenes, the absorption spectrum of the enzyme observed immediately after chemical modification does not change over a period of 1 week. The present data suggest that reaction of *p*-azobenzenearsonate with lysine in carboxypeptidase yields only the monoazolysyl derivative. The coincidence of the numbers of gram-atoms of arsenic incorporated with the total number of residues modified supports this view (Table I). In this regard, the product of the reaction of polylysine with diazotized sulfanilic acid under conditions favorable to the formation of the monoazolysyl derivative does not display an absorption maximum above 320 mμ (Burton and Waley, 1967).

Previous studies have indicated that lysine is not involved directly in the catalytic function of carboxypeptidase (Riordan and Vallee, 1964; Sokolovsky and Vallee, 1967). Consistent with these findings, N succinylation of the native enzyme does not prevent the catalytic changes accompanying azo coupling.

On the other hand, modification of tyrosyl residues of carboxypeptidase by a variety of reagents is known to markedly change its esterase and/or peptidase activities (Vallee and Riordan, 1968). Similarly, the effects of *p*-azobenzenearsonate on catalysis, though differing somewhat in detail from those observed in other instances, are consistent with the modification of tyrosine.

Spectral and amino acid analysis demonstrate diazo coupling of tyrosyl residues and successive chemical modifications relate the generation of azotyrosine to functional alterations. Thus, prior O acetylation of the tyrosyl residues prevents further activity changes upon diazo coupling[2] while preventing the formation of arsanilazotyrosine in the enzyme almost completely (Table II). Arsanilazo-*O*-acetylcarboxypeptidase, deacetylated with hydroxylamine, has enzymatic activities characteristic of the native enzyme. This further indicates that the formation of arsanilazotyrosine accounts for the functional consequences observed on modification with *p*-azobenzenearsonate.

A fourfold molar excess of tetranitromethane preferentially nitrates one rather than two of the tyrosyl residues which are acetylated by *N*-acetylimidazole (Riordan *et al.*, 1967b). *p*-Azobenzenearsonate does not alter the activities of nitrocarboxypeptidase nor does tetranitromethane appreciably alter the activities of arsanilazocarboxypeptidase (Table II). In this instance modification of a given tyrosyl residue by one reagent interferes with modification by another, though it cannot be stated at this time whether these are consequences of steric effects, direct substitution, or conformational changes. In contrast, both activities of the enzyme modified with an eightfold molar excess of diazonium-1H-tetrazole change significantly upon further modification with *p*-azobenzenearsonate. One explanation would be that these two azo reagents modify

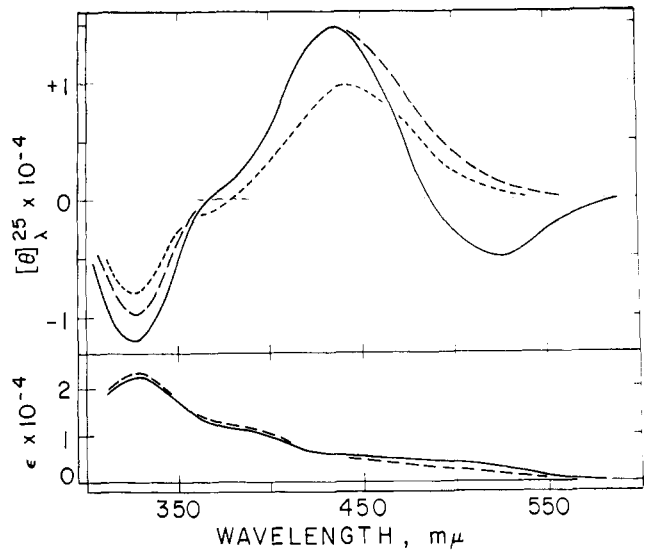


FIGURE 7: Circular dichroic and absorption spectra. Top: circular dichroic spectra of arsanilazocarboxypeptidase in the presence (— —) and absence (---) of 4.3 × $10^{-3}$ M glycyl-L-tyrosine. The circular dichroism measurements on apoarsanilazocarboxypeptidase (- - -) were performed subsequent to removal of zinc from the modified enzyme by 1,10-phenanthroline. Subsequent to the removal of the chelating agent, the apoenzyme employed contained 0.06 g-atom of zinc/mole. Bottom: absorption spectra of arsanilazocarboxypeptidase in the presence (— —) or absence (—) of 3.5 × $10^{-3}$ M glycyl-L-tyrosine; the absorption spectrum of apoarsanilazocarboxypeptidase is indistinguishable from that of the metalloenzyme to which glycyl-L-tyrosine has been added.

different tyrosyl residues. Such data are consistent with previous suggestions that diazonium-1H-tetrazole and tetranitromethane affect different and functionally distinct tyrosyl residues of carboxypeptidase (Riordan *et al.*, 1967b). Of course, protection of residues by more indirect mechanisms such as subtle conformational changes consequent to nitration or azo coupling cannot be dismissed. Ultimately, structural approaches will have to establish the basis of these effects directly. In this regard, peptide sequence work on nitro- and arsanilazocarboxypeptidase is now in progress to identify the specific residue modified (Bradshaw *et al.*, 1969; D. Behnke and B. L. Vallee, in preparation). It could be inferred from preliminary data that these two reagents interact with the same tyrosyl residue (D. Behnke and B. L. Vallee, in preparation; J. R. Riordan and R. A. Bradshaw, in preparation) though definitive assignment will have to await the completion of this work.

The catalytic changes consequent to modification with *p*-azobenzenearsonate differ quantitatively from those due to acetylation or nitration. Peptidase activity decreases by only about 50% in contrast with the greater than 90% loss of peptidase activity accompanying nitration. Moreover, the $K_m$ for HPLA hydrolysis is almost the same as that of the native enzyme while nitration increases $K_m$ approximately 25-fold. The basis of these quantitative differences in activities requires further study.

Extrinsic Cotton effects in enzymes generally have been observed on formation of reversible protein–chromophore complexes. In a few instances, these have been reported to result from covalent modifications of enzymes or other proteins (*e.g.*, Johnson *et al.*, 1968; Meloun *et al.*, 1968; Dowben and

[2] Studies with model compounds indicate that *p*-azobenzenearsonate does not alter the absorption spectrum of *N,O*-diacetyltyrosine or of *N*-acetyl-3-nitrotyrosine.

Orkin, 1967; Sigman *et al.*, 1969). Such extrinsic Cotton effects have been shown to offer particularly suitable opportunities for the study of structure–function relationships (Ulmer and Vallee, 1965).

The ellipticity bands of the modified protein arise most likely from azotyrosyl residues. Successive chemical modification supports this view. Thus, prior O acetylation prevents their formation while prior N succinylation does not. While modification of tyrosine apparently accounts for the circular dichroism spectrum, this is quite unlike that of azotyrosyl model compounds. The difference would seem to be due to conformational features of the azoenzyme, since in guanidine hydrochloride, the circular dichroism spectra of the protein and the model compound are almost identical. Indeed, azo chromophores have been shown to be generally suitable as structural probes for proteins (Fairclough and Vallee, 1969) and polypeptides (Goodman and Benedetti, 1968; Benedetti and Goodman, 1968; Benedetti *et al.*, 1968).

Certain features of the circular dichroism spectrum of azocarboxypeptidase would suggest that some of its detail might have more specific chemical implications. As pointed out above, the three-banded circular dichroism spectrum shown in Figure 3 is characteristic of a 10-fold molar excess of reagent. An increase in the molar excess of reagent enhances the amplitudes of two of the bands, but does not alter that at 525 mμ (Figure 5). Further, this latter band is the one most prominently perturbed by active center ligands.

The circular dichroism spectral changes produced by agents known to bind at the active center are both qualitative and quantitative and suggest that binding may induce direct or indirect conformational alterations of the arsanilazotyrosyl chromophore. The removal of zinc brings about a similar circular dichroism spectral change. Previous hydrodynamic and X-ray crystallographic studies have not revealed any gross conformational changes in carboxypeptidase A upon removal or replacement of the metal atom (Rupley and Neurath, 1960; Lipscomb *et al.*, 1968), although some evidence for metal-induced microscopic alterations has been obtained (Lipscomb *et al.*, 1968). The optical activity of the azotyrosyl chromophore of arsanilazocarboxypeptidase would appear to be a sensitive probe of such subtle microenvironmental alterations at the active site of this enzyme.

These circular dichroism spectral changes contrast with the minimal perturbation induced in the absorption spectrum by the same agents. The optical activity of the azotyrosyl chromophore, therefore, becomes a new and useful probe of the microchemical environment of the active site. The precise origin of these findings remains an intriguing question and is the subject of continued study.

The alteration of the azotyrosyl circular dichroism spectrum by active-site ligands as well as the activity changes caused by *p*-azobenzenearsonate are consistent with involvement of tyrosine in the function of carboxypeptidase, as inferred from other chemical modifications (Vallee and Riordan, 1968), the environmentally sensitive nitrotyrosyl absorption spectrum of nitrocarboxypeptidase (Riordan *et al.*, 1967a), and X-ray studies on crystals of carboxypeptidase $A_\alpha$ (Lipscomb *et al.*, 1968).

Chemical modification has been employed extensively in the study of structure–function relationships of enzymes and other proteins and has been particularly useful in assigning functional roles to specific amino acid residues. In certain instances chromophores can be "chemo-optical" probes (Edelman and McClure, 1968) when their absorption, nuclear magnetic resonance, or fluorescence spectra are environmentally sensitive. The optical activity of the covalently bound arsanilazochromophore which is sensitive to the microenvironment of the active center of carboxypeptidase as well as to the gross configuration of the protein molecule constitutes an important addition to this class of reagents. An optically active probe displaying these properties should be of great value in exploring the subtle interactions of a variety of substrates, inhibitors and modifiers with the active center of arsanilazocarboxypeptidase and, further, might be useful in broader explorations of the topology of the protein molecule.

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## References

Ando, A, Suzuki, M., Fuwa, K., and Vallee, B. L. (1969), *Anal. Chem.* (in press).

Anson, M. L. (1937), *J. Gen. Physiol. 20*, 663

Balls, A. K., and Wood, H. N. (1956), *J. Biol. Chem. 219*, 245

Benedetti, E., and Goodman, M. (1968), *Biochemistry 7*, 4242.

Benedetti, E., Kossoy, A., Falxa, M. L., and Goodman, M. (1968), *Biochemistry 7*, 4234.

Borek, F., Stupp, Y., Fuchs, S., and Sela, M. (1965), *Biochem. J. 96*, 577.

Bradshaw, R. A., Ericsson, L. H., Walsh, K. A., and Neurath, H. (1969), *Biochemistry 8*, 3859.

Burton, P. M., and Waley, S. G. (1967), *Biochem. J. 104*, 3P.

Coleman, J. E., and Vallee, B. L. (1960), *J. Biol. Chem. 235*, 390

Coombs, T. L., Omote, Y., and Vallee, B. L. (1964), *Biochemistry 3*, 653.

Djerassi, C. (1960), Optical Rotatory Dispersion, New York, N. Y., McGraw-Hill.

Dowben, R. M., and Orkin, S. H. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 2051.

Edelman, G. M., and McClure, W. O. (1968), *Accounts Chem. Res. 1*, 65.

Fairclough, Jr., G. F., and Vallee, B. L. (1969), *Fed. Proc. 28*, 763.

Fuwa, K., and Vallee, B. L. (1963), *Anal. Chem. 35*, 492.

Goodman, M., and Benedetti E. (1968), *Biochemistry 7*, 4226.

Gundlach, G., Köhne, C., and Turba, F. (1962), *Biochem. Z. 336*, 215.

Howard, A. N., and Wild, F. (1957), *Biochem. J. 65*, 651.

Johnson, G. F., Philip, G., and Graves, D. J. (1968), *Biochemistry 7*, 2101

Kagan, H. M., and Vallee, B. L. (1968), *Fed. Proc. 27*, 455.

Kagan, H. M., and Vallee, B. L. (1969), *Biochem. Biophys. Res. Commun. 34*, 654.

Lipscomb, W. N., Hartsuck, J. A., Reeke, Jr., G. N., Quiocho, F. A., Bethge, P. H., Ludwig, M. L. Steitz, T. A., Muirhead, H., and Coppola, J. C. (1968), *Brookhaven Sym. Biol. 21*, 24.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
McClure, W. O., Neurath, H., and Walsh, K. A. (1964), *Biochemistry 3*, 1897.
Meloun, B., Fric, I., and Sorm, F. (1968), *European J. Biochem. 4*, 112.
Metzger, H., Wofsy, L., and Singer, S. J. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 612.
Riordan, J. F., Sokolovsky, M., and Vallee, B. L. (1967a), *Biochemistry 6*, 358.
Riordan, J. F., Sokolovsky, M., and Vallee, B. L. (1967b), *Biochemistry 6*, 3609
Riordan, J. F., and Vallee, B. L. (1964), *Biochemistry 3*, 1768.
Rupley, J. A., and Neurath, H. (1960), *J. Biol. Chem. 235*, 609.
Sigman, D. S., Torchia, D. A., and Blout, E. R. (1969), *13th Ann. Meeting Biophys. Soc., Los Angeles, Calif.*, A-22.
Simpson, R. T., Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 616.
Snoke, J. E., Schwert, G. W., and Neurath, H. (1948), *J. Biol. Chem. 175*, 7.
Sokolovsky, M., and Vallee, B. L. (1967), *Biochemistry 6*, 700.
Spackman, D. H. Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Spies, J. R., and Chambers, D. C. (1949), *Anal. Chem. 21*, 1249.
Tabachnick, M., and Sobotka, H. (1959), *J. Biol. Chem. 234*, 1726.
Tabachnick, M., and Sobotka, H. (1960), *J. Biol. Chem. 235*, 1051.
Thiers, R. E. (1957), *Methods Biochem. Anal. 5*, 273.
Ulmer, D. D., and Vallee, B. L. (1965), *Advan. Enzymol. 27*, 37.
Vallee, B. L., and Riordan, J. F. (1968), *Brookhaven Sym. Biol. 21*, 91.

# Pyridyl Esters of Peptides As Synthetic Substrates of Pepsin*

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ABSTRACT: The synthesis of a series of new pepsin substrates of the type A-Phe-Phe-B is described, in which B is a pyridylalkyloxy group, A is benzyloxycarbonyl (Z), Z-Gly, or Z-Gly-Gly, and the Phe-Phe linkage is the only pepsin-sensitive bond. In their cationic form, these compounds are moderately soluble in aqueous solution at pH 2–4, and some of the compounds (*e.g.*, with A = Z-Gly-Gly) are among the most sensitive synthetic substrates hitherto found for pepsin. The determination of the kinetic parameters for their enzymic hydrolysis has shown that changes in the structure of the A and B groups of A-Phe-Phe-B may have very large effects on the value of $k_{cat}$ (*ca.* 500-fold change) with only small accompanying changes in the value of $K_M$. These kinetic data emphasize the importance, for the kinetic specificity of pepsin, of interactions between the A or B groups of the substrate with enzymic loci that are relatively distant from the site of catalytic action, and are consistent with the possibility that such secondary interactions may alter the conformation of catalytically important groups in the enzyme so as to alter greatly the efficiency of catalysis.

Recent work in this laboratory has been directed toward the delineation of the specificity of pepsin action on the X–Y bond of synthetic peptide substrates of the general type AX–YB. The study of the effect of varying the nature of the amino acid residue X or Y (the other is always L-phenylalanyl) has shown that when A = Z-His[1] and B = OMe or OEt, pepsin exhibits preference for the cleavage of X–Y units in which X = Phe and Y = Trp, Phe, or Tyr (Inouye and Fruton, 1967; Hollands *et al.*, 1969; Trout and Fruton, 1969). In the present study, the effect of altering the nature of the groups A and B in substrates of the type A-Phe-Phe-B has been examined; in these substrates, the Phe–Phe bond is the only one cleaved by the enzyme, and the initial rate of cleavage was determined by an automatic ninhydrin method. As in earlier studies, cationic substrates were employed, since they confer moderate solubility in the pH range 1–5 on peptide derivatives blocked at both the amino- and carboxyl-terminal residues. The present series of substrates contains a cationic group in the B portion of A-Phe-Phe-B, in the form of a pyridiniumalkyloxy group, and the substrates are therefore esters of pyridine alcohols. The 4-picolyl esters of peptides have recently been used with marked success in a repetitive method of peptide synthesis, introduced by Young and his colleagues (Garner *et al.*, 1968; Garner and Young, 1969). These investigators prepared the esters by the reaction of a benzyloxycarbonylamino acid with 4-picolyl chloride in the presence of an organic base, with dimethyl-

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 2485 (1966), are: $TyrI_2$, 3,5-diiodo-L-tyrosyl; $Phe(NO_2)$, *p*-nitro-L-phenylalanyl; OM3P, 3-pyridylmethoxy; OM4P, 4-pyridylmethoxy; OP3P, 3-(3-pyridyl)propyl-1-oxy; OP4P, 3-(4-pyridyl)propyl-1-oxy; In, isonicotinoyl; Osu, *N*-oxysuccinimido. Unless otherwise noted, the abbreviated designation of amino acid residues denotes the L form.