that ribofuranosylglyoxylic acid was the key C-7 α-keto acid used in the chemical synthesis of showdomycin (Kalvoda *et al.*, 1970).

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Chemical Modification of Carboxypeptidase A Crystals. Azo Coupling with Tyrosine-248†

J. T. Johansen,‡ D. M. Livingston,§ and B. L. Vallee*

ABSTRACT: Coupling of bovine carboxypeptidase A crystals with diazotized *p*-arsanilic acid modifies one tyrosyl residue. Cyanogen bromide cleavage of the enzyme and separation of the resultant fragments demonstrates virtually complete incorporation of the arsanilazo label into segment F_I containing residues 104–301. This fragment was solubilized by succinylation, digested with chymotrypsin, and the arsanilazotyrosyl-containing peptide was isolated by affinity chromatography using an antibody–Sepharose conjugate specific for the arsanilazotyrosyl moiety. The arsanilazotyrosyl peptide was purified by subsequent ion-exchange chromatography and recovered in 90% overall yield. Its amino acid composition, N-terminal threonine, and tryptophan content are uniquely compatible with the sequence of carboxypeptidase A containing residues 246–257 (Thr-Ile-Tyr-Gln-Ala-Ser-Gly-Gly-Ser-Ile-Asp-Trp). Tyrosine-248 is the residue labeled specifically by diazotized *p*-arsanilic acid in carboxypeptidase A crystals.

Diazonium salts have proven to be particularly useful reagents to introduce environmentally sensitive chromophores into proteins for the investigation of structure–function relationships (Fairclough and Vallee, 1970, 1971; Vallee *et al.*, 1971). In this regard diazotized *p*-arsanilic acid has been especially advantageous in studies of carboxypeptidase A. When crystals of the enzyme are treated with this reagent, one tyrosyl residue is labeled specifically (Johansen and Vallee, 1971a,b; J. T. Johansen and B. L. Vallee, in preparation). The resulting azotyrosyl chromophore has served to monitor changes in conformation as the enzyme changes from the solution to the crystalline state and *vice versa* (Johansen and Vallee, 1971b; J. T. Johansen and B. L. Vallee, in preparation). It has also been a valuable probe in assessing local conformational changes coincident with alterations in enzyme activity by limited proteolysis (Riordan and Livingston, 1971; D. M. Livingston and J. F. Riordan, in preparation).

Localization of the structural changes to a particular region of the protein required quantitative isolation and identification of the tyrosyl residue modified. Since conventional methods of peptide isolation usually involve many steps with consequent loss of material, we have employed affinity chromatography (Cuatrecasas *et al.*, 1968; Cuatrecasas and Anfinsen, 1971; Givol *et al.*, 1970) with specific antiarsanilazotyrosyl antibodies. By this procedure an arsanilazotyrosyl peptide is isolated in more than 90% yield. Its sequence demonstrates the fact that tyrosine-248 is the residue modified.

Materials

Carboxypeptidase A crystals prepared according to the procedure of Anson (1937) (Worthington Biochemical Corp.) were labeled with diazotized *p*-arsanilic acid and characterized

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as described previously (Johansen and Vallee, 1971b). α-Chymotrypsin, ovalbumin, and bovine γ-globulin were obtained from Worthington Biochemical Corp., *p*-arsanilic acid from Eastman Organic Chemicals, dansyl chloride from Sigma Chemical Co., and Sepharose 4-B and Sephadex G-75 from Pharmacia Fine Chemicals, Inc. Micropolyamide sheets were obtained from Schleicher & Schuell. All other chemicals were of reagent grade and were used without further purification.

Methods and Results

Analytical Procedures. Amino acid analyses were carried out with a Spinco Model 120C amino acid analyzer according to the procedures of Spackman *et al.* (1958). Samples were hydrolyzed with 6 N HCl in sealed evacuated tubes at 110° for 24 hr. The values reported are not corrected for losses during hydrolysis. Concentrations of arsanilazocarboxypeptidase were determined by absorbance at 278 nm using ϵ_{278} 7.32×10^4 M^{-1} cm^{-1} (J. T. Johansen and B. L. Vallee, in preparation). The concentration of azopeptides was determined at neutral pH from the absorption at 325 nm using ϵ_{325} 2.22×10^4 $mole^{-1}$ cm^{-1} (Tabachnik and Sobotka, 1959). A Zeiss M4-QIII spectrophotometer was used for absorption measurements. N-Terminal analysis was performed using the dansyl chloride method (Gray, 1967). Dansylamino acids were identified by thin-layer chromatography on polyamide sheets (Woods and Wang, 1967).

Antigens and Antibodies

Arsanilazoovalbumin and arsanilazobovine γ-globulin were prepared by coupling ovalbumin (7 mg/ml) and bovine γ-globulin (15 mg/ml) in 0.1 M $KHCO_3$ (pH 8.8), with a 50- to 100-fold molar excess of a freshly prepared solution of diazotized *p*-arsanilic acid (0.02 M) for 2 hr at 0°. The reaction was stopped with an 8-fold molar excess of aqueous phenol relative to the amount of diazotized arsanilic acid added. The modified proteins were then dialyzed against six changes of 150 volumes of 0.05 M Tris-Cl (pH 7.6), for 5 days and stored at −20° until used.

Antisera were generated by injecting two white, male New Zealand rabbits, both in the footpads and intramuscularly with 3.6 mg of arsanilazoovalbumin, emulsified 1:1 (v/v) with complete Freund's adjuvant. Further immunizations were performed with the same antigen similarly diluted and emulsified. After 4, 6, and 12 weeks the animals were bled and then given additional doses of 1, 22, and 7 mg of arsanilazoovalbumin, respectively. The sera from all bleedings were pooled and antiarsanilazo antibody activity was identified with arsanilazobovine γ-globulin by immunodiffusion. Antibodies were isolated by three successive fractionations with 50% $(NH_4)_2SO_4$ at room temperature. The precipitate was dissolved in 0.01 M phosphate–0.14 M NaCl (pH 7.4) and dialyzed against the same buffer overnight. The specific antiarsanilazo antibodies were isolated by adsorption on a column of an arsanilazobovine γ-globulin Sepharose conjugate. The column was washed with 0.01 M phosphate–0.14 M NaCl (pH 7.4) until the absorbance of the effluent at 280 nm was less than 0.05. The adsorbed antibodies were eluted from the column with 1 M ammonia and dialyzed immediately against several changes of phosphate buffer over a 24-hr period. A small amount of precipitate, formed during dialysis, was removed by centrifugation, and the antibodies were stored at −20°.

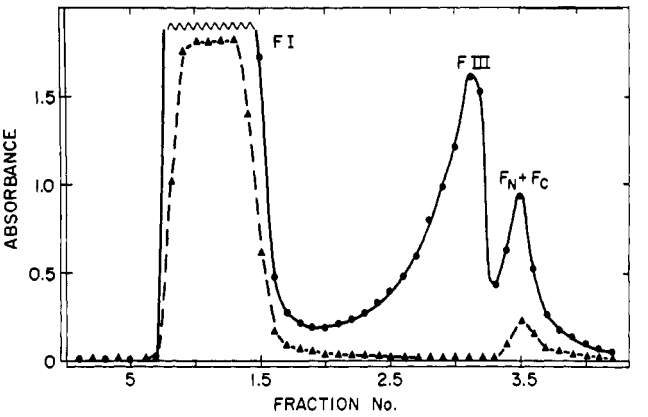

FIGURE 1: Separation of the cyanogen bromide fragments of arsanilazocarboxypeptidase. 300 mg was applied to a 2.5 × 80 cm column of Sephadex G-75, equilibrated and developed with 0.1 M propionic acid at 75 ml/hr, 23°. Fractions of 13 ml were collected and their absorbance measured at 280 nm (●) and 325 nm (▲).

Preparation of Modified Sepharose for Affinity Chromatography. Activation of Sepharose 4B with cyanogen bromide and covalent coupling of arsanilazobovine γ-globulin or antiarsanilazo antibodies to the activated Sepharose were performed as described (Cuatrecasas, 1970; Givol *et al.*, 1970). The Sepharose conjugates were equilibrated with 0.01 M phosphate–0.14 M NaCl (pH 7.4).

Cleavage of Arsanilazocarboxypeptidase A with Cyanogen Bromide and Separation of the Fragments. A typical preparation of arsanilazocarboxypeptidase contained 1.04 arsanilazotyrosyl residues per molecule as determined spectrophotometrically. Except for tyrosine, there were no other differences between the amino acid compositions of the native and the modified enzymes. In particular, the histidine and lysine contents of the two enzymes were identical. The values for tyrosine obtained by amino acid analysis were in close agreement with those obtained from spectral data. Furthermore, as measured by atomic absorption spectroscopy, the amount of arsenic incorporated was in good agreement with the number of tyrosyl residues modified, calculated from spectra and/or amino acid analysis.

The enzyme was cleaved by treatment with cyanogen bromide in 70% formic acid and the resulting fragments, F_I, F_{III}, and a mixture of F_N and F_C (Nomoto *et al.*, 1969) were separated on a 2.5 × 80 cm column of Sephadex G-75 equilibrated and developed with 0.1 M propionic acid (Figure 1). The fractions containing the arsanilazotyrosyl moiety, identified by absorption at 325 nm, were pooled and lyophilized. More than 95% of the azotyrosyl group was found to be associated with the F_I fragment (residues 104–301) as judged from the absorbance at 325 nm, and less than 5% in the $F_N + F_C$ pool (residues 8–22[1] and residues 302–307, respectively). Fragment F_{III} (residues 23–103) did not contain any modified tyrosyl residues. The amino acid composition of the F_I fragment was in excellent agreement with that of the F_I fragment of native carboxypeptidase A (Nomoto *et al.*, 1969) and that of the known sequence of carboxypeptidase A (Bradshaw *et al.*, 1969, 1971), except that 1.0 mole of tyrosine was replaced by 1.0 mole of arsanilazotyrosine.

[1] Residues 1–7 are present in carboxypeptidase prepared according to Cox *et al.* (1964) but are largely absent from the present preparation (Petra and Neurath, 1969).

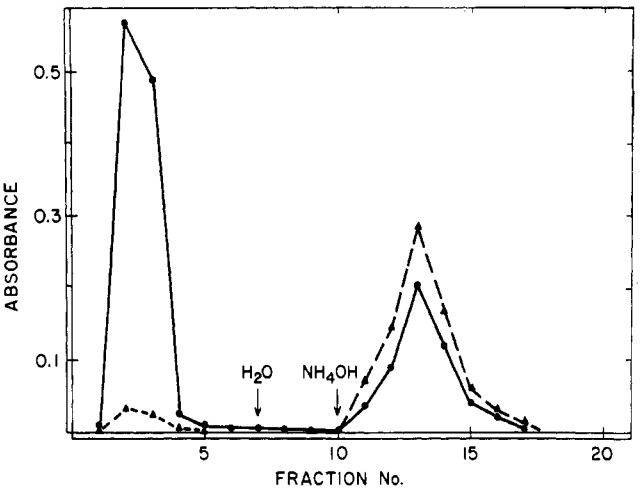

FIGURE 2: Isolation of the arsanilazotyrosyl peptides from arsanilazocarboxypeptidase. A sample, containing 0.06 μmole of azotyrosyl, from a chymotryptic digest of the succinyl-F_1 fragment, was applied to an antiarsanilazotyrosine antibody Sepharose column (1 × 4.5 cm), washed with 0.01 M phosphate–0.14 M NaCl (pH 7.4) and water. The arsanilazotyrosyl peptide was eluted with 1 M NH_4OH. Fractions of 3 ml were collected and their absorbance was determined at 280 nm (●) and 325 nm (▲).

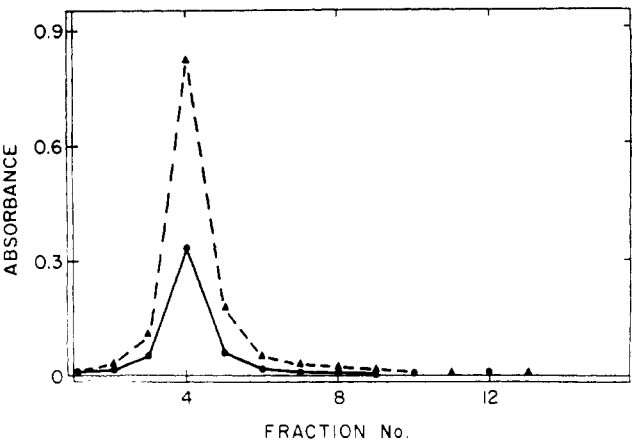

FIGURE 3: Chromatography of the arsanilazotyrosyl peptide (0.25 μmole) on a 0.9 × 20 cm column of PA-35 resin at 50°. The column was developed at a flow rate of 60 ml/hr with 0.2 M pyridine–acetate (pH 3.1). Fractions of 3 ml were collected and their absorbance measured at 280 nm (●) and 325 nm (▲).

Succinylation of F_I. F_I was solubilized by succinylation with a 2000-fold molar excess of crystalline succinic anhydride added in small portions to a suspension of F_I (5 mg/ml) in water; the pH was kept constant at 8.0 by addition of 1 N NaOH. Succinyl-F_I was precipitated at pH 2 by addition of 1 N HCl, collected by centrifugation, and then washed with water. It was treated with 2 M NH_2OH at pH 12 for 2 hr at room temperature to allow any modified seryl, threonyl, or tyrosyl residues to desuccinylate (Johansen, 1969). It was again isolated by precipitation with HCl and washed three times with water. The resulting *N*-succinyl-F_I was soluble in water at neutral pH.

Chymotrypsin Digestion of N-Succinyl-F_I and Peptide Isolation. *N*-Succinyl-F_I (10 mg/ml) was digested with chymotrypsin (2% by weight) in 0.1 M NH_4HCO_3 at 37°. An additional 1% of chymotrypsin was added after 6 hr and digestion was terminated after 24 hr by adjusting the pH to 2.5, followed by heating on a boiling water bath for 1 hr. A small amount of precipitate, which did not contain any azotyrosine, was removed by centrifugation.

The pH of the digest was adjusted to 7.4 and an aliquot containing 0.06 μmole of arsanilazotyrosine was applied to a 1 × 3 cm antiarsanilazotyrosine Sepharose column having a capacity of 0.08 μmole of arsanilazotyrosine. The column was washed with 0.01 M phosphate–0.14 M NaCl (pH 7.4) until the absorbance of the effluent at 280 nm had returned nearly to zero and then with 4–5 ml of water to avoid salt in the final peptide fraction (Figure 2). More than 90% of the yellow azotyrosine-containing peptide was adsorbed to the column while most of the material absorbing at 280 nm emerged unretarded. The adsorbed azopeptide was eluted with 1 M ammonia. The movement of an orange boundary served as a visible check on elution, since the yellow color of the azophenol changes to orange at alkaline pH. The column was washed immediately with phosphate buffer and was then ready to be used again. Four additional aliquots of the digest were separated similarly, and the fractions containing the azotyrosyl peptide were pooled and lyophilized.

The total yield was 94% of the amount applied to the column, as estimated from the absorbance at 325 nm. The amino acid composition of the azotyrosyl peptide was compatible with the sequence of carboxypeptidase A containing residues 246–256 (Bradshaw *et al.*, 1969). However, additional amino acids, not encountered in this sequence, were found in yields of 5–20% of the total present indicating contaminating peptides. Hence, the azopeptide was purified further by ion-exchange chromatography on a 0.9 × 23 cm column of PA-35 resin (Beckman), equilibrated, and developed at 60 ml/hr with 0.2 M pyridine–acetate (pH 3.1) at 50° (Benson *et al.*, 1966). The yellow peptide emerged unretarded (Figure 3) and the azotyrosine-containing fractions were pooled and lyophilized. The yield was 98%. The amino acid composition of this peptide is shown in Table I. The N-terminal residue was shown to be threonine by the dansyl chloride method. These data are uniquely compatible with the sequence of residues 246–256 of carboxypeptidase A (Bradshaw *et al.*, 1969, 1971). However, residue 257 in the sequence of carboxypeptidase A is tryptophan which would be destroyed during acid hydrolysis. Since the peptide is the result of chymotryptic digestion, tryptophan was thought to be the C terminus of the peptide. Magnetic circular dichroism analysis confirmed its presence.

Magnetic Circular Dichroism of the Purified Arsanilazotyrosyl Peptide. The tryptophan content of proteins and peptides can be determined by magnetic circular dichroism (Holmquist, 1971). The measurements were performed at 40 kG on a Cary Model 61 recording spectropolarimeter, equipped for magnetic circular dichroism. Corrections for the magnetic circular dichroism of the arsanilazotyrosyl residue itself were made employing arsanilazo-*N*-acetyl-TyrNH_2 as a model. The magnetic circular dichroism difference spectrum of the purified arsanilazotyrosine-containing chymotryptic peptide *vs.* arsanilazo-*N*-acetyl-TyrNH_2 is characteristic of tryptophan (Figure 4) (Holmquist, 1971; B. Holmquist and B. L. Vallee, in preparation). Based on a molecular magnetic ellipticity $[\theta]_M$ of 0.832° $(cm)^2$ $(dmole)^{-1}$ G^{-1} at 292 nm/tryptophanyl residue (B. Holmquist and B. L. Vallee, in preparation), the tryptophan content of the peptide was calculated to be 0.7 mole of Trp/mole of peptide.

These data show that the arsanilazotyrosyl peptide iso-

TABLE I: Amino Acid Composition of the Arsanilazotyrosine Peptide from a Chymotryptic Digest of the Succinyl-F_I Fragment.

Amino Acid	Arsanilazo Peptide	Theoretical[a,b]
Lysine		
Histidine		
Arginine		
Aspartic acid	0.9	1
Threonine	0.7	1
Serine	2.0	2
Glutamic acid	1.3	1
Proline		
Glycine	2.0	2
Alanine	1.1	1
Valine		
Isoleucine	2.0	2
Leucine	0.1	
Tyrosine	0.1	1
Arsanilazotyrosine	1.0	
Phenylalanine		
Tryptophan[c]	0.7	1

[a] Bradshaw *et al.* (1969, 1971). [b] Determined spectrophotometrically before acid hydrolysis. [c] Determined by magnetic circular dichroism.

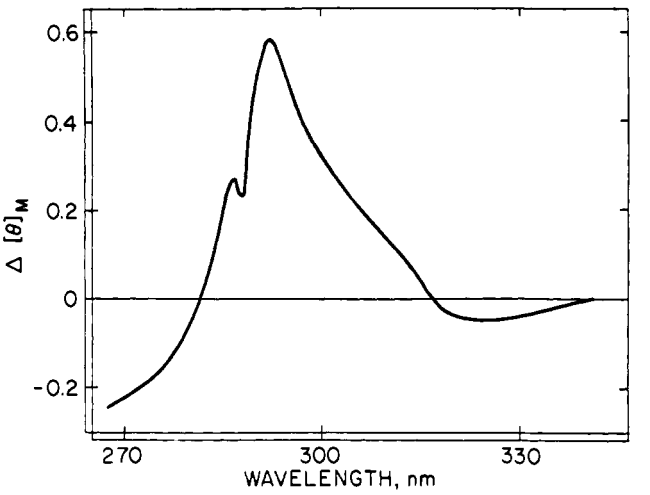

FIGURE 4: Magnetic circular dichroism difference spectrum of the purified arsanilazotyrosyl peptide *vs.* arsanilazo-*N*-acetyl-TyrNH$_2$. Both samples were dissolved in water (pH 7.5.) The spectra were recorded at 40 kG 23°.

lated corresponds to the sequence 246–257 (Thr-Ile-Tyr-Gln-Ala-Ser-Gly-Gly-Ser-Ile-Asp-Trp) of carboxypeptidase A (Bradshaw *et al.*, 1969, 1971). They demonstrate that tyrosine-248 is the arsanilazotyrosine present in this peptide. The overall yield of the peptide is high, *i.e.*, ~90%. This indicates that it is tyrosine-248 of carboxypeptidase A crystals which reacts specifically with diazotized arsanilic acid.

Discussion

The single tyrosyl residue of carboxypeptidase shown here to be modified with diazotized *p*-arsanilic acid has been thought to be a constituent of the active center of the enzyme based on iodination (Roholt and Pressman, 1967) and X-ray crystallography (Lipscomb *et al.*, 1968). Tyrosine-248 is the only tyrosyl residue found to come close to the hydrolyzable bond of the substrate and, hence, is thought to participate in the catalytic mechanism (Lipscomb *et al.*, 1968). In the crystalline state this residue is at the surface of the protein with its hydroxyl group in contact with the ambient environment. However, at least 15 of the other 18 tyrosyl hydroxyl groups are also accessible to solvent (Quiocho and Lipscomb, 1971). Only the hydroxyl group of tyrosine-238 and, to a much lesser extent, those of tyrosine-12 and -19 are not on the outside of the molecule. Substitution *ortho* to the hydroxyl group would seem to be sterically hindered for perhaps only 4 or 5 of the tyrosyl residues. Nevertheless, under the present conditions only one is modified to any significant degree. The NH of glycine-155, which is about 5 Å away, is the polar group of the enzyme closest to the hydroxyl group of tyrosine-248. It would seem unlikely that an interaction with this group would activate tyrosine-248 sufficiently to account for its unique modification by diazotized *p*-arsanilic acid. Moreover, when the crystalline enzyme is subjected to iodination, tyrosine-19, -42, -234, -238, -248, and -277 are all modified at least partially (F. A. Quiocho, 1967, unpublished results quoted in Quiocho and Lipscomb, 1971) indicating that the reactivities of these residues are similar at least toward iodine. Consequently, it would seem that other factors contribute to the specificity of the reaction of diazotized arsanilic acid with carboxypeptidase crystals.

The chemical properties of this reagent would have to be considered as one of the possible reasons. Thus, the phenylarsonic acid moiety resembles known inhibitors of carboxypeptidase and might be thought to direct the reagent toward the enzyme's active center, a possibility that deserves further study.

X-Ray studies on carboxypeptidase crystals have revealed that while the side chain of tyrosine-248 normally extends into the surrounding medium, in the presence of glycyl-L-tyrosine, there is a 120° rotation about the C_α–C_β bond moving the phenolic hydroxyl group 12 Å toward the glycyl-L-tyrosine peptide bond. These findings are basic to a proposal for the mechanism of action of carboxypeptidase (Lipscomb *et al.*, 1968). The identification of tyrosine-248 as the residue modified by diazotized arsanilic acid bears importantly on this suggestion.

In the crystalline state the modified enzyme is yellow but in solution it is bright red. The change in color is consistent with the formation of a red zinc–azophenol complex. In conjunction with the X-ray data this implies a change in conformation of the azotyrosine side chain on dissolution of the crystals (Johansen and Vallee, 1971a,b; J. T. Johansen and B. L. Vallee, in preparation). The interaction of azotyrosine-248 with zinc in the crystals and in solution, respectively, is an "all or none" phenomenon (Johansen and Vallee, 1971b). Glycyl-L-tyrosine turns the red color of the enzyme in solution to yellow. This cannot answer whether a structural change of the magnitude observed in the crystals also takes place when glycyl-L-tyrosine interacts with the enzyme in solution.

The present findings are also pertinent to properties of carboxypeptidase S, the product of limited proteolytic cleavage of the enzyme with subtilisin (Riordan and Livingston, 1971). Crystalline carboxypeptidase S has also been modified with diazotized arsanilic acid and affinity chromatography

has shown that tyrosine-248 is also modified in this instance. The general attributes of affinity chromatography have greatly facilitated the localization of the arsanilazotyrosyl residue in both these studies. The well-known antigenic specificity of phenylarsonic acid derivatives (Landsteiner, 1936) provides the basis for virtually quantitative recovery of arsanilazotyrosyl peptides from a digest of an arsanilazoprotein in a single step and in high yields. Chromatography on antibody Sepharose columns obviates the more laborious conventional procedures for peptide isolation which, because of their multistep nature, often result in low yields. This procedure was first employed by Givol *et al.* (1970) for the isolation of dinitrophenyl peptides. Later, and concurrent with this work, it was used for isolating arsanilazotyrosyl (Wilchek *et al.*, 1971) and nitrotyrosyl peptides (Helman and Givol, 1971). Arsanilazocarboxypeptidase modified in solution was among the proteins examined in the first of these studies (Wilchek *et al.*, 1971), but there was evidence for heterogeneity of tyrosine substitution. Tyrosine-19, -277, and -248 each were modified, but all these residues were substituted only partially. Moreover, it is known from other studies (Kagan and Vallee, 1969) that residues in addition to tyrosine are modified when the coupling reaction with diazotized *p*-arsanilic acid is carried out with the enzyme in solution. While differences in conditions could contribute to the altered reactivity and selectivity, the physical state of the enzyme during modification would likely seem to be a major factor.

In at least two other instances the physical state of proteins has been shown to affect their reactivities toward chemical modification. Marked differences in the reactivities of histidyl residues of both sperm-whale myoglobin and bovine pancreatic ribonuclease toward bromoacetate have been noted when modifications in the crystal and solution states were compared (Hugli and Gurd, 1970a,b; Bello and Nowoswiat, 1969). Similar comparisons of chemical modifications of other protein crystals *vs.* their solutions may well reveal analogous differences.

Acknowledgment

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