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Gas Chromatography–Mass Spectrometry for Probing the Structure and Mechanism of Action of Enzyme Active Sites. The Role of Glu-270 in Carboxypeptidase A†

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ABSTRACT: A new technique for the study of the mechanism of enzymes has been developed. An enzyme, modified by an active-site directed reagent, is digested by one or more proteases. The resulting mixture of oligopeptides is then analyzed directly by gas chromatography–mass spectrometry *without* the use of separation or isolation procedures. A comparison with unmodified enzyme identifies the modified residue as well as quantifies the reaction. This approach has been applied to the identification of Glu-270 in the active site of carboxypeptidase A using a carbodiimide as modification reagent. Studies on the possible incorporation of ^{18}O (from ^{18}O-enriched water) into Glu-270 or other acidic residues near the active site of carboxypeptidase A show that the oxygens of the carboxyl groups of these residues are not exchangeable.

Many methods have been developed for determining which amino acid residues in enzymes are involved in the catalytic process—serving either as substrate binding sites or in the bond-making and -breaking steps (Vallee and Riordan, 1969). One chemical approach involves the selective labeling of functional residues in an enzyme by active-site directed reagents. Subsequent digestion of the modified enzyme by proteases, isolation of the labeled peptide, and identification of the particular residue modified is a prerequisite to an eventual mechanistic interpretation. Such procedures, if possible, are often tedious, time consuming, and best suited to easily detectable labels, e.g., radioisotopes or chromophores.

We have developed a technique by which the digest of the labeled enzyme is transformed into a corresponding mixture of volatile peptide derivatives that can be analyzed directly by a gas chromatography–mass spectrometry (GC–MS)–computer system *without* further purification or isolation steps. Derivatization procedures are used which retain the label or transform it quantitatively and predictably. The resulting mixture of derivatives is amenable to gas chromatographic separation and the structure of the labeled oligopeptide is subsequently determined by mass spectrometry. Knowledge of the amino acid sequence of the enzyme permits the positioning of the modified residue.

This approach has been applied to the identification of Glu-270 in the active site of carboxypeptidase A using a water-soluble carbodiimide as the active-site directed reagent. Furthermore, it has been shown that because of the specificity of mass spectrometry, conditions can be used for labeling enzymes which are identical with those in biological systems, e.g., by the use of stable isotopes. We have applied this technique to the study of the possible incorporation of ^{18}O into side chain carboxyl groups by incubation in ^{18}O-enriched water. The results bear on the mechanism of action of carboxypeptidase A.[1]

Methods and Materials

Pyridine, dimethoxyethane (both "distilled in glass", Burdick and Jackson Lab.), and acetic anhydride ("Baker Analyzed" Reagent) were all redistilled before use. Lithium aluminum deuteride (Alpha-Ventron), methanol, chloro-

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[1] A preliminary account of portions of this work has been presented (Nau and Riordan, 1974).

form (both "Nanograde" from Mallinckrodt), trimethylsilyldiethylamine (Pierce Chem. Co.), and calcium acetate (Baker) were used without further purification. Ethereal diazomethane solution was obtained by distilling diazomethane generated from *N*-nitrosomethylurea (P and B Chemicals) into ether cooled by ice. Bovine carboxypeptidase A prepared according to the procedure of Anson (1937) was obtained from the Sigma Chemical Co. as an aqueous suspension of crystals. After washing three times with distilled deionized water, the crystals were dissolved in 10 ml of 3 *M* NaCl. Protein concentration was determined by diluting an aliquot into 0.05 *M* Tris–1 *M* NaCl (pH 7.5) and measuring absorbance at 278 nm with a Zeiss PMQ II spectrophotometer. The molar absorptivity on native carboxypeptidase A is $6.42 \times 10^4\ M^{-1}\ cm^{-1}$ (Simpson et al., 1963).

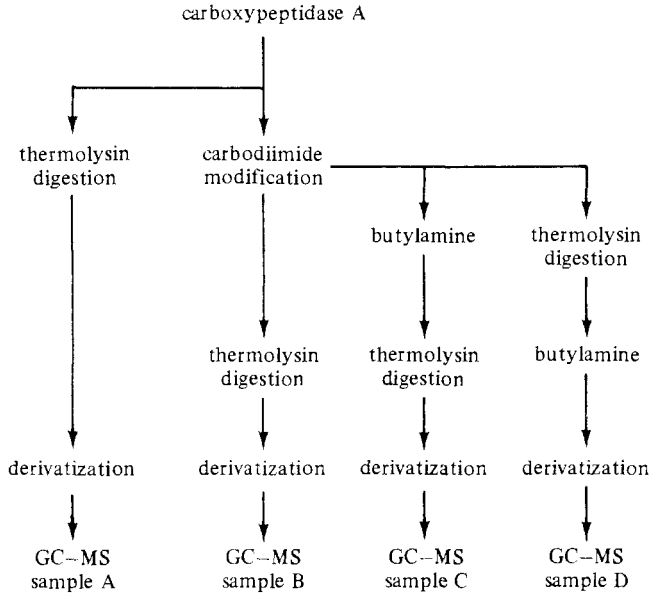
Digestion and Derivatization of Carboxypeptidase A. A sample of 0.3 μmol of carboxypeptidase A (sample A in Scheme I) suspended in water was lyophilized and the dry residue was taken up in 0.5 ml of 0.2 *M* NH_4HCO_3 buffer containing 2 m*M* $Ca(CH_3COO)_2$. Approximately 0.5 mg of thermolysin (3× krist., Serva, Heidelberg) was added and the mixture was stirred at 40° for 6 hr.[2] An additional 0.5 mg of thermolysin was added at that time and the mixture was again incubated at 40° for 12 hr.

After lyophilization, the resulting mixture of oligopeptides was dissolved in 1 ml of methanol and 100 μl of water and esterified by the rapid addition of ethereal diazomethane (within 1 min). The excess of diazomethane (yellow color) was destroyed immediately (to avoid N-methylation) by the addition of one drop of acetic acid. The reagents were removed in vacuo and the oligopeptide methyl esters were acylated at room temperature with 1 ml of 1:1 mixture of pyridine and acetic anhydride. After 45 min the solution was transferred into a 10-ml capacity glass bulb blown from 30 cm of 9-mm o.d. Pyrex tubing. The reagents were evaporated and 6 ml of 0.9 *N* $LiAlD_4$ was added. The bulb was sealed, sonicated for 2 min, and then stirred at 90° for 48 hr. The excess of $LiAlD_4$ was quenched by the dropwise addition of methanol (ice cooling) in a 50-ml flask and diluted with methanol to 25 ml. The aluminum salts were precipitated by the slow addition of 0.8 ml of water under stirring and sonication. The aluminum salts were filtered off and extracted with 15 ml of hot methanol (sonication). The combined extracts were evaporated in vacuo and then twice extracted with 15 ml of chloroform (sonication). The extracts were evaporated and the residue was treated for 1 hr at 55° with 200 μl of trimethylsilyldiethylamine in 400 μl of pyridine. The reagents were evaporated in vacuo and the oily residue was dissolved in 100 μl of benzene. An aliquot of this mixture was coinjected with three hydrocarbon standards into the gas chromatograph (vide infra).

Modification of Carboxypeptidase A by Carbodiimide. The 10 ml of stock enzyme solution, 16.8 mg/ml, 4.8 μmol, was diluted to 30 ml with 0.05 *M* 2-(*N*-morpholino)ethanesulfonate (Sigma)–1 *M* NaCl (pH 6.0) and cooled in an ice bath. A sample of 0.2 *M* 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (Aldrich) in the same buffer was added and reaction was allowed to proceed at 0–4° for an hour (Riordan and Hayashida, 1970). An aliquot was removed and assayed for esterase activity using hippuryl-L-phenyllactate as substrate (Davies et al., 1968). Under these conditions the activity of the modified enzyme was usually between 0 and 10% of that of the native enzyme. The rest of the sample was gel filtered through a 2.5 × 20 cm column of Bio-Gel P-4 (Bio-Rad) in 0.5 *M* 2-(*N*-morpholino)ethanesulfonate–1 *M* NaCl (pH 6.0). The eluate fractions containing enzyme were pooled and dialyzed exhaustively against distilled, deionized water at 4°. An aliquot of the resultant crystal suspension was dissolved in 0.05 *M* Tris–1 *M* NaCl (pH 7.5) and assayed for esterase activity. The rest was lyophilized in preparation for digestion. No restoration of activity occurred during the course of dialysis.

Digestion and Derivatization of Carboxypeptidase A Modified by Carbodiimide. A sample of 0.4 μmol of the lyophilized, modified enzyme (2.9% residual esterase activity) was digested with thermolysin and derivatized (sample B in Scheme I) using the same procedures as for the native enzyme. Another 0.4 μmol of the modified enzyme was treated with 0.5 ml of *n*-butylamine at 80° for 4 hr, lyophilized, and then digested with thermolysin and derivatized (sample C in Scheme I). Another sample (0.4 μmol) of the modified enzyme was first digested with thermolysin. After lyophilization, the mixture of oligopeptides was incubated with 0.5 ml of *n*-butylamine for 4 hr at 80° and then, following lyophilization, derivatized (sample D in Scheme I).

Scheme I



Studies on the Incorporation of ^{18}O into Carboxypeptidase A. A sample of 0.5 μmol of native enzyme was lyophilized at 0° and then suspended in 0.5 ml of ^{18}O-enriched water (Bio-Rad Labs) (43% enrichment as judged from the mass spectrum) in which 30 mg of dry NH_4HCO_3 had been dissolved. The substrate (50 μmol of Gly-Gly-Phe) was dissolved in 0.5 ml of ^{18}O-enriched water and the resulting solution was added to the enzyme suspension (enzyme/substrate = 1:100). The mixture was incubated at 25° for 2 hr, lyophilized (the ^{18}O-enriched water was recovered in a cooling trap), and then digested with thermolysin and derivatized as described above.

Gas Chromatograph–Mass Spectrometer–Computer System (GC–MS–Computer). The GC–MS–computer system consists of a Perkin-Elmer 990 gas chromatograph which is directly coupled to a low-resolution mass spectrom-

[2] Denaturation procedures (heat, 50% acetone, etc.) have been explored to increase the susceptibility of carboxypeptidase A to digestion by thermolysin. However, we have found that also native carboxypeptidase A is extensively degraded on incubation with thermolysin at 40°.

eter (Hitachi Perkin-Elmer RMU-6L) via a porous fitted glass separator (Watson and Biemann, 1965). A 3-ft or 6-ft glass column packed with 10% OV-17 on Gas Chrom Q (Applied Science Lab.) was used. The temperature was linearly programmed from 80 to 330° at 12°/min. An IBM 1800 computer was used for the continuous recording of the mass spectra (Hites and Biemann, 1968; Biller, 1972) (a mass spectrum was taken every 4.7 sec covering the mass range from *m/e* 30 to 755), for the processing of mass chromatograms (Hites and Biemann, 1970), and for the assignment of retention indices through automatic location of the coinjected hydrocarbon standards (Nau and Biemann, 1974).

Results and Discussion

General Principles. The present approach outlined in Scheme I aims at the identification of amino acid residues located in the active site of enzymes. A particular enzyme is digested by one or more proteases chosen to produce a large number of fragments which, most importantly, should also include one fragment containing the active residue. If the amino acid sequence of the enzyme under investigation is known and the modification of a single residue is to be determined (e.g., one which has been implicated to participate in the reaction mechanism by other techniques such as x-ray crystallography (Holmes and Blow, 1966)), the choice of protease is determined by the amino acid sequence around that residue. The oligopeptide produced should (1) contain the residue in question, (2) have a unique amino acid sequence which will allow its unambiguous location within the sequence of the enzyme molecule, and (3) be small enough so that it can be identified by GC–MS.

A more widely applicable technique has been developed for those cases where endopeptidases alone will not produce a unique fragment containing the active residue. An enzyme such as trypsin is used which, predictably and generally in high yield, cleaves specific peptide bonds. If these cleavages produce a primary degradation peptide which is too large to be analyzed directly by GC–MS, then this mixture is further digested by dipeptidyl aminopeptidase I (McDonald et al., 1968) which removes dipeptides sequentially from unblocked amino termini. The resulting peptide mixture will now contain the active residue as a dipeptide which can be identified by GC–MS following derivatization. This strategy has been applied to the identification of the aspartic acid residues in the active site of pepsin (H. Nau, unpublished). Since dipeptides often lack a unique sequence, it is, of course, preferable to use endopeptidases to produce a tri- or even tetrapeptide containing the active residue. This strategy will be exemplified below with carboxypeptidase A, where thermolysin is used to produce a fragment containing Glu-270, already implicated to play an important role in the catalytic mechanism (Lipscomb et al., 1968; Hass and Neurath, 1971; Petra, 1971).

The proteolytic digest will be an extremely complex mixture of oligopeptides with more than 100 fragments of very different polarity and size. Techniques which have been developed for (Förster et al., 1973) and applied to (Nau et al., 1973) the amino acid sequencing of polypeptides may also be used for the characterization of such complex hydrolysis mixtures. For example, digestion of denatured pepsin with native pepsin as well as with chymotrypsin and thermolysin has led to the detection of Ile-Val-Asp-Thr as well as Ile-Val-Asp in the resulting mixtures by GC–MS following derivatization (H. Nau, unpublished). These fragments contain the aspartic acid residue thought to participate in the active site of pepsin (Fry, 1968).

GC–MS Analysis of Carboxypeptidase A. The digestion of carboxypeptidase A, 0.3 μmol (sample A in Scheme I), was carried out with thermolysin, a protease with a very broad specificity (Matsubara et al., 1966; Ambler and Meadway, 1968; Matsubara and Feder, 1971) which should therefore produce a large number of fragments containing many of the acidic residues. Furthermore, this protease preferentially cleaves bonds involving the amino groups of Leu, Ile, Phe, and Val. The sequence around Glu-270, shown by x-ray crystallography (Lipscomb et al., 1968) and chemical studies (Hass and Neurath, 1971; Petra, 1971) to be part of the active site (—Phe—Thr—Phe—Glu 270—Leu—), also suggests that the dipeptide Phe-Glu-270 should be produced in high yield as will be Phe-Thr-268. Phe-Glu-270 is easily detected by GC–MS and has a sequence unique in the entire structure of carboxypeptidase A (Bradshaw et al., 1969).

The thermolysin digestion mixture was derivatized and analyzed by GC–MS as outlined in Methods and Materials. The total ionization–retention index plot (the computer-reconstructed gas chromatogram with the assigned retention indices (Nau and Biemann, 1974)) of this experiment is shown in Figure 1a. Numerous gas chromatographic peaks are present, many of them containing several peptide derivatives. The complete characterization of this mixture (the identification of all peptides present) would indeed be a time consuming and tedious task which was not undertaken since the sequence of the enzyme molecule is well known (Bradshaw et al., 1969).

Instead, attention was concentrated on the identification of specific residues, such as glutamic acid residues. It should be emphasized that the mass spectra of the derivatives employed exhibit abundant ions which indicate the position of a particular residue in the original oligopeptide (Förster et al., 1973; Nau et al., 1973) since the side chain masses of all amino acids that occur commonly in proteins (with the exception of Leu and Ile) are different. Thus, peptide derivatives containing an amino terminal phenylalanine always give mass spectra exhibiting a characteristic *m/e* 150 ion while those with a carboxyl terminal glutamic acid always give *m/e* 282 (Figure 2). No other N-terminal amino acid but Phe will give rise to a *m/e* 150 ion upon electron impact and no other C-terminal residue but Glu will produce a *m/e* 282 ion. If, therefore, the intensity of a particular ion is monitored throughout the gas chromatographic experiment, the maxima in the resulting plots, called "mass chromatograms" (Hites and Biemann, 1970), will indicate those gas chromatographic peaks containing a particular amino acid residue in a specific position. The mass chromatogram of ion *m/e* 150 (Figure 1b) indeed shows numerous maxima, each indicating a peptide with an amino terminal phenylalanine. These were identified conclusively by inspection of the mass spectra of the scans corresponding to the maxima of this mass chromatogram as well as of the mass chromatograms of other sequence-determining ions and retention indices (Nau et al., 1974).

Of the 16 phenylalanine residues (see Table I) present in carboxypeptidase A (Bradshaw et al., 1969), 14 are represented by the peptides labeled in the mass chromatogram shown in Figure 1b. However, not all are present in a peptide with a unique sequence. The two major fractions containing amino terminal phenylalanine are, as expected from the specificity of thermolysin and the sequence of the sub-

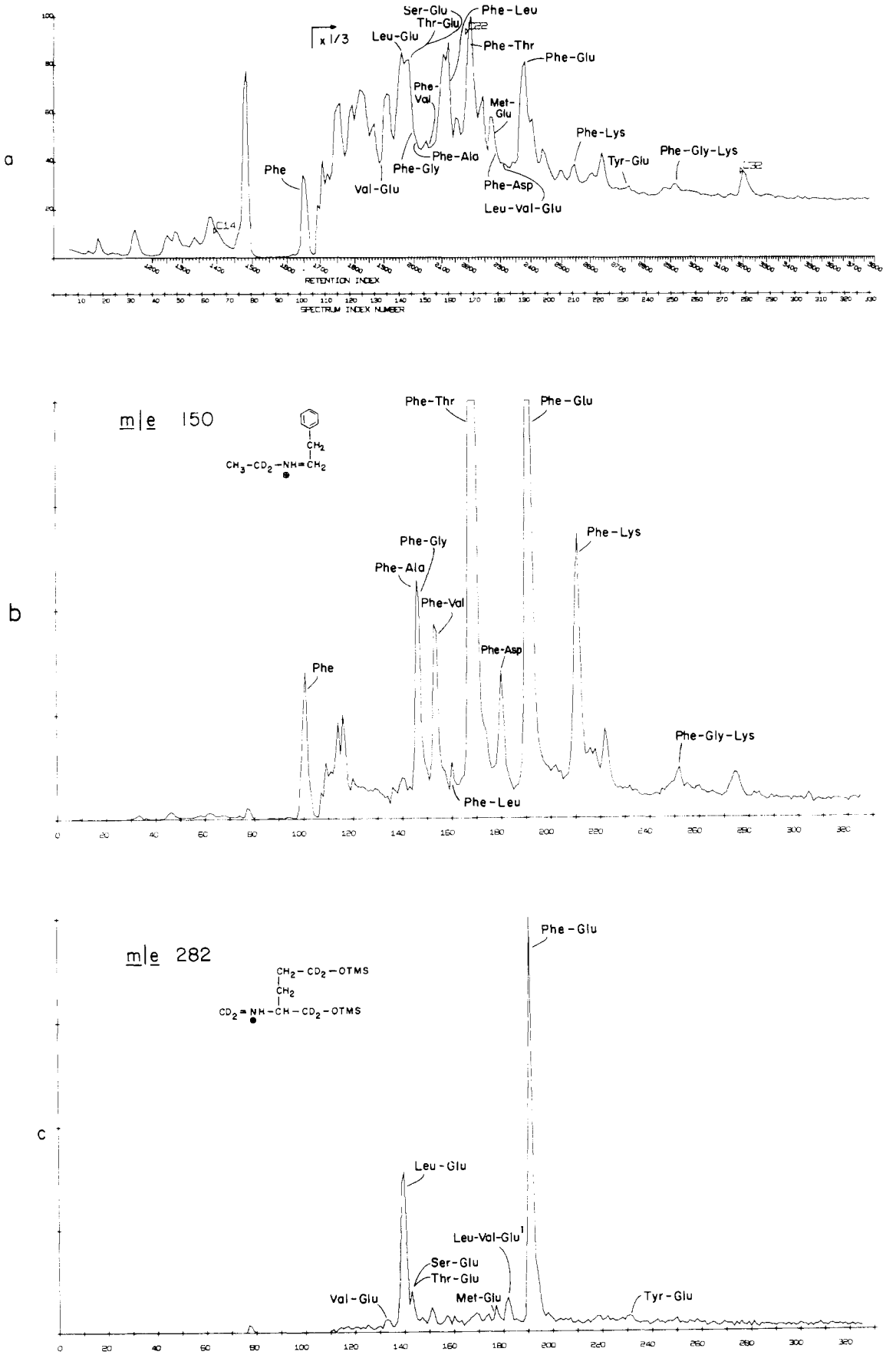


FIGURE 1: (a) Total ionization–retention index plot (C(14), C(22), C(32) . . . coinjected hydrocarbon standards) of a mixture of oligopeptide derivatives obtained by thermolysin digestion of carboxypeptidase A and subsequent derivatization (sample A). Only oligopeptides containing amino terminal phenylalanine or carboxyl terminal glutamic acid are assigned. The continuously recorded mass spectra are denoted by spectrum index numbers ("time axis" of the GC–MS experiment). (b) Mass chromatogram of ion *m/e* 150, i.e., computer-generated plot of the intensity of this ion vs. spectrum index number (time). (c) Mass chromatogram of ion *m/e* 282.

Table I: Tripeptide Segments of Carboxypeptidase A[a] Containing Amino Terminal Phenylalanine or Carboxyl Terminal Glutamic Acid.

Amino Terminal Phe	Carboxyl Terminal Glu
Phe-7-Asn-Tyr	Leu-Asp-Glu-17[b]
Phe-21-Met-Asp[b]	Val-Ala-Glu-28[b]
Phe-52-Ser-Thr[b]	Ser-Tyr-Glu-43
Phe-82-Ala-Lys	Ser-Arg-Glu-72[b]
Phe-86-Thr-Glu	Phe-Thr-Glu-88
Phe-96-Thr-Ala	Phe-Leu-Glu-108
Phe-106-Leu-Glu	His-Ser-Glu-122
Phe-116-Ala-Phe	Cys-Ser-Glu-163
Phe-118-Thr-His	Asn-Ser-Glu-173
Phe-151-Gly-Lys	Glu-Val-Glu-175
Phe-182-Val-Lys	Lys-Thr-Glu-218
Phe-189-Lys-Ala	Thr-Phe-Glu-270
Phe-192-Leu-Ser	Ala-Gln-Glu-292[b]
Phe-267-Thr-Phe	Ile-Met-Glu-302
Phe-269-Glu-Leu	
Phe-279-Leu-Leu	

[a] Based on the amino acid sequence reported by Bradshaw et al. (1969) and Petra et al. (1971). [b] These sequences were not represented in the mass spectral data. All others were present mostly as dipeptide derivatives (cf. Figure 1).

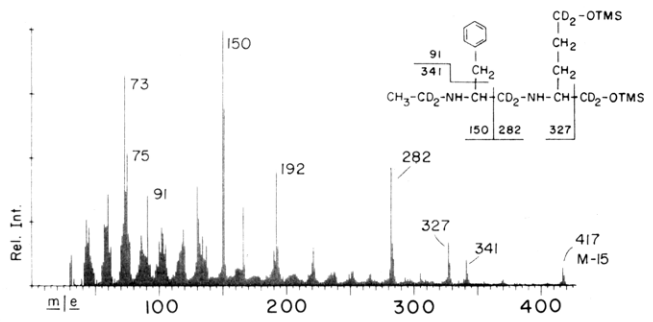


FIGURE 2: Mass spectrum of the scan having spectrum index number 193 from the GC–MS experiment of sample A (see Figure 1a) with assigned sequence ions of the derivative of Phe-Glu (structure shown in upper right corner).

strate carboxypeptidase A, the dipeptides Phe-267-Thr and Phe-269-Glu (vide supra).

Figure 1c shows the mass chromatogram of ion *m/e* 282 which indicates all peptides containing a carboxyl terminal glutamic acid residue. Ten out of the 14 glutamic acid residues (see Table I) which are present in carboxypeptidase A (Bradshaw et al., 1969) are represented. The major fraction in this mixture is, as expected, the derivative corresponding to Phe-Glu-270; all other fractions containing a peptide derivative with a carboxyl terminal glutamic acid are much less abundant.

The mass spectrum of the fraction corresponding to the Phe-Glu derivative (Figure 2) indeed shows abundant *m/e* 150 and 282 ions and also one at *m/e* 417 which is the M − 15 ion and indicates the molecular weight of this compound[3] (the structure is shown in the upper right corner of Figure 2). The retention index of this derivative (2405) is also very close to that predicted (Nau et al., 1975) for the derivative of Phe-Glu (2435).

GC–MS Analysis of Modified Carboxypeptidase A. In the next experiment (sample B in Scheme I), carboxypepti-

[3] Trimethylsilylated compounds usually exhibit weak molecular ions (M^+) but significant M − 15 ions, which are formed by the elimination of a methyl group from M^+.

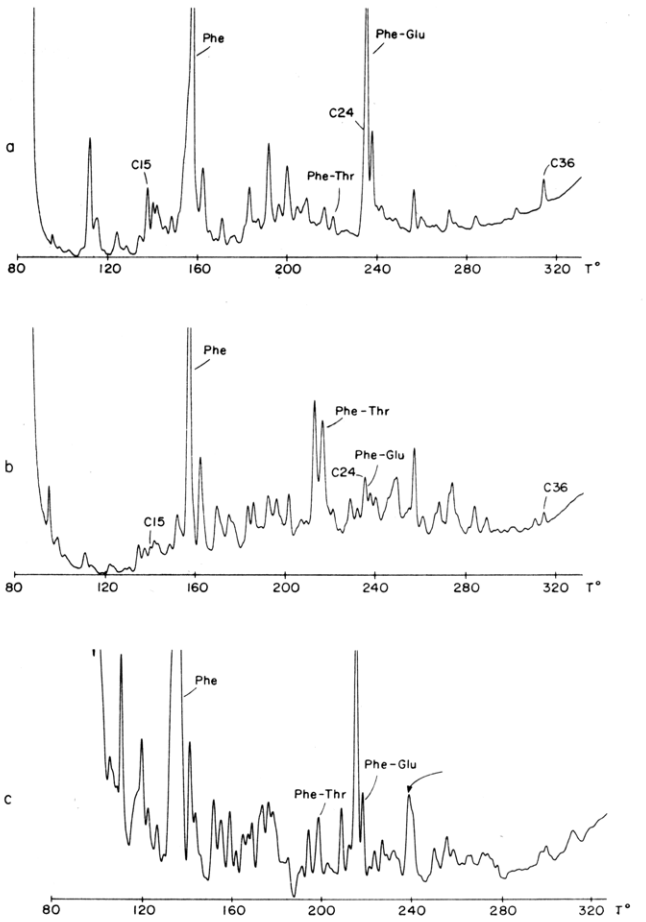
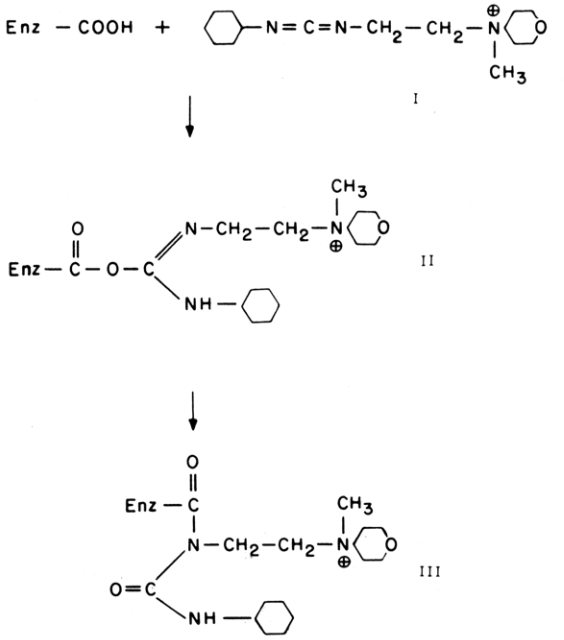


FIGURE 3: Gas chromatogram (using flame ionization detection) of the derivatized oligopeptide mixture obtained by (a) thermolysin digestion of unmodified carboxypeptidase A (sample A); (b) thermolysin digestion of carboxypeptidase A modified by the carbodiimide (sample B); (c) thermolysin digestion of a carboxypeptidase A sample which had been modified by the carbodiimide and subsequently treated with *n*-butylamine (sample C). The arrow in (c) points to the new peak. The gas chromatograms (a) and (b) were obtained on a 6-ft column (three *n*-alkanes denoted as C(15), C(24), and C(36) were also coinjected), the gas chromatogram (c) was obtained on a 3-ft column.

Scheme II



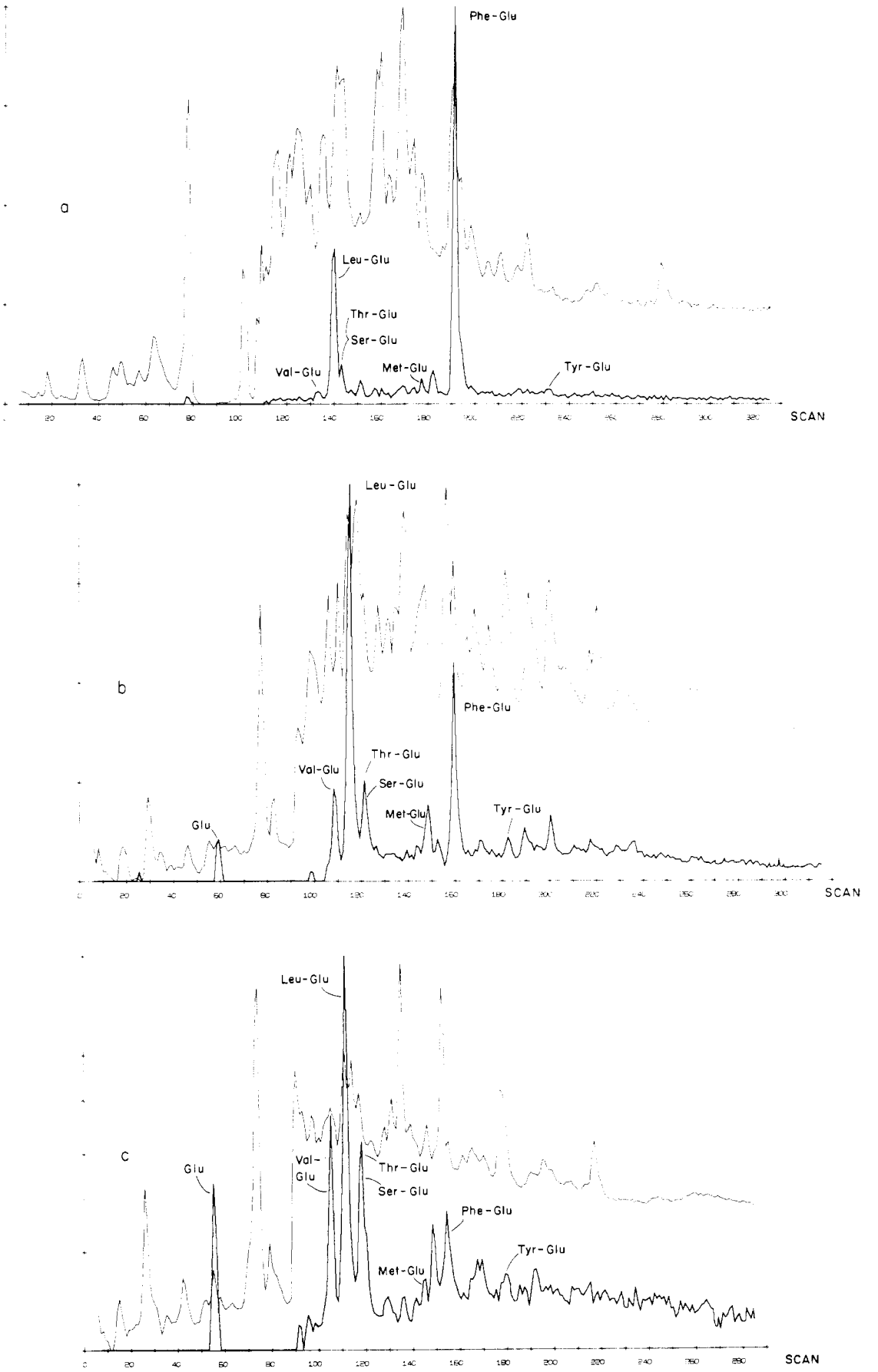


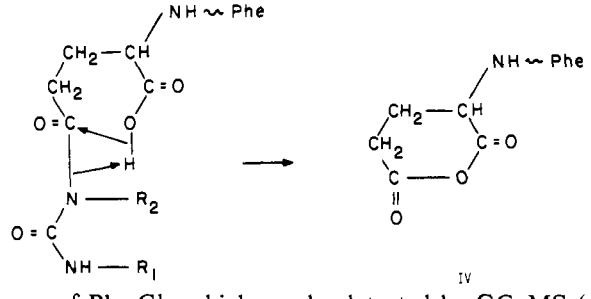
FIGURE 4: Overplots of ions *m/e* 282 (indicating carboxyl terminal glutamic acid), heavy lines, and the total ionization plots, light lines, of the GC–MS experiments performed on (a) sample A (thermolysin digest of unmodified carboxypeptidase A), (b) sample B (thermolysin digest of carboxypeptidase A modified by the carbodiimide) and (c) sample C (thermolysin digest of carboxypeptidase A modified by the carbodiimide and *n*-butylamine). A 6-ft column was used in (a) and a 3-ft column in (b) and (c).

dase A was first modified by the water-soluble 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide (I in Scheme II) using experimental conditions leading to the modification of a single residue (Riordan and Hayashida, 1970). After dialysis, the modified enzyme was digested by thermolysin and the resulting mixture derivatized and analyzed by GC–MS. The gas chromatogram of this mixture shows a variety of peaks, Figure 3b, with a pattern similar to that of the gas chromatogram obtained from sample A, Figure 3a, derived from the unlabeled enzyme. There is one apparent exception: the large peak in the gas chromatogram shown in Figure 3a and assigned to Phe-Glu (vide supra) is markedly diminished in the gas chromatogram shown in Figure 3b.

The greatly reduced recovery of Phe-Glu from sample B is also borne out by a comparison of the mass chromatograms of ion *m/e* 282 (corresponding to carboxyl terminal glutamic acid) from the two GC–MS experiments (shown in Figure 4a,b as overplots of these mass chromatograms and the corresponding total ionization plots). In sample A, Figure 4a, the peak in the mass chromatogram corresponding to Phe-Glu is the largest fraction derived from a peptide with a carboxyl terminal glutamic acid. However, in sample B, Figure 4b, this peak is considerably reduced while the peaks corresponding to all other fractions with a carboxyl terminal glutamic acid are relatively increased.

Identification of the Modified Residue. The reaction (Scheme II) of a carbodiimide (I) with a protein carboxyl group first forms an *O*-acyl isourea (II) which may then rearrange to the *N*-acyl urea (III) (Khorana, 1953). Cleavage of the Glu-270–Leu-271 bond of the carbodiimide-labeled carboxypeptidase A by thermolysin should produce a dipeptide with a free terminal carboxyl group. This carboxyl function could assist in the elimination of the substituted urea moiety through formation of an anhydride intermediate (IV, Scheme III) thereby accounting for the partial re-

Scheme III



IV

covery of Phe-Glu which can be detected by GC–MS (see Figures 3b and 4b). The mechanism shown in Scheme III is supported by the fact that, if the carbodiimide-modified enzyme is first digested by thermolysin and the resulting digest is then treated with *n*-butylamine (vide infra) (sample D in Scheme I), a significant amount of unmodified Phe-Glu can be detected.

The peptide containing the acidic amino acid residue which is modified by the carbodiimide cannot be identified directly by GC–MS since the positive charge on the carbodiimide precludes gas chromatography of the reaction product. Hence, a technique for the quantitative transformation of the reaction product into one more suitable to GC–MS was explored. Following the reaction with carbodiimide, another sample of carboxypeptidase A (sample C in Scheme I) was treated with *n*-butylamine, a strong nucleophile which can produce *n*-butylamides from either the *N*-acyl urea (III) or the *O*-acyl isourea (II) without producing side products, e.g., cleavage of peptide bonds. The resultant enzyme butylamide was then digested by thermolysin and the peptide mixture derivatized for gas chromatography. Indeed, the gas chromatogram of this sample, Figure 3c, exhibits only a very small peak corresponding to Phe-Glu relative to the peak of Phe-Thr or the other peptide derivatives present. However, one peak (labeled with an arrow in Figure 3c) is present only in sample C and not in sample A, Figure 3a, nor in sample B, Figure 3b, suggesting that this peak might correspond to the peptide derivative containing the butylamine-modified amino acid residue.

A GC–MS analysis of this sample gave the total ionization–retention index plot shown in Figure 5a. The overplot of the mass chromatogram of ion *m/e* 282 (carboxyl terminal glutamic acid) and the total ionization plot shows, Figure 4c, that the derivative of Phe-Glu has almost entirely disappeared. The mass spectrum, Figure 6, and the mass chromatogram of ion *m/e* 150, Figure 5b, indicate that the new fraction contains a derivative with an amino terminal phenylalanine. The mass chromatograms of ions derived from carboxyl terminal residues were then checked, but none was found to maximize at that particular scan. Instead, ion *m/e* 265 is abundant (see the mass spectrum in Figure 6) and also characteristic for this peak (see the mass chromatogram in Figure 5c). The other maxima in the mass chromatogram of ion *m/e* 265 correspond to derivatives of unmodified peptides, e.g., that at scan 90 corresponds to Val-Asp.[4] The new peak at scan 178 was identified conclusively as the derivative of the γ-*n*-butylamide of Phe-Glu (see structure shown in Figure 6) by other sequence-determining ions and the M − 15 ion (Figure 5a) which very specifically indicate that compound. No other derivative from a peptide containing either glutamic acid-γ-*n*-butylamide or aspartic acid-β-*n*-butylamide is detectable, in agreement with earlier findings that a single residue in carboxypeptidase A is modified by this carbodiimide (Riordan and Hayashida, 1970). Thus, Glu-270 is identified as the residue modified.

Quantitation of the Degree of Modification. A quantitation of the amount of Phe-Glu present in samples A, B, and C cannot be accomplished accurately simply by measuring the areas of the corresponding gas chromatographic peaks (Figure 3a–c), since each of these fractions may contain additional components. The derivative of Phe-Glu emerges in the tail of a small peak (see Figure 3b or 3c). This coelution with other peptide derivatives makes the measurement of the peak area corresponding to Phe-Glu meaningless.

On the other hand, as already seen above, mass chromatograms very specifically indicate components of complex mixtures and those of the sequence ions of Phe-Glu can be used for quantitation. The relative amount of Phe-Glu present in sample A (from the unmodified enzyme) as compared to the amounts of other derivatives containing a carboxyl terminal glutamic acid (e.g., Leu-Glu) is determined from the corresponding peaks in the mass chromatogram of ion *m/e* 282 in Figure 4a. The relative amount of Phe-Glu present in sample C (enzyme modified by carbodiimide and treated with *n*-butylamine) as compared to the amount of Leu-Glu present is then measured using the appropriate peaks in the mass chromatogram of ion *m/e* 282 in Figure 4c. By this means, 96% of Phe-Glu is found to be modified

[4] A particular sequence ion may sometimes correspond to more than one possible sequence, but a distinction between the possible structures can easily be made with the mass chromatograms of other sequence ions as well as retention indices.

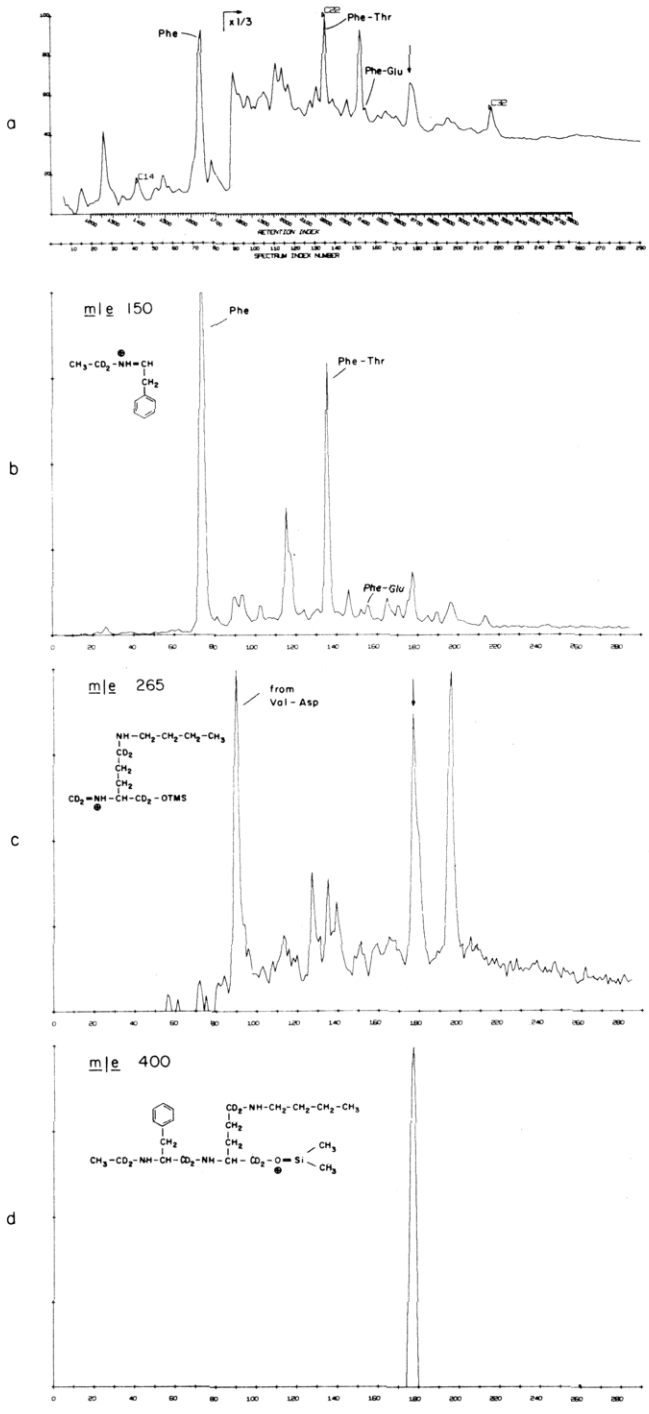


FIGURE 5: (a) Total ionization–retention index plot (C(14), C(22), C(32) . . . coinjected standard hydrocarbons) of a derivatized mixture of oligopeptide derivatives obtained by thermolysin digestion of carboxypeptidase A which had been modified by carbodiimide and *n*-butylamine (sample C); the arrow points to the new peak (see gas chromatogram shown in Figure 3c). Mass chromatograms of ions (b) *m/e* 150, (c) *m/e* 265, and (d) *m/e* 400.

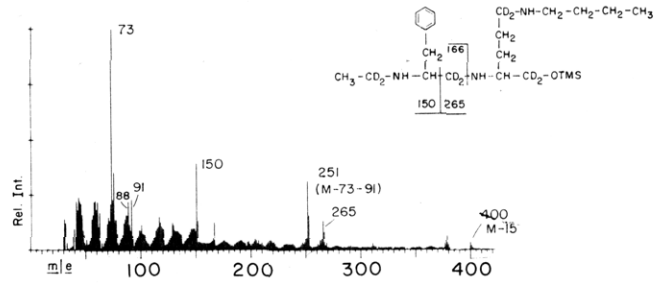


FIGURE 6: Mass spectrum of scan having spectrum index number 178 of the GC–MS experiment of sample C (Figure 5a) with assigned sequence ions of the derivative of Phe-Glu-γ-*n*-butylamide (structure shown in the upper right corner).

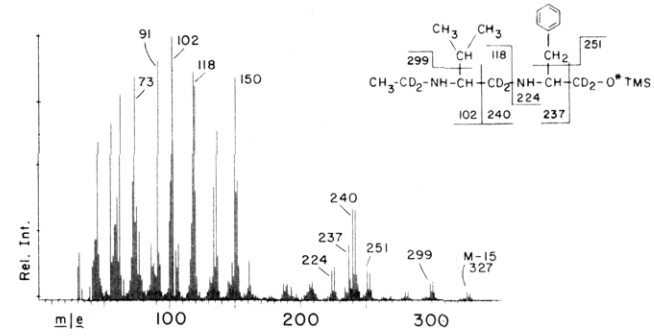


FIGURE 7: Mass spectrum of the derivative of Val-Phe obtained by ^{18}O-labeling of the dipeptide and subsequent derivatization. The position of the ^{18}O label is denoted by the asterisk.

since only 4% of the amount of Phe-Glu in sample A is present in sample C. This degree of modification agrees quite closely with the 97% loss of enzymatic activity found for this particular modified enzyme preparation.

Enzyme Labeling by Stable Isotopes. Many enzyme labels have been chosen in the past because of their easy detection—neglecting the fact that these labels might alter the structural properties of the enzyme and thus lead to distorted or perhaps erroneous conclusions. The high specificity of mass spectrometry allows the detection of stable isotopes which can be used to label enzymes under conditions which are virtually identical with those in biological systems. For example, ^{18}O-enriched water can be used to study the incorporation of an oxygen atom from the water into a specific residue of an enzyme.

It has been suggested (Lipscomb, 1972) that the γ-carboxyl group of Glu-270 may participate in the catalytic reaction mechanism of carboxypeptidase A by forming a mixed anhydride intermediate with the carboxyl group of the penultimate amino acid residue of a peptide substrate. Subsequent hydrolysis of this anhydride intermediate (if both carboxyl functions are attacked by water) would incorporate oxygen from water into the enzyme.

We have incubated the tripeptide, Gly-Gly-Leu, with carboxypeptidase A in ^{18}O-enriched water (see Materials and Methods) to explore if indeed an oxygen of water is incorporated into the active site of this enzyme. Following lyophilization, the enzyme was digested by thermolysin and the resulting mixture of oligopeptides was derivatized and analyzed by GC–MS. The mass spectrum corresponding to the fraction of the Phe-Glu derivative in this sample was identical with that shown in Figure 2 and no ^{18}O label was detected, e.g., the ions *m/e* 282 and 417 showed no appropriate isotope peaks. The cleavage product Gly-Gly-COOH (Gly-Gly-Phe-COOH $\xrightarrow{H_2{}^{18}O}$ Gly-Gly-C^{18}OOH + Phe-COOH) retained the label which was detected by GC–MS. Furthermore, a test peptide, Val-Phe, labeled with ^{18}O at the carboxyl terminus had been added to the sample. The mass spectrum of the derivative of this peptide (Figure 7) showed the expected isotope peaks. The sequence determining ions of portions of the molecule which contain the carboxyl terminus have isotope peaks displaced by two additional mass units.

Thus, it is concluded that either the anhydride mechanism is not functional in carboxypeptidase A or else the hypothetical anhydride intermediate is cleaved asymmetrically leading to the incorporation of ^{18}O only in the substrate. We have also looked for the possible incorporation of ^{18}O into a glutamic acid residue other than Glu-270 or into an aspartic acid residue. The three-dimensional model of carboxypeptidase A (Lipscomb et al., 1968) suggests that one aspartic acid residue, Asp-142, is particularly close to the bound substrate molecule and might participate in the mechanism of action. Indeed, we have found a record of this residue in the mass spectral data in the form of the unique tripeptide Val-Asp-Ala. However, the mass spectral analysis showed that the β-carboxyl group of Asp-142 was not labeled by ^{18}O.

Concluding Remarks. Numerous techniques are now available for chemically modifying specific residues in enzymes. Affinity labeling with active site directed reagents or simply judicious choice of reaction conditions with a group selective reagent can frequently result in the modification of a single unique residue. Differential labeling in the presence and absence of inhibitors or substrates can also be employed to specifically tag an amino acid residue of interest with, e.g., an isotopic derivative of the modifying reagent. If the amino acid sequence of the enzyme in question is known, it is virtually essential that the labeled residue be identified with respect to its location in the primary structure. Even if the sequence is not known, it is still very useful to localize the modified group to a specific peptide sequence in the molecule. Eventually the sequence information will become important for mechanistic interpretations. Methods for identification of such locations typically require proteolytic and/or chemical cleavage of the intact enzyme, separation and purification of the labeled peptide(s), and finally compositional and sequence analysis. Ion exchange, partition, and gel filtration chromatography remain the procedures most widely employed for peptide purification. However, immunoaffinity chromatography (Givol et al., 1970) can sometimes be helpful in the selective isolation of labeled peptides though this technique does not completely eliminate the need for additional chromatographic purification steps. Automated sequence analyzers have accelerated the acquisition of structural information but these still require pure peptides. In general, the standard approaches are time consuming and necessitate the use of labels with easily recognized characteristics such as color or radioactivity.

The new approach to the identification of modified amino acid residues in enzymes described here is based on a very efficient method for the characterization of extremely complex hydrolysis mixtures containing a large number of oligopeptides. Using a GC–MS–computer system, the components present in such mixtures can be separated by the gas chromatograph and identified by the mass spectrometer in a single experiment, thus obviating the tedious and time-consuming procedures conventionally employed. The vast amount of data generated in such an experiment is stored by the computer and can be presented in the form of mass spectra, mass chromatograms, and retention indices. These data lead, in a very straightforward way, to the identification of labeled residues. A quantitative comparison of the mass spectral data from two experiments, one performed on the native enzyme, the other on the labeled enzyme—all other parameters in the two experiments being identical—allows deduction of the extent of the modification (Figures 3 and 4).

A wide variety of labels can be used in this approach including, most importantly, stable isotopes. Incorporation of these labels are easily detected and quantified by the appropriate isotope peaks present in the mass spectra of the compounds in question (Figure 7). Therefore, various new labels can be employed which are difficult to use with classical techniques since efficient detection methods other than mass spectrometry are not available. The most important ones among these are ^{15}N and ^{18}O isotopes, since radioactive isotopes of these atoms are not accessible. Thus, for example, we have been able to examine the possible incorporation of ^{18}O from water into the various carboxyl side chains of carboxypeptidase A.

A GC–MS–computer experiment provides a record of many—ideally all—amino acid residues of an enzyme under investigation which are present in the hydrolysis digest. Thus, a single experiment provided mass spectra records of most of the glutamic acid (Figure 1b) and phenylalanine residues (Figure 1c) of carboxypeptidase A (see also Table 1). If records of the remaining few residues had been desired, another digest of the enzyme could have been performed using a different protease.

Not all oligopeptides present are generated in high yield. Nevertheless, identification of labeled amino acid residues and quantitation of the modification reaction can be made even when the pertinent peptides are present only as minor components (1% or less of the major ones) or are not separated from other components of the mixture, a frequent event (Figures 1a and 3).

About 0.3 μmol of enzyme was used in each hydrolysis experiment. However, only a small amount of the derivatized hydrolysate of such a mixture was actually used for the GC–MS–computer experiment. In principle, the amount of sample required could be reduced by a factor of 10, especially if further improvements in the gas chromatographic and mass spectrometric techniques can be made.

It should be emphasized, however, that the present technique is limited only to certain peptides. This limit is determined by both the size of the oligopeptide (molecular weight) and the polarity of the constituent amino acids (Gly and Ala are very nonpolar, Trp and His are very polar). Although all dipeptides and the majority of the tri- and tetrapeptides can be identified by the technique discussed here, a few very polar tripeptides such as Trp-Trp-Trp are not amenable to gas chromatography. On the other hand, much larger peptides composed of the nonpolar amino acids (e.g., hexaalanine) can be readily analyzed by GC–MS. In fact, recently developed new peptide derivatives (Nau, 1974), which are more volatile than those used in the work presented here, should expand the application of our technique to the detection of larger and more polar peptides.

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The Interaction of Bovine Erythrocyte Superoxide Dismutase with Hydrogen Peroxide: Inactivation of the Enzyme†

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ABSTRACT: Bovine erythrocyte superoxide dismutase was slowly and irreversibly inactivated by hydrogen peroxide. The rate of this inactivation was directly dependent upon the concentrations of both H_2O_2 and of enzyme, and its second-order rate constant at pH 10.0 and 25° was 6.7 M^{-1} sec^{-1}. Inactivation was preceded by a bleaching due to rapid reduction of Cu^{2+} on the enzyme, and following this there was a gradual reappearance of a new absorption in the visible region, which was coincident with the loss of catalytic activity. Inactivation of the enzyme was pH-dependent and indicated an essential ionization whose pK_a was approximately 10.2. Replacement of H_2O by D_2O raised this pK_a but did not diminish the catalytic activity of superoxide dismutase, measured at pH 10.0. Several compounds, including xanthine, urate, formate, and azide, protected the enzyme against inactivation by H_2O_2. Alcohols and benzoate, which scavenge hydroxyl radical, did not protect. Compounds with special affinity for singlet oxygen were similarly ineffective. The data were interpreted in terms of the reduction of the enzyme-bound Cu^{2+} to Cu^+, by H_2O_2, followed by a Fenton's type reaction of the Cu^+ with additional H_2O_2. This would generate Cu^{2+}–OH· or its ionized equivalent, Cu^{2+}–$O\cdot^-$, which could then oxidatively attack an adjacent histidine and thus inactivate the enzyme. Compounds which protected the enzyme could have done so by reacting with the bound oxidant, in competition with the adjacent histidine.

Superoxide dismutases catalyze the reaction $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$. By thus scavenging O_2^- they serve to protect respiring cells against its deleterious reactivities.

The superoxide dismutase of bovine erythrocytes is characteristic of the corresponding enzymes found in the cytosols of eukaryotes and it is the most thoroughly studied of these enzymes (Fridovich, 1974, 1975). Its molecular weight is 32000 and it is made up of two identical subunits, each of which contains one Cu^{2+} and one Zn^{2+}. X-ray diffraction analysis has shown (Richardson et al., 1975) that the Cu^{2+} and Zn^{2+} are in close proximity, as was predicted by Fee and Gaber (1972). They are, in fact, joined by a common ligand, which is the imidazole ring of histidine-61. In addi-

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