STRUCTURE OF 2-KETO-3-DEOXY-6-PHOSPHOGLUCONATE ALDOLASE: SEQUENCE OF AN ACTIVE SITE PEPTIDE OF 50 AMINO ACIDS

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ABSTRACT

STRUCTURE OF 2-KETO-3-DEOXY-6-PHOSPHOGLUCONATE ALDOLASE:

SEQUENCE OF AN ACTIVE SITE PEPTIDE OF 50 AMINO ACIDS

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David Ding-tsair Tsay

Determination of the three-dimensional structure of 2-keto-3-deoxy-6-phosphogluconate aldolase from Pseudomonas putida by X-ray crystallography requires a knowledge of the primary structure, that is, the amino acid sequence of the polypeptide chain. As a first step in total sequence determination, the primary structure of the active site region was investigated. The active site region was labeled by incubating the aldolase with ^{14}C-pyruvate and cyanoborohydride, thereby producing a covalent bond between the ε-nitrogen of lysine residue of enzyme and the α-carbon of pyruvate. As a basic strategy for fragmentation and sequencing, the ^{14}C-pyruvate-labeled, reduced and carboxymethylated enzyme was first cleaved with cyanogen bromide, and a putative set of eight peptides was distributed into fractions by ion exchange column chromatography. The active site peptide of 50 amino acids, recognized by its radioactivity, was subsequently isolated. The active site peptide was further cleaved with trypsin, and the tryptic fragments were first separated by gel filtration and then each purified by ion exchange column chromatography. This resulted in isolation of 6 unique peptides which together accounted for all the amino acid residues of the active site peptide. The amino

acid sequence of each tryptic fragment was established by either Edman degradation or carboxypeptidase cleavage. The alignment of the tryptic fragments in sequence was facilitated by the isolation of 2 tryptic fragments following modification of the arginine residues of the active site peptide with 1,2-cyclohexanedione. The complete sequence of the active site peptide of KDPG aldolase was thus elucidated to be the following: Gly-Tyr-Ala-Leu-Gly-Tyr-Arg-Arg-Phe-Lys*-Leu-Phe-Pro-Ala-Glu-Ile-Ser-Gly-Gly-Val-Ala-Ala-Ile-Lys-Ala-Phe-Gly-Gly-Gly-Pro-Phe-Asn-Ile-Arg-Phe-Cys(SCm)-Pro-Thr-Gly-Asx-Gly-Val-Ala-Pro-Asn-Val-Arg-Tyr-Asn-Met. The asterisk indicates the amino acid labeled with ^{14}C-pyruvate.

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By

David Ding-tsair Tsay

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DEDICATION

To My Parents and Parents-in-law

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

The abbreviations used in the text are: KDPG, 2-keto-3-deoxy-6-phosphogluconate; PTH, phenyl thiohydantoin; TPCK, L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone: CHD, 1,2-cyclohexanedione; and SCm, S-carboxymethyl.

The three-letter symbols for the amino acid residues are those recommended by the International Union of Biochemistry, as published in the Biochemical Journal, 102, 23 (1967). They are: Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Cys, cysteine; Glu, glutamic acid; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine; and Asx, asparagine or aspartic acid.

INTRODUCTION

In 1952, an alternative pathway of the so-called hexose monophosphate shunt was first discovered in Pseudomonas saccharophila by Entner and Doudoroff (1). They demonstrated that 6-phosphogluconic acid was cleaved to pyruvate and 3-phosphoglyceraldehyde when incubated with Pseudomonas saccharophila cell extracts. The entire scheme was postulated, and later confirmed (2-4), to involve two enzymes: 6-phosphogluconate dehydrase (EC 4.2.1.12), which converted 6-phosphogluconate to 2-keto-3-deoxy-6-phosphogluconate (KDPG); and KDPG aldolase (EC 4.2.1.14), which cleaved KDPG to pyruvate and D-glyceraldehyde-3-phosphate. Thereafter, this gluconate pathway was shown to operate exclusively among eubacteria, in which it plays a major catabolic role in the utilization of hexoses (1,5), hexonates (6-9), and hexuronates (10-13).

It has been shown that KDPG aldolase is inactivated by treatment with pyruvate in the presence of borohydride, but it is not inactivated by treatment with glyceraldehyde-3-phosphate plus borohydride, or borohydride alone (14). The reduced adduct was identified as ε-N-(2-n-hydroxypropyl)lysine (15). Therefore, this enzyme is mechanistically related to the Class I aldolases which function by means of a Schiff base mechanism (16,17). However, Roseman (18), using piperidine-2-carboxylic acid as a Schiff base model compound, argued that Schiff base formation alone is not a sufficient driving force for aldolytic cleavage. It is believed, then, that besides the azomethine-forming lysyl residue, an additional nucleophilic

residue may be required to assist in the overall catalytic process (16,19).

In this connection, several experiments were previously conducted to investigate the nature of the additional nucleophilic group. Ingram and Wood (15) demonstrated that 1,2-dinitrofluorobenzene reacted with lysyl residues, which are distinct from the azomethine-forming lysine, of KDPG aldolase with accompanying inactivation of the enzyme activity. This dinitrophenylation of lysyl residues could be prevented by KDPG, glyceraldehyde-3-phosphate, and phosphate, but not by pyruvate. They concluded, therefore, that those dinitrophenylated lysyl residues functioned in binding the phosphate moiety of KDPG. However, Barran and Wood (20) reasoned that the decrease of enzyme activity was due to conformational changes which resulted in impaired catalysis rather than impaired substrate binding, since the dinitrophenylated enzyme had nearly the same binding affinity for KDPG as the native enzyme.

Possible involvement of histidine residues in the catalytic function was also studied by photooxidation of the aldolase at high light intensities (21). Inactivation of enzyme and destruction of histidine were coincident. However, no decisive conclusion could be reached since under the experimental conditions, destruction of the histidine residues was accompanied also by destruction of both Schiff base formation and proton exchange.

Meloche (22) has shown that inactivation of enzyme by a substrate analog, bromopyruvate, was paralelled by esterification of a carboxylate group or alkylation of mercaptyl ion, depending on the conformational state of the enzyme. It was proposed that each of these bases may play a role in the aldolytic reaction, one being involved in

activating the pyruvate proton-exchange reaction and the other functioning in the cleavage or condensation reaction. Recently, Meloche et al. (23) obtained evidence from kinetic and stereochemical studies to demonstrate that the substrate analog did react within the active site region.

KDPG aldolase is extremely interesting in view of its molecular structure. Hammerstedt et al. (24) showed that the native enzyme has a molecular weight of 73,000 ±2,000, as calculated from the Svedberg equation, while the subunit has a molecular weight of 24,000 ± 2,000. These data were in good agreement with those obtained by disc gel electrophoresis and amino acid composition analysis (25). These results strongly indicate that KDPG aldolase is a trimeric enzyme, consisting of three identical or nearly identical subunits. Further substantiation of the trimeric structure was obtained from following observations. (a) The native enzyme contains 12 cysteine, but no cystine. After carboxymethylation with ^{14}C-iodoacetate and subsequent trypsin digestion, 4 radioactive peptides were isolated. Three of the radioactive peptides contained a 1:1 ratio of carboxymethylcysteine to either lysine of arginine, whereas the fourth contained carboxymethylcysteine alone. (b) The native enzyme contains 66 lysine and arginine residues. Following tryptic digestion and peptide-mapping, the number of peptides as detected with specific reagents for histidine, tyrosine, cysteine, tryptophan, and arginine, were consistent with three identical subunits. (c) Three moles of carboxy-terminal asparagine were released by digestion of S-carboxymethylated enzyme with carboxypeptidase A. (d) Three moles of ^{14}C-pyruvate per mole of enzyme were covalently

bound by borohydride reduction. (e) Hybridization using native and maleylated enzyme indicated 4 hybrid species as detected by disc gel electrophoresis (25). More recently, X-ray crystallographic data showed that the crystal of KDPG aldolase existed in the cubic system with a symmetric space group of $P2_13$ which has 12 equivalent positions (26). This suggested that the molecules of KDPG aldolase are assemblages of trimers in the crystal with the subunits related by a 3-fold rotation axis.

However, the proposal of a three-subunit molecular model for KDPG aldolase is made against an adverse background of experience and belief. This is because of the rarity of proteins containing odd-numbered subunits (27,28). Further, fructose-1,6-P_2 aldolase was once believed to be a well-documented 3-subunit enzyme (29-32), but this was later retracted in favor of a structure composed of 4 identical subunits (33-36). Nevertheless, another example of trimeric enzyme has also appeared as in the case of Δ^5-3-ketosteroid isomerase from Pseudomonas testosteroni. Boyer and Talalay (37) demonstrated that tryptic digestion of the crystalline isomerase resulted in a total of 11 fragments, and that the sum of the amino acid residues from these tryptic fragments was equivalent to one-third of the total amino acid composition of the enzyme, thus indicating 3 identical subunits. The primary sequence of these subunits has already been established (38).

Despite the fact that the current available evidence overwhelmingly supports a trimeric structure for KDPG aldolase (19), special efforts are now under way to further establish the structure. This will

involve determination of the 3-dimensional structure of KDPG aldolase by X-ray crystallography and establishment of the primary structure of its subunits by chemical and enzymatic methods. To this end, Robertson et al. (39) recently isolated a major radioactive hexadecapeptide from KDPG aldolase following incubation with ^{14}C-pyruvate and borohydride, carboxymethylation, and digestion of the derivatized enzyme with trypsin. This hexadecapeptide contained the unique azomethine-forming lysyl residue, and its sequence was determined as Phe-ε-N-(1-carboxyethyl)Lys-Leu-Phe-Pro-Ala-Glu-Ile-Ser-Gly-Gly-Val-Ala-Ala-Ile-Lys. Since this peptide accounted for more than 66% of radioactivity of the derivatized enzyme, it was concluded that only one active site-derived tryptic peptide was formed, and therefore, the sequences around all the catalytic sites of the three subunits are identical.

The present work reported here is an extension of the above sequence study using a different experimental approach. The ^{14}C-pyruvate labeled, reduced, and S-carboxymethylated KDPG aldolase was cleaved with cyanogen bromide. A major radioactive peptide containing 50 amino acid residues was isolated, and its primary structure was subsequently established using a combination of peptidase digestions and Edman degradations. The significance of the structural and functional aspects of this active site peptide will be discussed.

EXPERIMENTAL PROCEDURE

MATERIALS

Trypsin (TRL, twice crystallized, bovine pancreas), carboxypeptidase A (COADFP, bovine pancreas), and carboxypeptidase B (COBDFP, hog pancreas) were obtained from Worthington Biochemical corp., Freehold, N.J. Leucine aminopeptidase, aminopeptidase M, lactic dehydrogenase (Type III), and deoxyribonuclease were purchased from Sigma Chemical Co., St. Louis, Mo. Iodoacetic acid was also from Sigma, and was recrystallized from carbon tetrachloride:benzene (1:2, v/v) before use. Cyanogen bromide was product of Eastman Kodak, Rochester, N.Y. Pyruvate-3-^{14}C, 10.3 mCi per mole, was obtained from Amersham-Searle Corp., Des Plaines, Ill. Sephadex gels were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Dowex 50-X2 resin was obtained from Bio-Rad Lab., Richmond, Calif. L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone was product of Cal-Biochem., La Jolla, Calif. Sodium cyanoborohydride was obtained from Ventron Corp., Alfa Products, Beverly, Mass., and was purified by the procedure of Borch et al. (40). Calcium phosphate gel was prepared by the method of Colowick (41). 1,2-cyclohexanedione was obtained from Aldrich Chemical Co., Milwaukee, Wis., and was recrystallized from petroleum ether. Potassium gluconate was product of Charles Pfizer and Co., New York, N.Y. Fluorescamine was purchased from Hoffmann-La Roche, Inc., Nutley, N.J. Cheng-Chin polyamide sheets, PTH-amino acid standard kit, and 2-(4'-t-butylphenyl)-5-(4'-biphenyl)-1,3,4-oxdiazole was obtained from

Pierce Chemical Co., Rockford, Ill. The chemicals used for Edman degradation, including phenyl isothiocyanate, benzene, trifluoroacetic acid, and pyridine, were all Sequanal grade and obtained from Pierce Chemical Co. Pyridine used for column chromatography was product of Mallinckrodt Chemical Works, St. Louis, Mo., and was refluxed with ninhydrin and distilled before use. All other chemicals were reagent grade, available from commercial sources.

METHODS

Growth of Bacteria: The stock culture of Pseudomonas putida, strain A 3.12 (ATCC 12633), was maintained on a nutrient agar slant (Difco), and was transferred at monthly intervals. For mass production of cells, the bacteria were cultivated stepwise in a liquid gluconate medium (pH 7.0) containing the following ingredients: $MgSO_4 \cdot 7H_2O$, 0.1%; citric acid, 0.15%; $(NH_4)_2HPO_4$, 0.6%; K_2HPO_4, 0.6%; $FeCl_3$, 0.0005%; and potassium gluconate, 2% (24). One loopful of fresh slant culture was inoculated into 10 ml of liquid medium, and was incubated with constant shaking at 37°C for 12 hrs. One ml of above cell suspension was added to 100 ml of medium which, in turn, was used to expand cell growth in 1 liter of medium in Fernbach flasks. Finally, a total of 5 liters of culture, together with 300 ml of 20% antifoam B (Dow Chemical Co.), were added to 100 liters of medium in a 130-liter New Brunswick Fermenter. The culture was grown at 28°C under aeration at a rate of 1 liter of air per minute. The culture was harvested after reaching the stationary phase of growth (about 11 hrs.). The average yield per run was around 2 kg.

Enzyme Purification: KDPG aldolase was purified and crystallized by following the method of Hammerstedt et al. (24) with a slight modification. All steps of purification were carried out at 4°C.

1. Crude Extract: Two kg of frozen cell paste was thawed overnight at 4°C, and was then suspended in 3 liters of cold water. The cell suspension, after addition of 6 mg of DNAase, was passed through a

pre-chilled Manton-Gaulin homogenizer. Crushed ice was added to the cell suspension during the homogenization process. The homogenate was then diluted to a total of 6 liters with cold water, and was used in the next step without centrifugation.

2. Acid treatment: Three hundred ml of 4 N HCl was added dropwise, while stirring vigorously, to the crude extract over a 15-minute period. After standing for another 15 minutes, the precipitate was removed by centrifugation. The pH of the supernatant was about 1.5.

3. Ammonium sulfate fractionation: As a general procedure, an appropriate amount of mechanically ground ammonium sulfate powder was slowly added to the supernatant over a period of 30 minutes, and after stirring for another 10 minutes, the precipitate was removed by centrifugation at 9000 rpm for 30 minutes. The large portion of undesired material in the supernatant was removed by increasing the ammonium sulfate concentration, first to 0.8 M, and then to 1.2 M. The KDPG aldolase in the 1.2 M supernatant was then precipitated by raising the ammonium sulfate concentration to 1.9 M. The precipitate was dissolved in 150 ml of 0.01 M phosphate buffer, pH 6.0. Solid potassium bicarbonate was added to adjust the pH of the solution to 6.0.

4. Dialysis: The protein solution was dialyzed against 2 liters of 0.01 M phosphate buffer, pH 6.0, to remove residual ammonium sulfate. Three changes of dialysis buffer were made at 8-hour intervals. A small amount of precipitate in the dialyzed protein solution was removed by centrifugation.

5. Calcium phosphate gel absorption and elution: Gel, prepared as under Materials section, was added to the solution in a ratio of 1 mg gel per 200 units of enzyme activity. After gentle stirring for 10 minutes, the gel suspension was centrifuged, and the supernatant was discarded. In this way, about 95% of the enzyme activity was absorbed on the gel. The gel was then washed twice with 0.01 M phosphate buffer, pH 6.0. Following this, the enzyme was eluted with 0.075 M phosphate buffer, pH 6.0, with gentle stirring for 30 minutes. This elution procedure was repeated twice, and all eluates were pooled for the next step.

6. Ammonium sulfate precipitation: Powdered amminoum sulfate was added, following the general procedure described above, to the gel eluate to a concentration of 2.5 M. The pellet was then dissolved in 0.01 M potassium phosphate buffer, pH 6.0, to a protein concentration of 12 mg per ml.

7. Crystallization: The protein solution was adjusted to 0.9 M in ammonium sulfate; while maintaining moderate stirring, the cold-saturated ammonium sulfate solution was added dropwise to induce slight turbidity. The turbid solution was then kept at $4^{o}C$ for 2 to 3 days. The crystals so obtained were collected by brief centrifugation. They were then dissolved in 0.1 M potassium phosphate buffer, pH 7.0, and were stored at $4^{o}C$.

Enzyme Assay: The KDPG aldolase activity was assayed at $28^{o}C$ by measuring the decrease of absorbance at 340 nm (42). The assay mixture

(0.15 ml) contained imidazole buffer, pH 8.0, 53 mM; NADH, 0.53 mM; KDPG, 3 mM; and excess lactic dehydrogenase. One unit of activity was defined as the amount of aldolase which, under conditions of the coupled assay, catalyzed an absorbance change of 1.0 per minute in a microcuvette of 1 cm light path.

Protein Determination: Protein concentration was determined either by the method of Lowry et al. (43) using crystalline bovine serum albumin as the standard, or by the 280/260 spectrophotometric method using the following relationship: Protein (mg/ml)= $1.55 \times A_{280} - 0.76 \times A_{260}$ (44).

Amino Acid Analysis: The composition of KDPG aldolase or its derivatives was determined by using a noncommercial ultrasensitive amino acid analyzer (45). Appropriate amounts of salt-free protein solution (0.1 to 0.5 mg) were transferred to the hydrolysis vial and then lyophilized. After addition of 0.5 ml of constant-boiling HCl and 10 mg of crystalline phenol, the vial was degassed and sealed in a vaccumn below 50 μ. The hydrolysis was performed at 110°C for 24 hrs. The hydrolysate was then dried with a rotary evaporator, and was redissolved in 0.1 ml of 0.2 M citrate buffer, pH 2.0. An aliquot of hydrolysate containing the equivalent of about 25 μg of protein was analyzed in the amino acid analyzer, with norleucine as an internal standard.

Cysteine was determined as S-carboxymethyl cysteine for the iodoacetate-treated protein or as cystine for the native protein using an air oxidation procedure of Moore and Stein (46). Tryptophan was estimated by the spectrophotometric method (47). Corrections for

hydrolytic loss were generally made for threonine (5%) and serine (10%).

For detection of free amino acid residues released by chemical or enzymatic cleavage of peptides, the reaction mixture was acidified to pH 2.0, then directly applied to the amino acid analyzer without prior acid hydrolysis.

Disc Gel Electrophoresis: Polyacrylamide gel electrophoresis was performed at pH 8.3 according to the procedure of Davis (48). The gels (7.5%, in 6 x 85 mm glass tubes) were first electrophoresed in 5 mM tris-glycine buffer, pH 8.3, for 1 hour at 3 ma per tube. A 10 µl sample (100 µg) of crystalline KDPG aldolase solution was mixed with equal part of 20% glycerol, and the mixture was applied to the gel. The electrophoresis was then continued for 45 minutes at 5 ma per tube. The gel was stained with 0.05% Coomassie Brilliant Blue R250 in 12.5% trichloroacetic acid by the method of Chrambach et al. (49). The protein band was scanned at 550 nm in a Beckman DU spectrophotometer equipped with a Gilford linear transport system.

High Voltage Paper Electrophoresis: The purity of peptide fragments was examined at both pH 2.0 and 6.5 in 0.124 M pyridine-acetate buffer at 2500 volts for 1 to 2 hrs. The peptides were detected by dipping the paper strip in 0.25% ninhydrin in acetone (50). In the case of radioactive peptides, the paper strip was examined for radioactivity in a Packard paper strip scanner.

Radioactivity Measurement: Carbon-14 radioactivity was determined by a Packard Tri-Carb liquid scintillation spectrometer (Model 3000). The XDC scintillation solution of Bruno and Christian (51) was used. Wherever accurate measurement of radioactivity in a peptide solution was

necessary, corrections were made for possible interferences in counting, such as chemiluminescence and quenching.

Determination of Peptide with Ninhydrin: The location and amount of amino nitrogen in peptides were determined by a modification of the procedure of Hirs (52). Samples containing 5-50 nmoles of peptide in 1 x 10 cm polyethylene tubes were dried at 80°C in an oven. 0.15 ml of 13.5 N NaOH was added to each tube, and alkaline hydrolysis was subsequently conducted in an autoclave at 121°C for 20 minutes. After cooling, each hydrolysate was neutralized with 0.25 ml of glacial acetic acid, and each received 0.5 ml of 2% ninhydrin reagent prepared according to Hirs (52). Color development was performed in a water-bath at 95°C for 20 minutes. The reaction mixture was cooled in ice water and was diluted with 2.5 ml of 50% ethanol, followed by vigorous mixing. After 10 minutes, the absorbance at 570 nm was measured with a Gilford 300N spectrophotometer. L-Leucine was used as a standard.

Fluorometric Assay of Peptides: Peptides were also determined by the procedure described by Böhlen et al. (53). A sample containing 0.1 to 10 nmoles of peptide in a 1 x 7.5 cm glass tube was placed in a 80°C oven for 1 to 3 hrs until dried. The peptide was then redissolved in 1.5 ml of 0.05 M sodium phosphate buffer, pH 8.0. While vigorously shaking on a vortex mixer, 0.5 ml of 0.03% fluorescamine in acetonitrile was rapidly added to the peptide solution by means of a syringe. The fluorescence was measured, within an hour, in a filter fluorometer (Aminco, American Instrument Co.) using a 390 nm filter for excitation

and a 475-490 nm filter for emission.

Labeling the Enzyme with ^{14}C-Pyruvate: Crystalline KDPG aldolase (650 mg, or 9 μmoles) was incubated at 4°C with pyruvate-3-^{14}C (81 μmoles, 150 μCi) in 99 ml of 0.1 M potassium phosphate buffer, pH 6.0. After 30 minutes of incubation with constant gentle stirring, $NaBH_3CN$ (36 μmoles in 1 ml) was added to reduce the azomethine intermediate of the enzyme-substrate complex (14,15). The extent of reduction was monitored by assaying the residual enzyme activity. About 98% of starting enzyme activity was lost over a 2-hour period of incubation, and the reaction was then terminated. The labeled enzyme was precipitated with 2.5 M ammonium sulfate, and was subsequently re-dissolved in 40 ml of 0.2 M ammonium bicarbonate. It was then dialyzed thoroughly against 0.2 M ammonium bicarbonate to remove residual ^{14}C-pyruvate and phosphate, and then lyophilized.

Preparation of Reduced and S-Carboxymethylated Enzyme: The radioactive enzyme was reduced and converted to S-carboxymethyl derivative according to the procedure of Crestfield et al. (54). The reaction volume was scaled up 8-fold to 60 ml, so that it contained 28.9 g of urea, 2.4 ml of 5% EDTA, 24 ml of 1.44 M Tris buffer, pH 8.6, and 600 mg of lyophilized radioactive enzyme. The reaction mixture was stirred for 2 hrs to completely dissolve the enzyme. After flushing with nitrogen, the reduction reaction was initiated by addition of 0.8 ml of 2-mercapto-ethanol. The reaction was performed in the dark at room temperature for 4 hrs, while nitrogen was maintained over the surface of the solution. Following this, 2.2 g of iodoacetic acid in 8 ml of 1 N NaOH was added to

carboxymethylate the SH group of cysteine residues of the enzyme. After 20 minutes of stirring, the excess iodoacetic acid and other reagents were removed by dialysis in the dark against 3 liters of 0.05 M ammonium bicarbonate, pH 8.1. The dialyzed enzyme solution was then lyophilized, and the extent of carboxymethylation was examined by amino acid analysis of the acid hydrolysate of the modified enzyme. Since no cysteine (determined as cystine) could so be detected (Table 2), the carboxymethylation reaction was presumably complete.

Cyanogen Bromide Cleavage of Reduced and S-Carboxymethylated Enzyme: Cyanogen bromide cleavage was performed in 70% formic acid essentially as described by Steers et al. (55). To the reduced and S-carboxymethylated enzyme (500 mg, 7 μmoles) in 70% formic acid at a concentration of 10.2 mg per ml, a 50-fold molar excess of cyanogen bromide over methionine residues (about 147 μmoles) in the enzyme was added. The reaction was allowed to proceed in the dark at room temperature in a glass-stoppered flask. After 24 hrs the reaction solution was diluted 4-fold with glass-distilled water, and the excess reagents were removed in a rotary evaporator, followed by lyophilization. Amino acid analysis indicated that essentially all of the methionine residues had disappeared (Table 2).

Dowex-50 Column Chromatography: Since, in the present work, column chromatography with Dowex 50-X2 cationic exchange resin was utilized extensively for peptide purification, a general description of the procedure is given. The procedure was based on that of Schroeder et al. (56) with 2 major modifications: (a) resin was suspended in 4 M

pyridine-acetate buffer, pH 5.3, and was subsequently packed to the column in the same buffer; and (b) the column was developed by ascending rather than descending flow of the gradient (except for one case). These modifications were made to minimize the problem of resin shrinkage which occurred during the higher concentration gradient elution, thereby giving a smoother gradient.

The column, packed by the above method, was then equilibrated with 2 column-volumes of 0.2 M pyridine-acetate buffer, pH 3.1. Peptides were generally dissolved in an appropriate volume of 0.2 M pyridine-acetate buffer, pH 3.1, and were then applied to the bottom of the column. Development of the chromatogram usually involved 2 consecutive gradient systems. The first contained 4.5 column-volumes of 0.2 M pyridine-acetate buffer, pH 3.1, in the mixing chamber, and 9 column-volumes of 2 M pyridine-acetate buffer, pH 5.0, in the reservoir chamber. The second system contained 1.35 column-volumes of 2 M pyridine-acetate buffer, pH 5.0, in the mixing chamber, and 2.7 column-volumes of 8.5 M pyridine-acetate buffer, pH 5.6, in the reservoir chamber. At the conclusion of the second gradient, the column was, if necessary, further subjected to 0.3 column-volumes of 2 N NaOH to remove very strongly absorbed materials. The peptide elution profile was established by assaying appropriate aliquots of eluate from every other fraction by either the ninhydrin method, or the fluorometric method as described in the preceding sections. Fractions comprising a distinct peak were pooled, and the volatile solvents were removed in a rotary evaporator.

Purification of the Active Site Peptide CB-14GD: Isolation and purification of the azomethine-forming peptide fragment was greatly facilitated by the fact that it contained covalently bound ^{14}C radioactivity, and also by the preliminary observation that it was larger in size than the other cyanogen bromide peptides.

1. Descending column chromatography on Dowex 50: Cyanogen bromide peptides (450 mg, 18.75 μmoles each) were dissolved in 10 ml of 50% formic acid, and then diluted to 30 ml with 0.2 M pyridine-acetate buffer, pH 3.1. They were subsequently separated on a 4 x 84 cm Dowex 50-X2 column by descending flow of a pyridine-acetate gradient. The first gradient contained 4 liters of 0.2 M buffer, pH 3.1, and 8 liters of 2 m buffer, pH 5.0; while the second gradient consisted of 2 M buffer, pH 5.0, and 4 liters of 8.5 M buffer, pH 5.6. The flow rate was 120 ml per hour and fractions of 10 ml were collected. Those fractions constituting the major radioactive peak were pooled and designated as CB-14 (Figure 2).

2. Column chromatography on Sephadex G-50: The CB-14 fraction (93 mg) was dissolved in 5 ml of 50% pyridine, and was applied to a 1.5 x 210 cm column of Sephadex G-50 (medium particle size) previously equilibrated with 50% pyridine. The column was eluted by upward flow with 50% pyridine at a constant rate of 16 ml per hour. Fractions of 2 ml were collected, and 10-μl aliquots from every other fraction were withdrawn for peptide detection and for radioactivity measurement. Those fractions comprising the major radioactive peak were combined and

designated as CB-14G (Figure 3).

3. Ascending column chromatography on Dowex 50: Fraction CB-14G (65 mg) was dissolved in 1 ml of 70% formic acid, and subsequently diluted to 5 ml with 0.2 M pyridine-acetate buffer, pH 3.1. It was then chromatographed on a 0.9 x 100 cm Dowex 50-X2 ascending column using a gradient of pyridine-acetate. The first gradient was made of 333 ml of 0.2 M buffer, pH 3.1, and 666 ml of 2 M buffer, pH 5.0; while the second gradient used 100 ml of 2 M buffer, pH 5.0, and 200 ml of 8.5 M buffer, pH 5.6. Fractions of 2 ml were collected at a rate of 12 ml per hour. Aliquots of 10 µl taken from every other fraction were assayed for radioactivity and ninhydrin reactivity. Those fractions, designated as CB-14GD (Figure 4), were combined.

Tryptic Digestion of the CB-14GD Peptide: Trypsin was treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK) according to the procedure of Carpenter (57) to eliminate chymotryptic activity. Prior to use, the TPCK-treated trypsin was dissolved in 0.001 N HCl to a concentration of 0.5% and was kept at $4^{\circ}C$ for 2 hrs (58). Tryptic digestion of CB-14GD peptide (20 mg in 2 ml of 0.1 M NH_4HCO_3 buffer, pH 7.9) was then initiated by addition of 0.2 mg of the TPCK-treated and acid-incubated trypsin. The hydrolysis was conducted at $30^{\circ}C$, and the pH was maintained at 7.9 by addition of 1 M NH_4OH. At the conclusion of hydrolysis (about 4 hrs) the reaction mixture was immediately lyophilized. A portion of the tryptic digest was then redissolved in 0.2 M citrate buffer, pH 2.0, and was applied to the amino acid analyzer to determine any free amino acids liberated by trypsin.

Purification of Tryptic Peptides of CB-14GD:

1. Ascending column chromatography on Sephadex G-50: The tryptic digest of CB-14GD peptide was dissolved in 1 ml of 0.1 N NH_4OH, and was then applied to the bottom of a Sephadex G-50 column (1.5 x 210 cm) previously equilibrated with 0.1 N NH_4OH. Separation of peptides was carried out by upward chromatography using 0.1 N NH_4OH as eluent. Fractions of 2 ml were collected at a rate of 14 ml per hour. Aliquots of effluent were taken from every other fraction for measuring ninhydrin color value and for determining radioactivity. Those fractions composing a distinct peak (Figure 5) were pooled and subjected to lyophilization. This yielded 4 distinguishable peak fractions: TG-1, TG-2, TG-3, and TG-4.

2. Ascending column chromatography on Dowex 50: Each peak fraction from the above chromatogram was further purified by chromatography on Dowex 50-X2. Preliminary observations indicated that all of the tryptic peptides could be eluted by the first pyridine-acetate gradient system, therefore, the second pyridine-acetate system was eliminated. Each peak fraction (TG-1, TG-2, TG-3, or TG-4) was dissolved in 1 ml of 0.2 M pyridine-acetate buffer, pH 3.1, and was then applied to a Dowex 50-X2 column (0.9 x 100, or 0.6 x 54 cm). Separation of peptides was further achieved by a pyridine-acetate convex gradient, consisting of 333 ml of 0.2 M buffer, pH 3.1, and 666 ml of 2 M buffer, pH 5.0. Fractions of 2 ml were collected at a constant rate of 18 ml per hour. Peptides in the effluent were detected by means of either ninhydrin color reaction or fluorescence with fluorescamine.

By a combination of the above two types of column chromatography, five homogeneous tryptic peptide fragments derived from the parent CB-14GD peptide were obtained (Figure 6 through 8).

Digestion with Carboxypeptidase A and B: A suspension of carboxypeptidase A (10 nmoles) was washed twice with 1 ml of cold glass-distilled water, and then dissolved in 0.1 ml of 10% $LiCl_2$. Carboxypeptidase B was used without further treatment. No free amino acids were detectable when 5 nmoles of either enzyme preparation was examined in the amino acid analyzer.

Both carboxypeptidases were added to the reaction mixture in a substrate to enzyme molar ratio of 50:1 to 1000:1, as specified in each experiment. Hydrolysis was performed at 30°C in 0.2 M N-ethylmorpholine-acetate buffer, pH 8.5. Aliquots containing 10 to 20 nmoles of peptide were withdrawn from the reaction mixture at each time interval, and the reaction was immediately terminated by dilution with 0.2 M citrate buffer, pH 2.0, followed by freezing. These were stored at -20°C until final analysis for the presence of amino acids.

Edman Degradation: The peptide (300-600 nmoles) in 20% formic acid was transferred to a 1 x 7.5 cm glass test tube and lyophilized. It was redissolved in 0.5 ml of freshly prepared 70% pyridine, and degassed for 1 minute with a moderate stream of N_2 through a capillary tube immersed in the solution. 40 µl of phenyl isothiocyanate was added, and N_2 bubbling was continued for another 30 seconds. The tube was then capped with parafilm, and the coupling reaction was allowed to proceed at 45°C

for 1 hour. The excess volatile reagents and by-products were removed in a desicator over NaOH pellets under reduced pressure. Non-volatile by-products, such as diphenylthiourea, were removed by extraction 4 times with 1 ml of benzene. The residue was then taken to complete dryness under reduced pressure, redissolved in 0.6 ml of anhydrous trifluoroacetic acid, and bubbled with N_2 for 2 minutes. The cyclization reaction was performed under N_2 atmosphere at 40°C for 15 minutes. Following this, trifluoroacetic acid was removed under reduced pressure. The residue was dissolved in 0.7 ml of 0.2 M acetic acid, incubated at 60°C for 10 minutes, and then extracted 4 times with 1 ml portions of benzene to remove the phenyl thiohydantoin (PTH) amino acid. The remaining solution which contained the residual peptide was lyophilized, redissolved in 70% pyridine, and an aliquot (usually 10-20 nmoles) removed for amino acid composition analysis. The volume was restored by addition of 70% pyridine, and the sample was subjected to another degradative cycle.

In some cases, the PTH-amino acid in benzene extract was dried under reduced pressure, and its identity was examined by methods described in the next sections.

Alkaline Hydrolysis of Phenyl Thiohydantoin Amino Acid: The procedure is essentially that of Africa and Carpenter (59). 0.5 ml of 0.4 N NaOH (oxygen-free) was added to a hydrolysis vial containing 50 nmoles of PTH-amino acid sample. The vial was sealed under vacuum, and then placed in an oven at 120°C for 12 hrs. The hydrolysate was neutralized with 1 N HCl, and dried at 35°C on a rotary evaporator. The residue was

redissolved in 0.2 ml of 0.2 M citrate buffer, pH 2.0, and an aliquot taken for amino acid analysis.

Direct Identification of Phenyl Thiohydantoin Amino Acid on Polyamide Sheet: The thin-layer chromatographic method recently published by Summers et al. (60) was followed to identify PTH-amino acid at sub-nanomole level. About 0.2 nmole of unknown PTH-amino acid was dissolved in methanol, and was spotted to the left-hand corner of a 5 x 5 cm polyamide sheet (about 7 mm from the bottom and left sides). A mixture of authentic PTH-amino acids (0.2 nmoles each) was applied to the other side of the sheet in a location exactly coincident with the above spot. The sheet was then developed in the first solvent system (toluene:n-pentane:glacial acetic acid; 60:30:35, v/v) containing 0.025% of 2-(4'-t-butylphenyl)-5-(4'-biphenyl)-1,3,4-oxdiazole as a fluorescent indicator. When the solvent front reached the top edge (about 11 minutes), the sheet was removed and dried in a hot air stream. Chromatography in the second dimension was then conducted in 35% aqueous acetic acid. Again, the chromatogram was removed as soon as the solvent front reached the top edge of the sheet (about 13 minutes), and dried in hot air. The chromatogram was inspected under short-wavelength ultraviolet illumination, and the spots where fluorescence was quenched on both sides of the sheet was circled with pencil. The unknown PTH-amino acid was then identified by matching the spots with standards on the other side of the sheet.

Cleavage of TGD-2E Peptide with Hydroxylamine: TGD-2 peptide was successively cleaved by 5 Edman degradative cycles. The residual peptide,

TGD-2E (about 80 nmoles), was dissolved in 0.5 ml of 0.1 N acetic acid and passed through a sintered glass filter. The filtrate was lyophilized and redissolved in 1 ml of 2 M hydroxylamine in 0.2 M K_2CO_3, pH 9.0 (61). The cleavage reaction was performed at 45°C for 4 hrs. The reaction mixture was then chromatographed on an ascending Sephadex G-15 column (0.9 x 100 cm) previously equilibrated with 0.2 M NH_4HCO_3, pH 8.0. The column was developed with the same buffer at a constant rate of 20 ml per hour and 1-ml portions of effluent were collected. An aliquot of 0.1 ml was taken from every other fraction for peptide detection by the fluorometric method.

Modification of CB-14GD Peptide with 1,2-Cyclohexanedione: The procedure of Toi et al. (62) was adopted to modify the arginyl residues of the active site peptide. CB-14GD peptide (300 nmoles) was dissolved in 1 ml of 0.2 N NaOH, and a 0.12 ml of 0.1 M 1,2-cyclohexanedione (CHD) in 0.2 N NaOH was added. The reaction mixture was incubated at 25°C for 3 hrs, and was subsequently neutralized by addition of 0.23 ml of 1 N HCl. The reaction product was then desalted on a Sephadex G-15 column (0.9 x 100 cm) using 0.1 N NH_4OH as eluent. The flow rate was 20 ml per hour, and 2-ml portions were collected. The radioactive fractions (CHD-modified peptide) emerging at the void volume were pooled and lyophilized.

Isolation of Tryptic Fragments of Cyclohexanedione-Modified Peptide: The CHD-modified CB-14GD peptide (290 nmoles) was digested with trypsin using the same procedure outlined in the previous section. The tryptic peptide fragments were first separated from the undigested parent peptide

by chromatography on a Sephadex G-50 column (1.5 x 220 cm) using 0.1 M NH_4OH as eluent. The flow rate was 16 ml per hour, and fractions of 2-ml each was collected. The tryptic peptides, emerging as a single peak (see Results section), were concentrated by lyophilization. These were subsequently fractionated on a Dowex 50-X2 column (0.6 x 54 cm) using a modified pyridine-acetate gradient, consisting of 100 ml of 1 M buffer, pH 5.0, and 200 ml of 2 M buffer, pH 6.0. Each 1-ml portion of effluent was collected at a rate of 11.3 ml per hour. Distinct peptide peaks were taken separately to dryness on a rotary evaporator.

RESULTS

Preparation of Crystalline KDPG Aldolase: In seeking to obtain an adequate quantity of pure KDPG aldolase for the purpose of this sequence work, about 40 kg of Pseudomonas putida were grown in 20 fermenter runs. On the average, there is about 200 mg of KDPG aldolase activity in the crude extract from 1 kg of cell paste, and about 30 mg of crystalline enzyme can subsequently be obtained through the purification procedure. A typical result of enzyme purification is summarized in Table 1. The crystalline enzyme is assumed to be homogeneous on the basis of following information. (a) The specific activity of the twice crystallized enzyme (13,356 units/mg protein) is essentially the same as that reported by Meloche and Wood (42) under similar assay conditions; (b) polyacrylamide gel electrophoresis of the crystalline enzyme, up to 100 µg, indicated a single protein component (Figure 1); and (c) as shown in Table 2, the amino acid composition of the crystalline enzyme is in good agreement, within experimental error, to the data reported by Robertson et al. (25).

Purification of Active Site Peptide CB-14GD: The aldolase was treated with pyruvate and cyanoborohydride, carboxymethylated, and then cleaved with cyanogen bromide as described under Methods section. The cyanogen bromide cleavage mixture was first fractionated on a Dowex 50-X2 column as shown in Figure 2. One major peak (CB-14) as well as some minor peaks were associated with the radioactivity. The CB-14 contains about

Table 1. Purification of KDPG Aldolase from Pseudomonas putida.

The data are based on 2 kg of cell paste. Details of the purification procedure appear in the Methods section. Assays of KDPG aldolase activity are conducted at 28°C, using KDPG as primary substrate and lactic dehydrogenase as coupling enzyme. One unit of enzyme activity is defined as the amount of aldolase which catalyzes an absorbance of 1.0 per minute in a microcuvette of 1 cm light path.

Step	Volume	Total activity	Specific activity	Purification	Recovery
	ml	units ($\times 10^5$)	units/mg protein	fold	%
Crude extract	6,000	54.4	11		100
Acid treatment	4,375	44.1	62	5.6	81
Ammonium sulfate precipitate	104	26.2	1,370	124	48
Dialysis	125	23.0	1,800	164	42
Calcium phosphate gel elution	150	18.0	10,158	924	33
Crystallization					
first crystal	7	11.9	13,110	1,181	22
second crystal	6	10.5	13,356	1,214	19

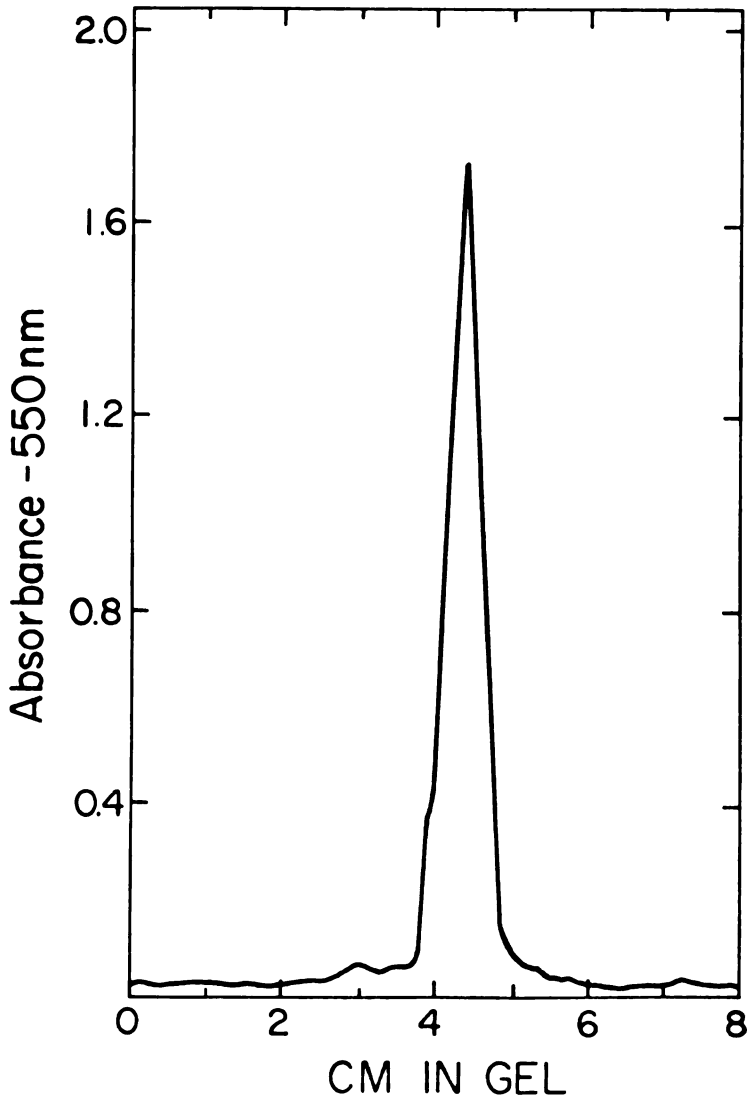


Figure 1. Disc Gel Electrophoresis of Crystalline KDPG Aldolase at pH 8.3.

Polyacrylamide gels (7.5%) were pre-electrophoresed for 1 hour in 5 mM Tris-glycine buffer, pH 8.3. Samples (100 µg) were applied in 10% glycerol, and electrophoresis was continued for 45 minutes at 5 ma per gel. Protein was stained with Coomassie Brilliant Blue, and its absorbance was subsequently scanned at 550 nm.

Table 2. Amino Acid Composition of KDPG Aldolase and Its Derivatives.

The columns indicated by Roman numerals contain the following enzyme preparations: I, carboxymethylated enzyme as reported by Robertson et al. (25); II, native enzyme; III, pyruvate-labeled, reduced, and carboxymethylated enzyme; and IV, cyanogen bromide cleavage products of pyruvate-labeled, reduced, and carboxymethylated enzyme. A 0.4 mg of KDPG aldolase or its derivatives was hydrolyzed at 110°C for 24 hrs with 0.5 ml of HCl as detailed in the Methods section. The hydrolysates (25 μg or an appropriate portion) were examined by an ultrasensitive amino acid analyzer. Cysteine was determined as SCm-cysteine (I,III, and IV) or cystine (II, expressed as cysteine). Corrections for hydrolytic losses were made for threonine (5%) and serine (10%). Methionine was absent in IV, and instead, its derivatives (homoserine and homoserine lactone) were determined and expressed together as in parenthesis. Tryptophan was estimated by the spectrophotometric method (47). The presence of ε-N-(1-carboxyethyl)-lysine was indicated in III and IV, but was not quantified. The percent yield is expressed as sum of weights of recovered amino acids, after correction for water addition in hydrolysis, divided by weight of sample before acid hydrolysis (25 μg). The data were obtained from the average of at least three analyses. Numbers represent residues based on 9 residues of tyrosine per mole of enzyme.

Table 2.

Amino acid	I	II	III	IV
Cysteine	12.32	12.67	11.62	12.32
Aspartic	50.17	50.32	50.80	48.35
Threonine	29.94	30.98	29.17	29.38
Serine	25.86	27.06	26.43	28.23
Glutamic	61.07	59.21	58.78	60.76
Proline	47.28	45.14	43.48	46.25
Glycine	61.53	62.34	58.98	60.90
Alanine	92.51	91.45	88.56	89.04
Valine	39.67	40.31	40.61	39.67
Methionine	20.56	19.80	21.99	(18.56)
Isoleucine	52.86	52.24	53.09	51.67
Leucine	60.34	59.73	60.40	59.76
Tyrosine	9.00	9.00	9.00	9.00
Phenylalanine	19.78	20.74	19.66	19.78
Tryptophan	11	11.56	11.78	11.95
Histidine	3.16	2.88	3.04	3.01
Arginine	43.94	41.95	42.89	41.78
Lysine	21.36	20.14	18.17	17.84
ε-N(1-carboxyethyl)-lysine			+	+
NH_3	10	n.d.	n.d.	n.d.
Yield, %	99.28	94.28	95.29	93.60

Figure 2. Descending Chromatography of Cyanogen Bromide Peptides on a Dowex 50-X2 Column.

The ^{14}C-labeled, reduced, and S-carboxymethylated KDPG aldolase was cleaved by CNBr, and the resultant peptide mixture (about 18.75 μmoles of each) was applied to a Dowex 50 column (4 x 84 cm) as described in the text. The column was developed with a concave gradient of pyridine-acetate buffer. Gradient system I consisted of 4 liters of 0.2 M buffer (pH 3.1) and 8 liters of 2 M buffer (pH 5.0); while gradient system II contained 2 liters of 2 M buffer (pH 5.0) and 4 liters of 8.5 buffer (pH 5.6). The point of change of the gradient system is indicated in the figure. Fractions of 10 ml were collected at a rate of 120 ml per hour. Samples (0.1 to 0.4 ml) were taken from alternate fractions for radioactivity measurement and ninhydrin color determination after alkaline hydrolysis. Appropriate peak fractions were pooled and designated as follows. CB-1, fraction #35-70; CB-2, #80-100; CB-3, #117-135; CB-4, #144-156; CB-5, #157-178; CB-6, #179-202; CB-7, #203-245; CB-8, #246-280; CB-9, #281-350; CB-10, #351-427; CB-11, #428-512; CB-12, #640-730; CB-13, #1100-1145; CB-14, #1146-1304; CB-15, #1305-1330.

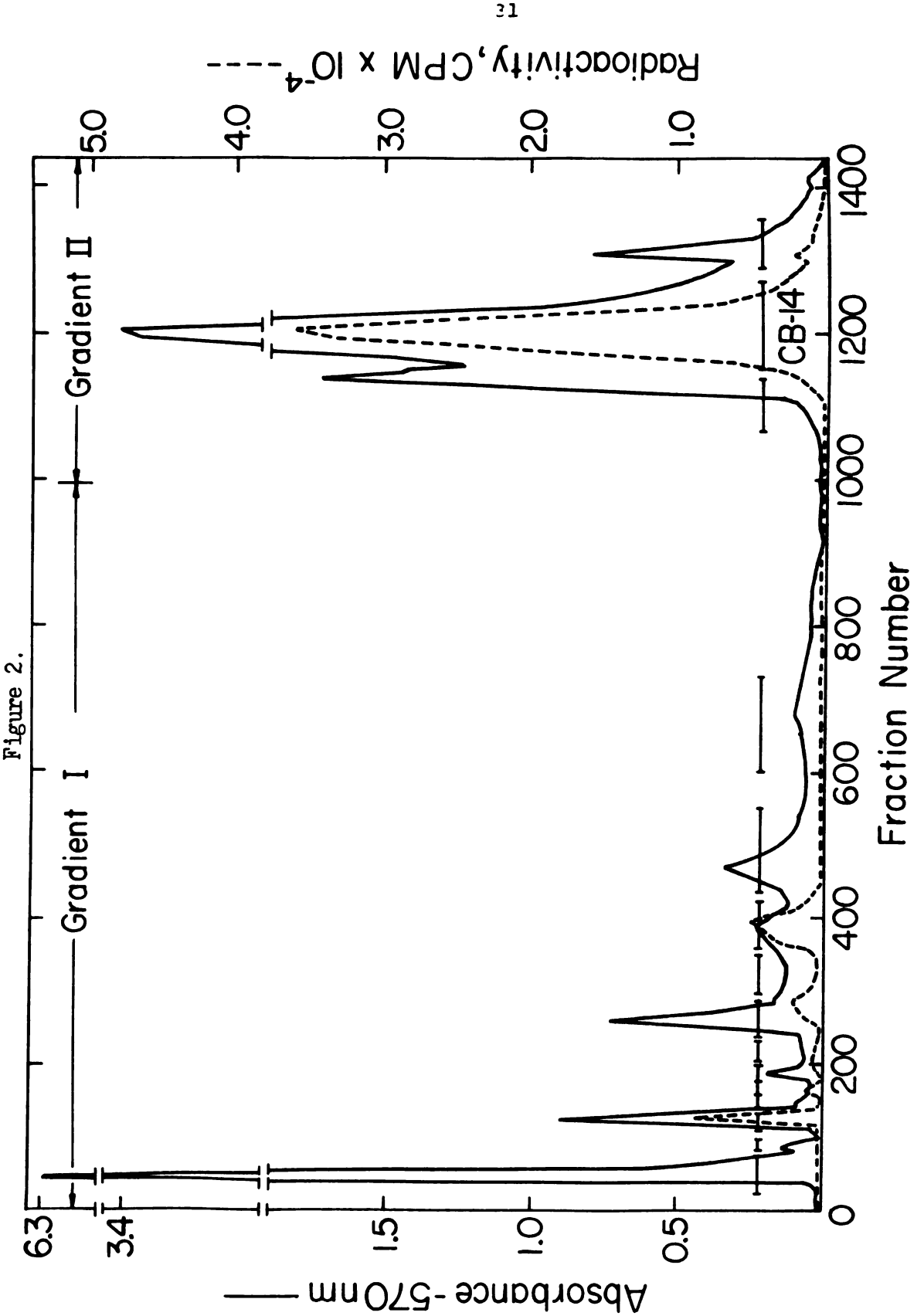

Absorbance -570nm
Radioactivity, CPM x 10^{-4}
Fraction Number
Gradient I
Gradient II
CB-14
6.3
3.4
1.5
1.0
0.5
5.0
4.0
3.0
2.0
1.0
0
200
400
600
800
1000
1200
1400

Figure 2.

85% of the initial radioactivity, and presumable contains the active site peptide as one of its components. For the purpose of present work, therefore, the CB-14 fractions were further purified, while other pooled fractions were dried and kept for sequence studies in the future.

The chromatographic pattern of CB-14 fractions on Sephadex G-50 column is shwon in Figure 3. The racioactivity was eluted from the column mainly as a single major peak. Nearly complete recovery of initial radioactivity was obtained in this step. As revealed by both ninhydrin color and radioactivity profiles, the major peak appeared to be slightly contaminated at the front and tail regions of the peak. Thus, those fractions underlined by a bar (CB-14G) were pooled for the final purification.

Figure 4 shows the Dowex 50 column chromatography of CB-14G fractions. About 97% of the starting radioactivity was located in CB-14GD, while 2.5% of the radioactivity was associated with a minor peak eluted just ahead of CB-14GD region. The amino acid composition of the major radioactive peak (CB-14GD) is shown in Table 3. Tryptophan was found to be absent in this peptide as examined by the spectral method of Edelhoch (47). It is apparent that the number of each residue per mole of peptide is close to integral. Furthermore, high voltage paper electrophoresis of CB-14GD at pH 2.0 and 6.5 indicated a single ninhydrin-positive spot which is coincident with the radioactivity. Based on this information as well as analysis of the terminal amino acid residues (presented in the following section), it is

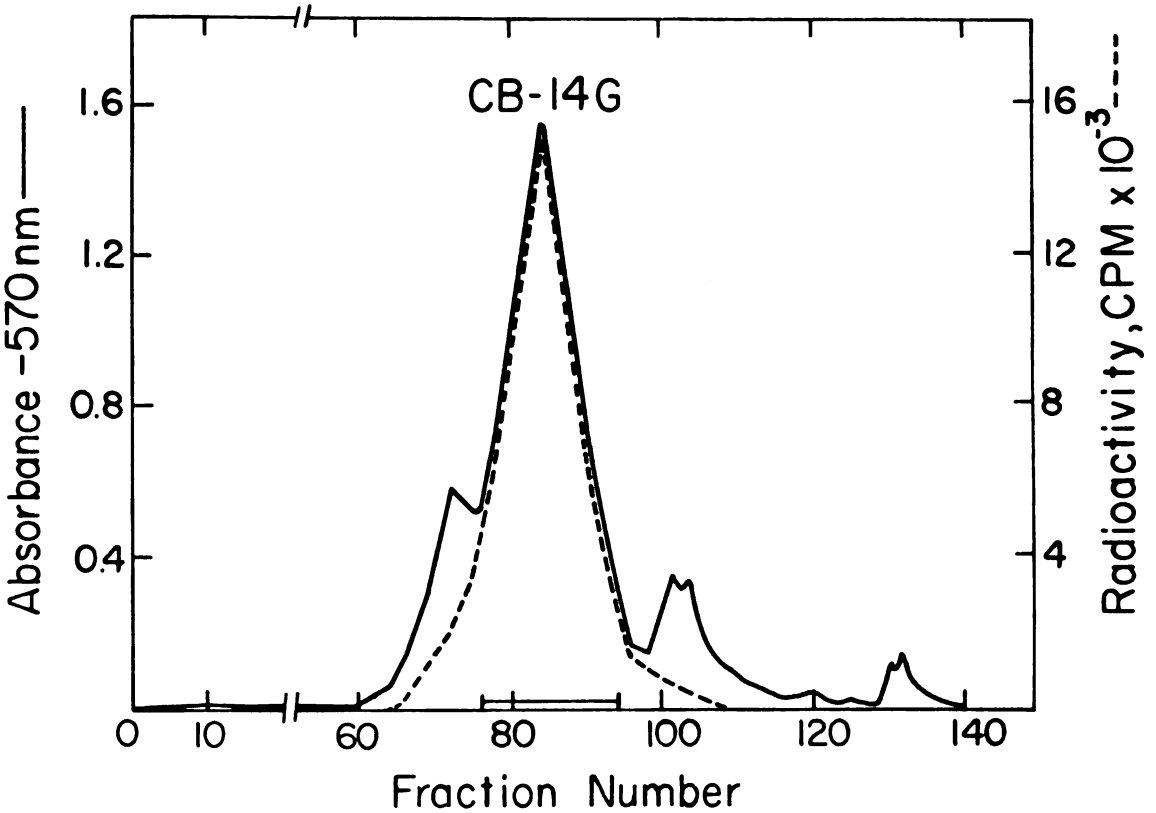


Figure 3. Ascending Chromatography of CB-14 Fraction on a Sephadex G-50 Column.

A 93 mg sample was fractionated on the Sephadex column (1.5 x 210 cm) with 50% pyridine as eluent at a flow rate of 16 ml per hour. Fractions of 2 ml were collected, and 10-µl aliquots of effluent from every other fraction were withdrawn for peptide detection and for radioactivity measurement. The barred fractions (#76-95, designated as CB-14G) were combined for further purification.

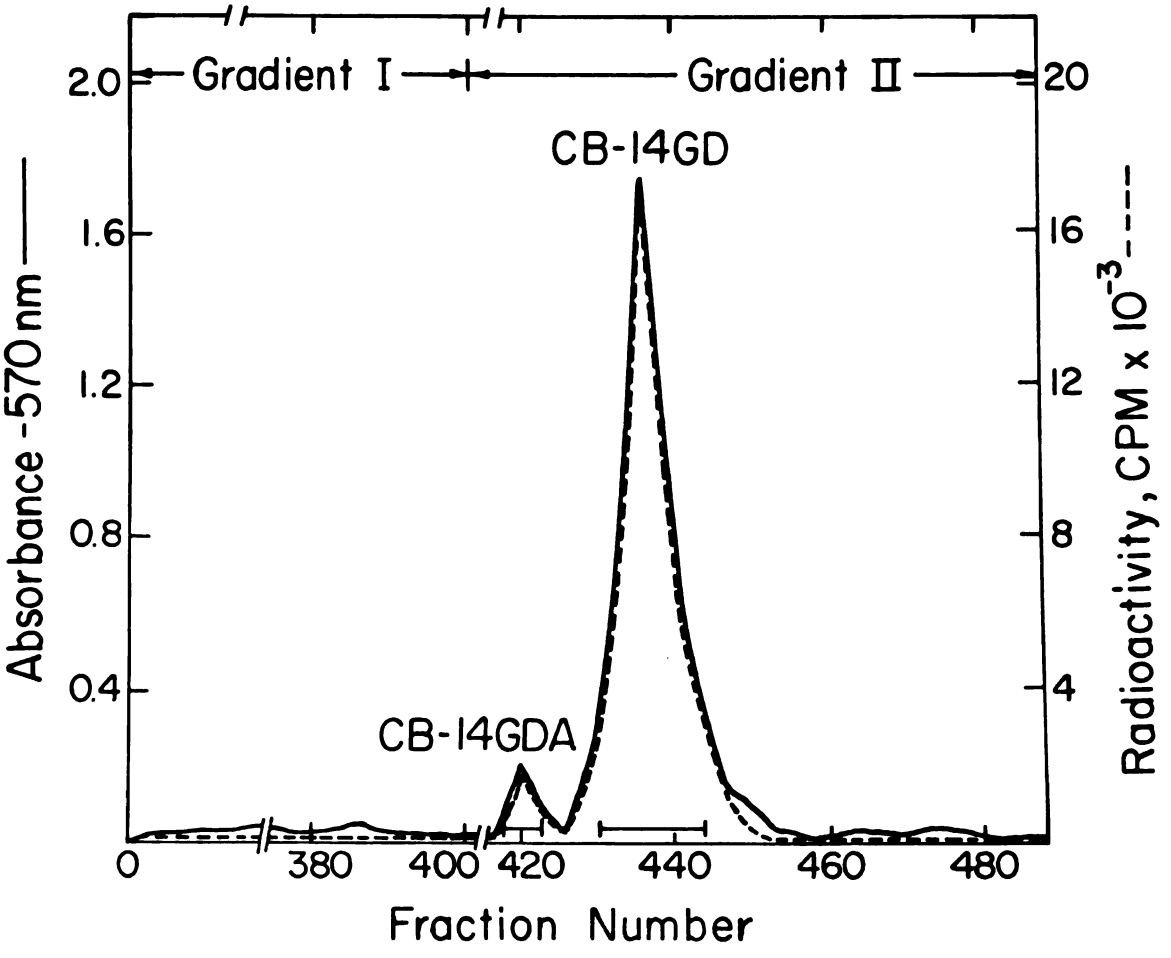


Figure 4. Ascending Chromatography of CB-14G Fraction on a Dowex 50-X2 Column.

Column size: 0.9 x 100 cm. The column was treated with two concave gradients of pyridine-acetate buffer. Gradient I: 333 ml of 0.2 M buffer (pH 3.1) and 666 ml of 2 M buffer (pH 5.0). II: 100 ml of 2 M buffer (pH 5.0) and 200 ml of 8.5 M buffer (pH 5.6). Effluents were collected in 2-ml portions at a flow rate of 12 ml per hour. Appropriate samples (10 to 100 µl) from every other fraction were assayed for ninhydrin-positive material and radioactivity. The CB-14GD fractions (#430-445) and CB-14GDA fractions (#417-425), as underlined by a bar, were pooled.

Table 3. Amino Acid Composition of the Active Site Peptide (CB-14GD) and Its Tryptic Derivatives.

Peptide samples (10 to 25 nmoles) were hydrolyzed at 110°C for 24 hrs with constant-boiling HCl as detailed in the Methods section. The composition of each peptide is expressed in terms of the molar ratio of the constituent amino acids based on valine or arginine. The integral values in parenthesis are the assumed number of residues per mole of peptide. Threonine and serine were corrected for the 5 and 10% hydrolytic destruction, respectively. Methionine was determined as homoserine plus homoserine lactone. The TG-5 was contaminated with TGD-4 and TGD-5 at a level of 5 and 12%, respectively. Without acid hydrolysis, only arginine was detected in the TG-5 sample. All of the data represent the average value of duplicate runs.

Table 3.

Amino acid	CB-14GD	TGD-1	TGD-2	TGD-3	TGD-4	TGD-5	TG-5	CB-14GDA	TGD-1A	TGD-2A
SCm-cysteine	0.81(1)		0.96(1)					0.68(1)		0.60(1)
Aspartic	4.03(4)		2.08(2)	1.05(1)		0.92(1)		4.02(4)		2.09(2)
Threonine	1.05(1)		0.98(1)					1.08(1)		1.01(1)
Serine	1.06(1)	0.99(1)						1.21(1)	0.78(1)	
Glutamic	1.35(1)	1.14(1)						1.47(1)	0.78(1)	
Proline	4.39(4)	1.05(1)	2.21(2)	1.18(1)				4.48(4)	0.85(1)	2.11(2)
Glycine	9.06(9)	2.30(2)	2.02(2)	2.86(3)	1.85(2)			8.56(9)	2.01(2)	1.86(2)
Alanine	6.16(6)	3.02(3)	1.01(1)	0.96(1)	0.99(1)			5.81(6)	2.52(3)	1.01(1)
Valine	3.00(3)	1.00(1)	2.00(2)					3.00(3)	1.00(1)	2.00(2)
Methionine	0.81(1)					0.54(1)		0.85(1)		
Isoleucine	2.99(3)	1.94(2)		0.88(1)				2.81(3)	1.56(2)	
Leucine	2.05(2)	1.15(1)			0.98(1)			2.45(2)	0.95(1)	
Tyrosine	2.89(3)				1.85(2)	1.00(1)		2.54(3)		
Phenylalanine	4.94(5)	1.99(2)	0.86(1)	1.73(2)				4.88(5)	1.68(2)	0.94(1)
ε-N-(1-carboxy ethyl)-lysine	1.37(1)	1.14(1)						1.09(1)	0.75(1)	
Lysine	0.86(1)	0.99(1)						0.97(1)	0.78(1)	
Arginine	4.04(4)		1.04(1)	1.00(1)	1.00(1)		1.00(1)	3.79(4)	0.96(1)	0.96(1)
Total residues	50	16	13	10	7	3	1	(50)	(17)	(13)

concluded that CB-14GD peptide is in a highly homogeneous state. It should also be noted that the minor radioactive peak (CB-14GDA) bears a similar amino acid composition to that of CB-14GD (Table 3).

Amino-Terminal and Carboxy-Terminal Analysis of CB-14GD: The amino-terminus of CB-14GD peptide was established by a single step of Edman degradation, followed by analysis of PTH derivative. When the alkaline hydrolysate of the PTH derivative was examined on amino acid analyzer, only glycine was detected. Also, chromatography of the PTH derivative on a thin-layer polyamide sheet showed a single spot corresponding to the authentic PTH-glycine. Thus, glycine was identified as the amino-terminal residue.

A partial sequence at the carboxy-terminus of CB-14GD was obtained by enzymatic digestion (Table 4). Sequential cleavage of CB-14GD for 4 hrs with carboxypeptidase A resulted in the liberation of 1 residue each of homoserine, asparagine, and tyrosine (exp. 1). When digested first with carboxypeptidase A, then with carboxypeptidase B (exp. 2), two additional amino acids, 0.9 residue of arginine and 0.7 residue of valine, were released. Based on the rate of release of amino acids by carboxypeptidase A and the fact that carboxypeptidase B, but not carboxypeptidase A, will cleave the arginyl residue at carboxy-terminus (63), the partial structure at the carboxy-terminus of CB-14GD is concluded to be -Val-Arg-Tyr-Asn-Met. It should also be noted that the excessive yield of asparagine (13.74 residues, exp. 2) suggests the existence of another asparagyl residue at the position

Table 4. Determination of Carboxy-terminal Sequence of CB-14GD Peptide with Carboxypeptidase A and B.

Digestion was performed at 30°C in 0.2 M N-ethylmorpholine acetate buffer, pH 8.5. In experiment 1, CB-14GD peptide was digested with carboxypeptidase A (molar ratio of enzyme to substrate, 1:250) for the time periods indicated. In experiment 2, the peptide was digested with carboxypeptidase A (1:250 molar ratio) for 3 hrs; then carboxypeptidase B (1:500 molar ratio) was added, and the digestion continued for another hour. All values of released amino acids are expressed as nmoles per 10 nmoles of CB-14GD peptide.

	Time	Amino acid released				
		Valine	Arginine	Tyrosine	Asparagine	Homo-serine
	Hour					
Exp. 1	0.5			1.90	4.36	5.36
	1.5			2.08	6.16	7.08
	3.0			5.50	6.64	9.01
	4.0			9.59	11.18	9.15
Exp. 2	3.0			7.28	7.36	9.56
	4.0	7.29	8.93	10.10	13.74	9.35

proximal to valine.

Purification of Tryptic Peptides of CB-14GD: A portion of the tryptic digest of CB-14GD was examined on the amino acid analyzer without acid hydrolysis. Approximately 0.75 residue of arginine per mole of peptide was detected, indicating that arginine was released from CB-14GD as a free amino acid. This would be expected if arginine is next to another basic amino acid in the CB-14GD peptide.

The tryptic digest mixture was first chromatographed on a Sephadex G-50 column as shown in Figure 5. The fraction designated as TG-5 (#220-232) contained mainly free arginine as revealed by amino acid analysis of either the pooled material or its acid hydrolysate. This finding is in accord with the result just discussed above. This fraction was thus set aside without further study. Other distinct peak fractions, TG-1 through TG-4, were individually purified further by chromatography on a Dowex 50-X2 column.

The radioactive TG-1 fraction was separated into 2 peaks, each associated with radioactivity (Figure 6). The major TGD-1 peak contains 75% of initial radioactivity applied to the column, while the minor TGD-1A peak contains only 24% of the starting total radioactivity. The amino acid composition analysis of TGD-1 (Table 3) indicates that its composition is identical to that of the hexadecapeptide previously sequenced by Robertson, Altekar, and Wood (39). Analysis of TGD-1A also reveals that it bears the same amino acid composition as TGD-1 except that it contains an additional arginine residue. As discussed later,

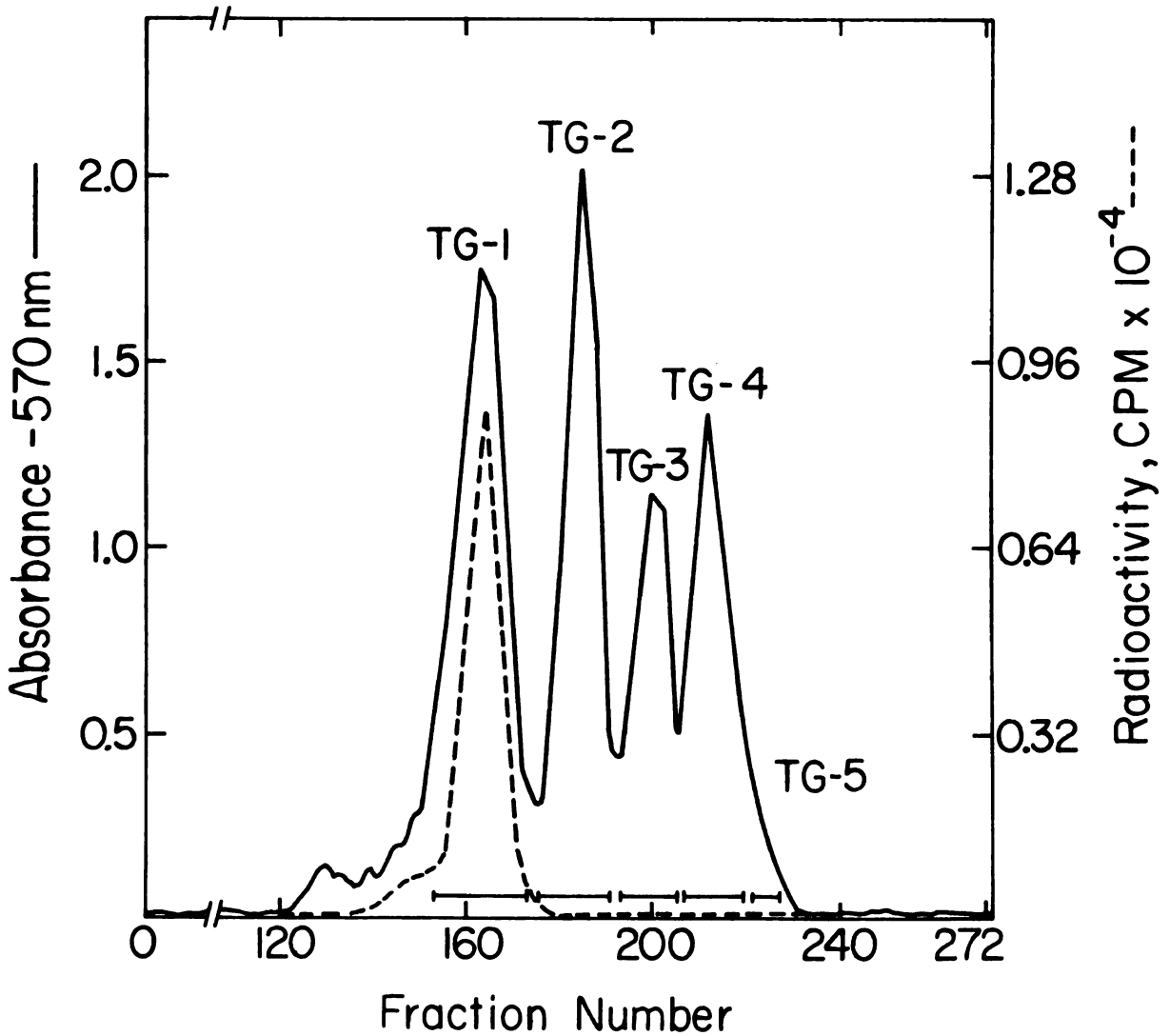


Figure 5. Separation of the Tryptic Digest of CB-14GD Peptide on A Sephadex G-50.

A 20 mg of active site peptide was digested for 4 hrs with 0.2 mg of trypsin as described in the Methods section. The digest was chromatographed in ascending mode on the Sephadex G-50 column (1.5 x 210 cm) using 0.1 N NH_4OH as developer. The flow rate was 14 ml per hour, and the volume of each fraction was 2 ml. Appropriate samples were taken from every other fraction to assay for ninhydrin-positive material and radioactivity. Those fractions indicated by a bar were pooled and designated as follows: TG-1 (#155-175), TG-2 (#176-192), TG-3, (#193-205), TG-4 (#206-219), and TG-5 (#220-232).

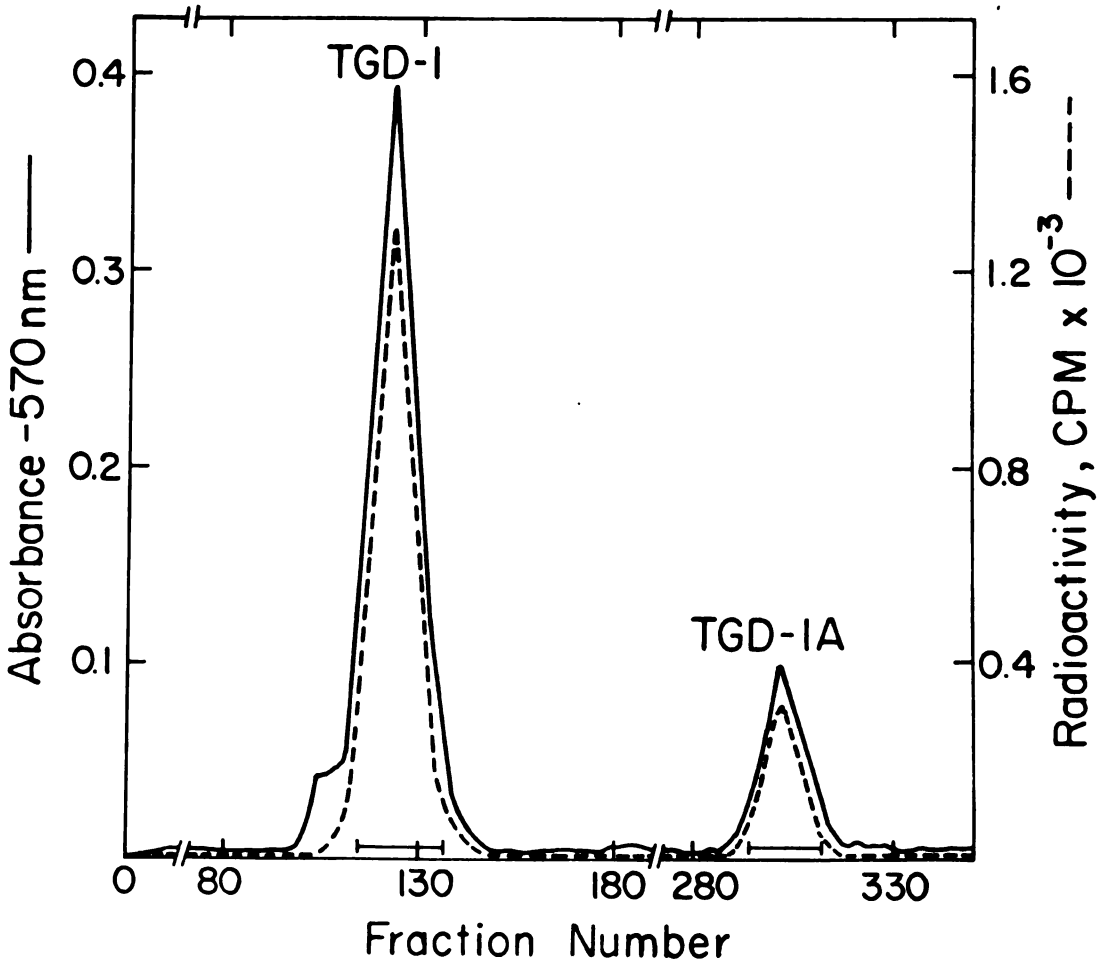


Figure 6. Purification of TG-1 Fraction on Dowex 50-X2.

The sample was dissolved in 1 ml of 0.2 M pyridine-acetate buffer (pH 3.1) for application to the column (0.9 x 100 cm). The column was developed by upward flow with a pyridine-acetate gradient containing 333 ml of 0.2 M buffer (pH 3.1) and 666 ml of 2 M buffer (pH 5.0). Fractions of 2-ml were collected at a rate of 18 ml per hour. Aliquots of the effluent from every other tube were assayed for radioactivity and for ninhydrin color value after alkaline hydrolysis. Those underlined fractions were combined and assigned as TGD-1 (#123-133) and TGD-1A (#295-320).

this unique peptide is believed to be an incomplete tryptic digest product, and it aids in assigning the free arginine to its position in the sequence of CB-14GD. It should also be noted that Robertson, Altekar, and Wood (39) previously obtained a radioactive heptadecapeptide (designated peptide 4) from a Dowex-50 column chromatogram of the tryptic fragments of ^{14}C-pyruvate labeled, reduced, and carboxymethylated KDPG aldolase. This heptadecapeptide has the same amino acid composition as TGD-1A peptide reported here.

Chromatography of TG-2 and TG-3 fractions yielded a major peak each, TGD-2 and TGD-3, respectively, as shown in Figure 7 and 8. Their amino acid compositions are listed on Table 3. Thus, TGD-2 is a tridecapeptide containing the sole SCm-cysteine and threonine residues of CB-14GD; while TGD-3 is a decapeptide. Also shown in Figure 7, there is a minor peak (TGD-2A) which is eluted just ahead of TGD-2 peak. It is of considerable interest that this minor peak resembles TGD-2 in amino acid composition (Table 3).

Figure 9 shows the chromatographic result of TG-4 fraction. Two distinct peaks, TGD-4 and TGD-5, were well separated by the concave gradient of pyridine-acetate buffer. TGD-4 is an arginine-containing heptapeptide, and TGD-5 is a tripeptide containing the unique homoserine residue (Table 3). Since the partial carboxy-terminal sequence of CB-14GD was established as -Val-Arg-Tyr-Asn-Met, the homoserine-containing TGD-5 is assumed to have a primary structure as Tyr-Asn-Met. This conclusion eliminates the necessity of any further analysis on this

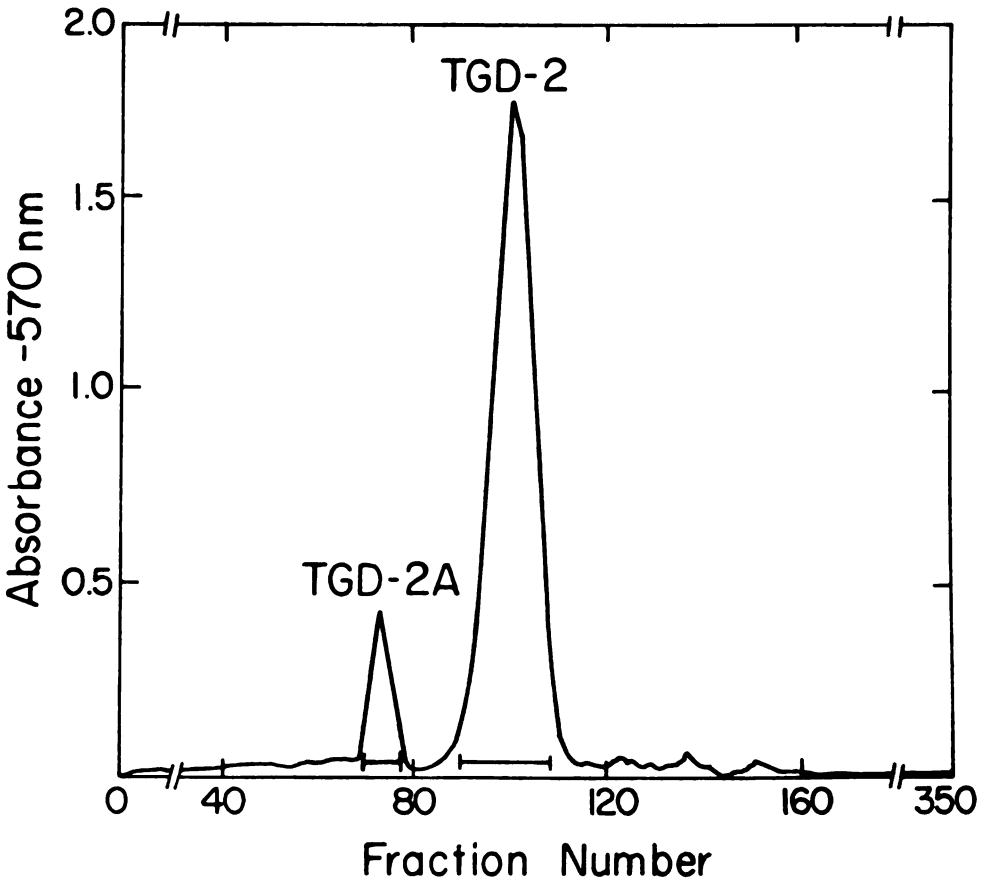


Figure 7. Purification of TG-2 Fraction on Dowex 50-X2. The conditions used were as described in Figure 6. Fractions #94 to 100 were assigned as TGD-2, while fractions #68 to 80 were assigned as TGD-2A.

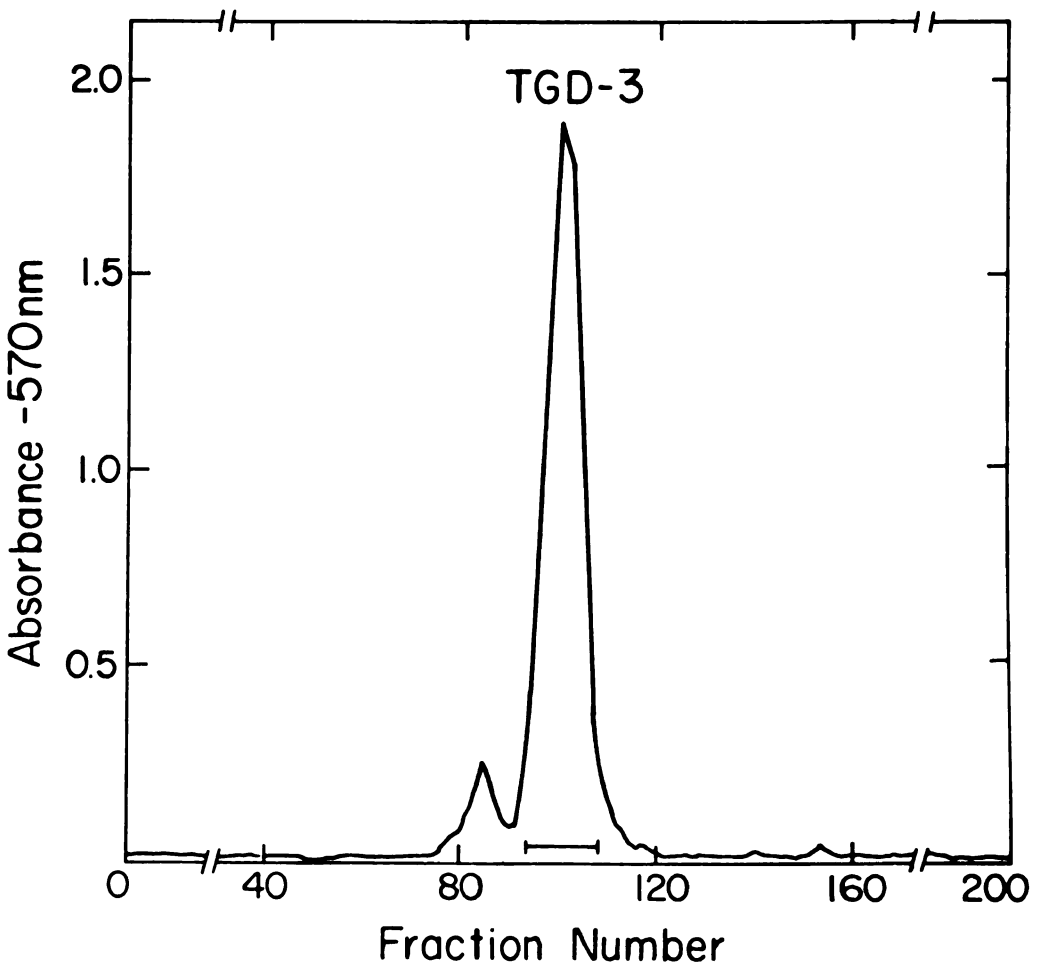


Figure 8. Purification of TG-3 Fraction on Dowex 50-X2.

The sample was dissolved in 1 ml of 0.2 M pyridine-acetate buffer (pH 3.1), applied to the column (0.6 x 54 cm). The column was subsequently treated in the ascending mode with a concave gradient of pyridine-acetate buffer containing 83 ml of 0.2 M buffer (pH 3.1) and 166 ml of 2 M buffer (pH 5.0). The flow rate was 11 ml per hour, and 1-ml fractions were collected. Aliquots of effluent from every other tube were assayed for ninhydrin-positive material after alkaline hydrolysis. Fractions #93 to 109 were pooled and designated as TGD-3.

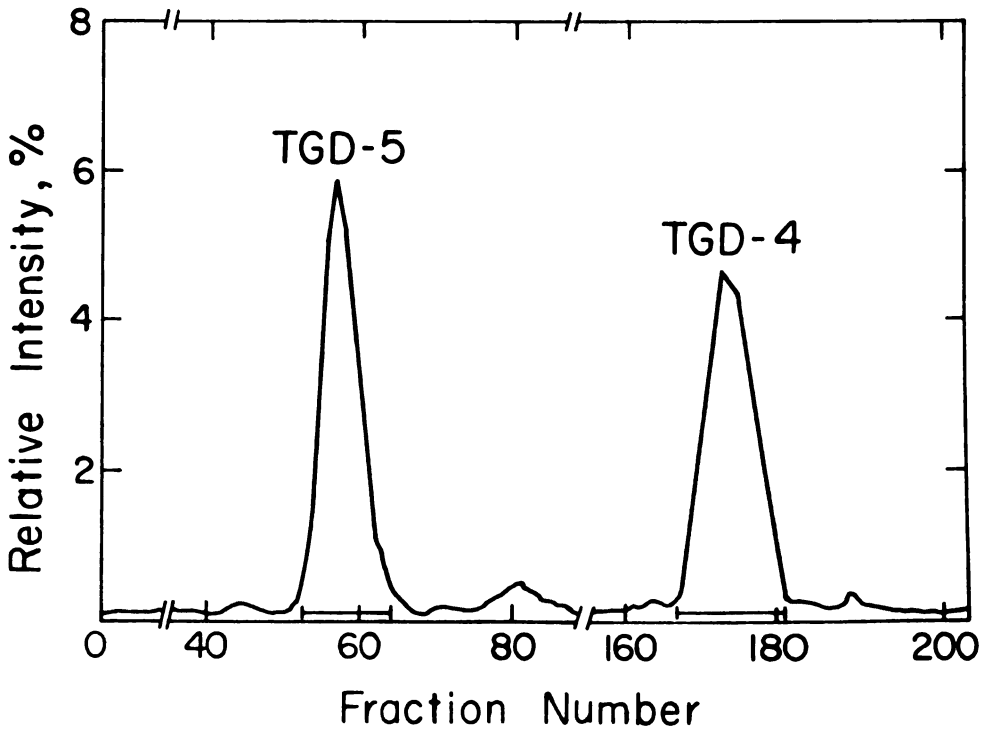


Figure 9. Purification of TG-4 Fraction on Dowex 50-X2.

The procedure was the same as described in Figure 8, except that peptides were detected by the fluorometric method. Pooled fractions were designated as TGD-4 (#51-65) and TGD-5 (#165-180).

tripeptide.

When the amino acid composition was ascertained, it becomes apparent that the sum of amino acid residues of all tryptic fragments, namely, TGD-1, TGD-2, TGD-3, TGD-4, TGD-5, and free arginine (TG-5), accounts for all of the residues of the parent CB-14GD peptide (Table 3).

The overall recovery of each tryptic fragment is presented in Table 5. As indicated, the incomplete digest fragment, TGD-1A, was recovered in 22% yield, while the TGD-1 and arginine fragments were recovered in 71% and 75% yield, respectively, after correction for losses in the purification steps. Since CB-14GD peptide contains 4 arginine residues, 3 of which are quantitatively recovered in TGD-2, TGD-3, and TGD-4, it is reasonable to assume that the remaining arginine residue was the one found in the tryptic digest as free arginine and also found in the TGD-1A peptide. Therefore, it seems most likely that the TGD-1A peptide consists of both TGD-1 and the arginine fragment.

Sequence Studies on the Hexadecapeptide TGD-1: In the first Edman degradation step, the peptide TGD-1 yielded PTH-Phe as identified by the alkaline hydrolysis method (59) and a residual peptide containing one less phenylalanine residue than TGD-1. This suggests that phenylalanine is the amino-terminal residue. This peptide was not sequenced further; however, it is concluded to have a sequence identical to that of the hexadecapeptide previously reported by Robertson et al. (39) on the basis of following considerations. Both peptides are tryptic fragments derived from the same active site region of KDPG aldolase; and both are

Table 5. Recovery of Tryptic Peptides Derived from CB-14GD.

The amount of each peptide recovered was calculated from the amount of purified material which was obtained from Dowex column chromatography (Figures 6 through 9). Corrections were made for losses in sampling and handling during experimental processes. The amount of free arginine was that amount detected in the tryptic digest of CB-14GD. The percent recovery was then expressed as the adjusted amount of each peptide to the total amount of CB-14GD initially used for trypsin digestion.

Tryptic fragment	Recovery, %
Arginine	75
TGD-1	71
TGD-1A	22
TGD-2	94
TGD-3	95
TGD-4	92
TGD-5	92

identical in amino acid composition, and in containing an amino-terminal phenylalanine as well as a carboxy-terminal lysine. The complete sequence of TGD-1 is thus assumed to be the following: Phe-Lys*-Leu-Phe-Pro-Ala-Glu-Ile-Ser-Gly-Gly-Val-Ala-Ala-Ile-Lys. The Lys* is to indicate the azomethine-forming lysyl residue.

Sequence Studies on TGD-1A and Placement of the Arginine Fragment: Two consecutive Edman degradation steps were performed on TGD-1A peptide, and the PTH derivatives were examined by thin-layer chromatography on a polyamide sheet. This identified PTH-Arg and PTH-Phe, in that order, as the degradation products. The partial amino-terminal sequence of TGD-1A was thus established as Arg-Phe-. This result supports the previous suggestion that TGD-1A is an incomplete tryptic digest product containing TGD-1 and arginine. The arginine fragment is placed, therefore, to link to the amino-terminal residue (phenylalanine) of the TGD-1 fragment.

Sequence Studies on the Tridecapeptide TGD-2: Carboxy-terminal sequences: Incubation of TGD-2 peptide with carboxypeptidase A for 2 hrs did not release any amino acid residues. This is in accord with the specificities of both trypsin and carboxypeptidase A, namely, that TGD-2 is a tryptic peptide containing an arginyl residue at the carboxy-terminus. The rate of release of amino acid residues using a combination of carboxypeptidase A and B is shown in Table 6. The result permits unambiguous assignment of the partial carboxy-terminal sequences as -Asn-Val-Arg.

Table 6. Determination of Carboxy-terminal Sequence of TGD-2 Peptide with Carboxypeptidase A and B.

Digestion was conducted at 30°C in 0.2 M N-ethylmorpholine acetate buffer (pH 8.5) for a series of time intervals as indicated. A combination of carboxypeptidase A and B at an enzyme to substrate molar ratio of 1:100 and 1:1000, respectively, was used. The amount of amino acid released is expressed as nmoles per 10 nmoles of peptide.

Time	Amino acid released		
	Asparagine	Valine	Arginine
Min.			
10	0.9	8.0	8.1
20	1.8	8.1	8.2
40	2.9	8.4	8.8
540	6.3	9.8	9.9

Edman degradation: Table 7 summarizes the results of 6 consecutive steps of Edman degradation, each with a subsequent analysis of the amino acid composition of the remaining peptide. The first and second Edman degradation resulted in the complete removal of Phe and SCm-cys, one at a time in that order, from the parent peptide. At the third Edman step, a decrease of about 0.6 residue of proline per mole of peptide was observed, while other amino acid residues remained in stoichiometric molar quantities. The incomplete removal of proline at this and next two Edman steps could be attributed to any of the following: (a) incomplete Edman reactions; (b) inadequate extraction of PTH-Pro from the remaining peptide; and (c) the greater analytical error due to a smaller proline peak (lower color value) when 5 to 10 nmoles of acid hydrolysate of peptide were analyzed. In spite of this sequencing problem with proline, threonine and glycine were cleaved from the remaining peptide to a significant extent at the fourth and fifth Edman cycle, respectively. Therefore, the partial amino-terminal sequence of TGD-2 peptide is deduced as Phe-Cys(SCm)-Pro-Thr-Gly-.

However, at the sixth step and even upon performing two more Edman degradations, no significant decreases of any amino acid in the remaining peptide could be demonstrated. The apparent resistance to Edman degradation suggests that a cyclized aspartyl residue exists as the new amino-terminus of the residual peptide as has been reported by Smyth *et al.* (64), Weber and Konigsberg (65), and other workers (61,66-69). It was postulated that the phenylthiocarbamyl peptide formed between phenyl

Table 7. Partial Sequence Determination of TGD-2 Peptide by the Subtractive Edman Degradation Method.

A 600 nmoles of TGD-2 peptide were subjected to Edman degradation as detailed in the Methods section. The amino acid composition, expressed in integral values, of TGD-2 is obtained from Table 3. The acid hydrolysate of residual peptide (5-10 nmoles) from each degradation cycle was examined on an amino acid analyzer for its constituents. The percent yield is expressed as the amount of residual peptide recovered to the amount of peptide used in that particular degradation cycle. The number "0" or blank indicates that the particular amino acid was undetectable when 5 nmoles of peptide hydrolysate was examined on amino acid analyzer.

Cycle	Yield %	Amino acid composition									Residue assigned
		Phe	Cys(SCm)	Pro	Thr	Gly	Asp	Val	Ala	Arg	
0		1	1	2	1	2	2	2	1	1	
1	92	0	0.86	2.34	1.01	2.17	2.03	1.73	1.06	1.00	Phe
2	95		0	2.02	0.84	1.86	1.85	1.71	1.01	1.00	Cys(SCm)
3	99			1.45	0.84	2.01	1.97	1.84	1.05	1.00	Pro
4	84			1.41	0.34	1.99	2.01	1.80	1.09	1.00	Thr
5	95			1.41	0.23	1.36	1.89	1.76	0.90	1.00	Gly
6	72			1.25		1.06	1.96	1.84	1.05	1.00	

isothiocyanate and aspartyl imide at the amino-terminus of a peptide would not undergo rapid cyclization since the required transition state would involve the sterically unfavorable fusion of two five-membered rings (65). In this case, Edman degradation would not result in removal of the amino-terminal aspartyl imide residue, and subsequent HCl hydrolysis of the phenylthiocarbamyl peptide would cleave the phenylthiocarbamyl group to regenerate aspartic acid, thus explaining the failure to observe any decrease in the number of aspartyl residue.

It has been reported that the aspartyl cyclic imide found in protein can be cleaved by hydroxylamine (61,68). This procedure was adopted to cleavage of the putative cyclic imide blocking further Edman degradation. In order to facilitate the isolation of cleavage products, the TGD-2 peptide was degraded by 5 Edman cycles to expose the resistant residue. The remaining peptide (TGD-2E) was then treated with hydroxylamine as detailed in the Methods section. A new peptide, TGD-2EH, was subsequently purified from a Sephadex G-15 column as shown in Figure 10. Amino acid analysis indicates that TGD-2EH contains the following residues: Glycine (1.30), Valine (2.14), Alanine (1.14), Proline (0.98), Aspartic (1.16), and Arginine (1.00). When this composition is compared with that of the residual peptide obtained from the sixth Edman degradation step (Table 7), it is evident that the hydroxylamine treatment results in removal of one aspartyl residue from TGD-2E. These data thus agree with the proposal that hydroxylamine specifically cleaves the cyclic imide bond of anhydroaspartylglycine (61,68). Therefore, the

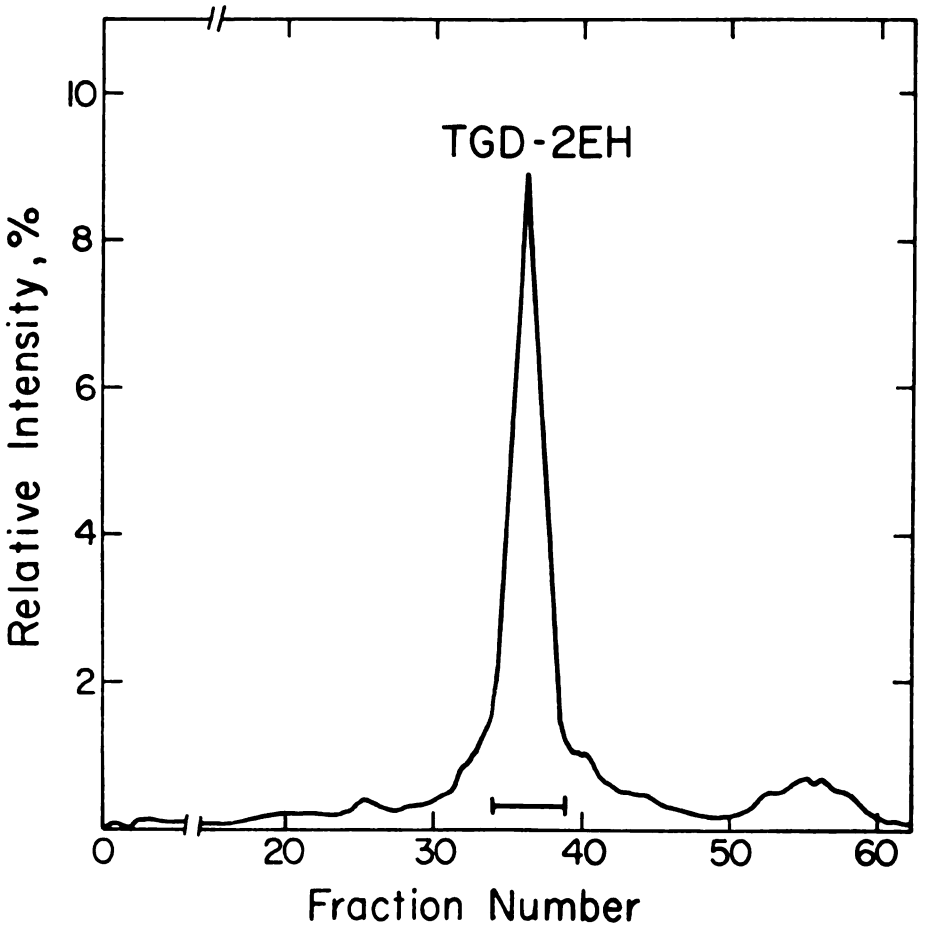


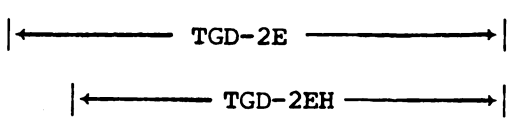
Figure 10. Gel Filtration on Sephadex G-15 of Products Resulting from Hydroxylamine Cleavage of TGD-2E.

Column size: 0.9 x 100 cm. Conditions: The column was developed with 0.2 M NH_4HCO_3 (pH 8.0) at a flow rate of 20 ml per hour. The effluent was collected in 1-ml portions, and aliquots of effluent (0.15 to 0.5 ml) from every other fraction were assayed by reacting with fluorescamine (see Methods section). Major peak fractions (#34-39) was assigned as TGD-2EH.

cyclic aspartyl residue was placed as the amino-terminus of TGD-2E peptide. However, it is uncertain whether the cyclic aspartyl residue is originally derived from an asparagyl or aspartyl residue of TGD-2 peptide, although the asparagyl residue is generally believed to enter into cyclic imide formation (68). For this reason, Asx was chosen to represent the anhydroaspartyl residue of TGD-2E peptide.

The TGD-2EH peptide was subjected to 4 consecutive Edman degradations, and the PTH derivatives were identified by thin-layer chromatography on polyamide sheets. As a result, the partial sequence of TGD-2EH was established as Gly-Val-Ala-Pro-(Asp, Val, Arg). Since this arginine-containing heptapeptide is derived from TGD-2 and thus contains the same carboxy-terminal sequence of the parent peptide, its complete sequential structure is deduced as Gly-Val-Ala-Pro-Asn-Val-Arg.

Based on the above results, the complete primary structure of peptide TGD-2 is summarized as follows:



Phe-Cys(SCm)-Pro-Thr-Gly-Asx-Gly-Val-Ala-Pro-Asn-Val-Arg

Sequence Studies on the Decapeptide TGD-3: This peptide was subjected to 9 steps of Edman degradation. The progressive decrease in one amino acid residue of the residual peptide after each degradation step is unambiguous (Table 8). After 9 degradation steps, the residual

Table 8. Complete Sequence Determination of TGD-3 Peptide by the Subtractive Edman Degradation Method.

TGD-3 peptide (500 nmoles) was subjected to Edman degradation using conditions described in the Methods section. Aliquots of residual peptide (5-10 nmoles) from each degradation step was hydrolyzed and examined on amino acid analyzer for its composition. Notations used in the table are the same as those in Table 7. Asn, instead of Asp, was assigned to the 8th position according to the information obtained from carboxypeptidase digestion (detailed in the text). After the 9th cycle of degradation, the residual material was examined on amino acid analyzer without prior acid hydrolysis. Free arginine was detected.

Cycle	Yield %	Amino acid composition							Residue assigned
		Ala	Phe	Gly	Pro	Asp	Ile	Arg	
0		1	2	3	1	1	1	1	
1	74	0	1.95	3.19	0.86	1.24	0.92	1.00	Ala
2	100		1.14	3.03	0.98	1.20	0.92	1.00	Phe
3	80		0.93	2.20	0.85	1.16	0.93	1.00	Gly
4	96		1.02	1.67	1.03	1.17	0.98	1.00	Gly
5	99		0.83	0.23	0.80	1.31	0.98	1.00	Gly
6	79		0.86		0.38	1.24	0.81	1.00	Pro
7	74		0.12			1.23	1.01	1.00	Phe
8	79					0.60	1.08	1.00	Asn
9	45						0	1.00	Ile-Arg

material was applied to the amino acid analyzer without prior acid hydrolysis. Free arginine was detected.

To investigate the identity of the Asx residue, TGD-3 peptide was digested with a combination of carboxypeptidase A and B for one hour. The amino acids so liberated are Phe (0.39), Asn (0.87), Ile (0.90), and Arg (1.00). Therefore, asparagine was assigned to the structure of TGD-3. The complete sequence of this decapeptide was thus concluded to be Ala-Phe-Gly-Gly-Gly-Pro-Phe-Asn-Ile-Arg.

Sequence Studies on the Heptapeptide TGD-4: Results of 6 cycles of Edman degradation are shown in Table 9. In all cases, amino acid residues were unequivocally assigned to their appropriate position. The excessive decrease of tyrosine at the second degradation was believed to be due to the incomplete deaeriation at the time sample was prepared for acid hydrolysis. After the sixth degradation step, the unhydrolyzed residual material was directly identified as free arginine by amino acid analyzer. These data permit assignment of the complete sequence of TGD-4 peptide as Gly-Tyr-Ala-Leu-Gly-Tyr-Arg.

Construction of the CB-14GD Sequence: As previously presented, the CB-14GD peptide contains glycyl and methionyl (homoseryl) residues at the amino- and carboxy-terminus, respectively. Partial sequence of CB-14GD at the carboxy-terminal side has also been established as -Val-Arg-Tyr-Asn-Met (Table 4). Further, tryptic digestion of CB-14GD peptide resulted in 6 small fragments which together accounted for all the amino acid residues of the parent peptide (Table 3). On the basis

Table 9. Complete Sequence Determination of TGD-4 Peptide by the Subtractive Edman Degradation Method.

A 300 nmoles of TGD-4 peptide was used for this sequence study. Conditions and notations in the table are the same as in Tables 7 and 8.

Cycle	Yield %	Amino acid composition					Residue assigned
		Gly	Tyr	Ala	Leu	Arg	
0		2	2	1	1	1	
1	70	1.16	1.78	0.97	0.94	1.00	Gly
2	83	0.96	0.77	1.01	0.76	1.00	Tyr
3	68	1.29	1.03	0.43	0.96	1.00	Ala
4	78	1.21	0.95		0.26	1.00	Leu
5	63	0.55	1.23		0.25	1.00	Gly
6	50		0			1.00	Tyr-Arg

of these data, it is possible to place 3 tryptic fragments in appropriate positions on the CB-14GD structural map: (a) TGD-4 is the only tryptic fragment having a glycyl residue as amino terminus, it is therefore assigned to the amino-terminal position of CB-14GD; (b) the tripeptide TGD-5 occupies the carboxy end of CB-14GD because it contains the sole homoseryl residue; and (c) the tridecapeptide TGD-2 is placed proximal to TGD-5, because it contains the unique -Val-Arg sequence at the carboxy-terminus (Table 6). Besides this, isolation of an incomplete tryptic digest product, TGD-1A, led to the conclusion that TG-5 (arginine) links to the amino-terminus of TGD-1 as discussed in a previous section. Therefore, the remaining task of constructing the complete sequence of CB-14GD lies in the positioning of both the TGD-1A and TGD-3 fragment as following:

(TGD-4)-{ (Arg)-(TGD-1), (TGD-3) }-(TGD-2)-(TGD-5)

|← TGD-1A →|

To this end, CB-14GD peptide was treated with 1,2-cyclohexanedione to modify its arginyl residues. The modified peptide was subjected to trypsin digestion as detailed in the Methods section. Isolation of the tryptic digest products on a Sephadex G-50 column is presented in Figure 11. The first radioactive peak was eluted at the breakthrough volume, and was probably the parent CHD-peptide. The second radioactive peak (CHD-1,2) presumably contained the trypsin cleavage products, since homoserine and lysine residues were liberated in nearly equal amounts when the peak material was digested with

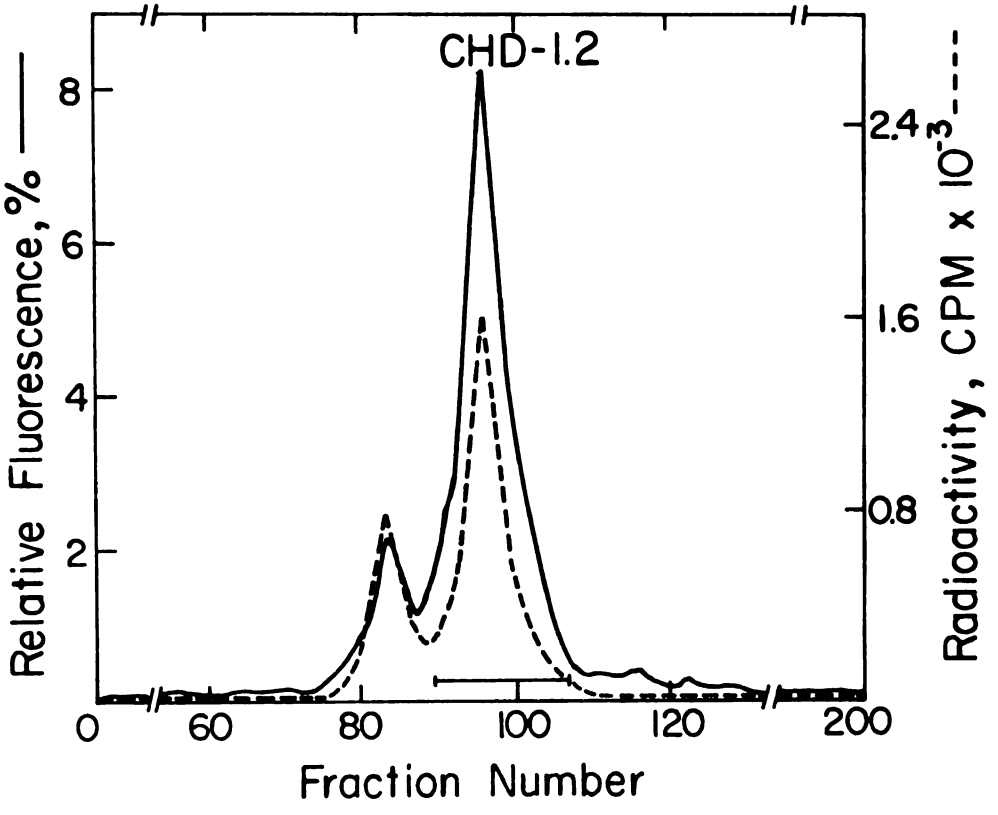


Figure 11. Chromatography of the Tryptic Digest of CHD-Modified CB-14GD Peptide on a Sephadex G-50.

The tryptic fragments (280 nmoles) were separated by upward flow on a Sephadex column (1.5 x 220 cm) using 0.1 M NH_4OH as eluent. Fractions of 2 ml were collected at a rate of 16 ml per hour. Aliquots of 40 µl were withdrawn from every other tube for monitoring peptide and radioactivity. The fractions under the bar (#90-108) were pooled and designated as CHD-1,2.

carboxypeptidase A. The inability to separate the cleavage products on the Sephadex column suggests their approximate similarity in molecular size. Figure 12 shows the fractionation of CHD-1,2 materials on a Dowex 50-X2 column. Two recognizable peaks, CHD-1 and CHD-2, were obtained by eluting with a relatively more gradual increase in concentration and pH of the pyridine-acetate buffer system. The amino acid composition of both peaks is shown in Table 10. It is apparent that both CHD-1 (24 residues) and CHD-2 (26 residues) together account for all the amino acid residues of the CHD-modified CB-14GD peptide. By matching their constituents to those of known tryptic peptides (Table 3), it is obvious that CHD-1 is made of TGD-4 and TGD-1A, while CHD-2 contains TGD-2, TGD-3, and TGD-5. Furthermore, as revealed by carboxypeptidase A digestion, CHD-1 has a lysyl residue as the carboxy-terminus, and in contrast, CHD-2 contains homoserine as the carboxy-terminal residue. Therefore, the tryptic fragments of CB-14GD can be arranged in sequential order as follows:

(TGD-4)-(Arg)-(TGD-1)-(TGD-3)-(TGD-2)-(TGD-5)

|←——— CHD-1 ———→|←——— CHD-2 ———→|

Finally, the arrangement of tryptic peptides as depicted above is further confirmed by the following observation. Partial digestion of CB-14GD peptide with aminopeptidase M results in the liberation of the unique azomethine-forming lysyl residue, together with other amino acids which can be accounted for by those residues of TGD-4 and TGD-1A (Table 11). Figure 13 gives the sequence of the active site peptide of 50 amino acids (CB-14GD) as established in the present work.

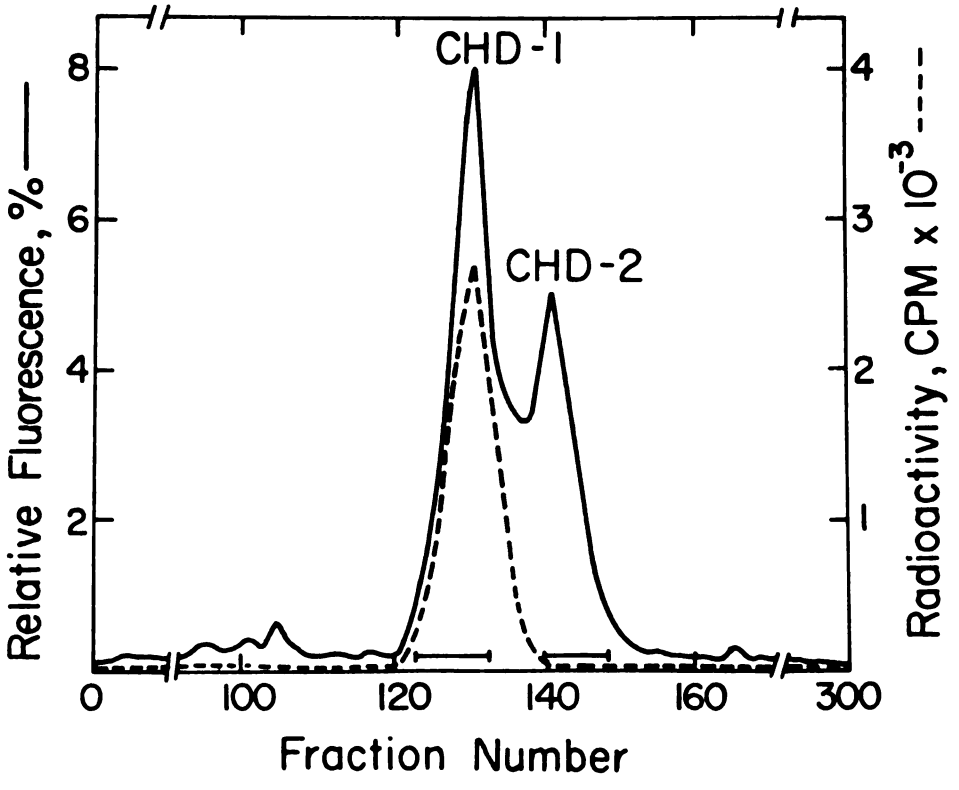


Figure 12. Separation of CHD-1,2 Fraction on Dowex 50-X2.

The sample (360 nmoles) was fractionated in ascending mode on a Dowex column (0.6 x 54 cm) by a concave gradient of pyridine-acetate buffer containing 100 ml of 1 M buffer (pH 5.0) and 200 ml of 2 M buffer (pH 6.0). Each 1-ml portion of effluent was collected at a rate of 11.3 ml per hour. Aliquots of 50 µl from every other tube were taken for radioactivity determinations and fluorescence measurements. Those fractions, underlined by bars, were combined and designated as CHD-1 (#122-132) and CHD-2 (#140-148).

Table 10. Amino Acid Composition of CHD-1 and CHD-2 Peptides.

Approximately 10 nmoles of peptide were hydrolyzed at 110°C for 24 hrs with constant-boiling HCl and subsequently analyzed for the amino acid constituents according to the procedure described in the Methods section. Numbers in the table represent residues of each amino acid based on 1 residue of valine per mole of CHD-1 and 2 residues of valine per mole of CHD-2. The integral values in parenthesis are the assumed number of residues per mole of peptide. Homoserine and ε-N-(1-carboxyethyl)-L-lysine were not determined.

Amino acid	CHD-1	CHD-2
SCm-cysteine		1.00 (1)
Aspartic		4.04 (4)
Threonine		0.90 (1)
Serine	0.97 (1)	
Glutamic	1.10 (1)	
Proline	1.26 (1)	3.47 (3)
Glycine	4.30 (4)	5.34 (5)
Alanine	4.10 (4)	2.06 (2)
Valine	1.00 (1)	2.00 (2)
Methionine (as Homoserine)		n.d. (1)
Isoleucine	2.08 (2)	1.03 (1)
Leucine	2.16 (2)	
Tyrosine	2.07 (2)	0.93 (1)
Phenylalanine	1.90 (2)	2.80 (3)
ε-N-(1-carboxyethyl)-Lysine	n.d. (1)	
Lysine	1.02 (1)	
Arginine (as CHD-arginine)	1.69 (2)	1.62 (2)
Total residues	24	26

Table 11. Digestion of CB-14GD Peptide with Aminopeptidase M.

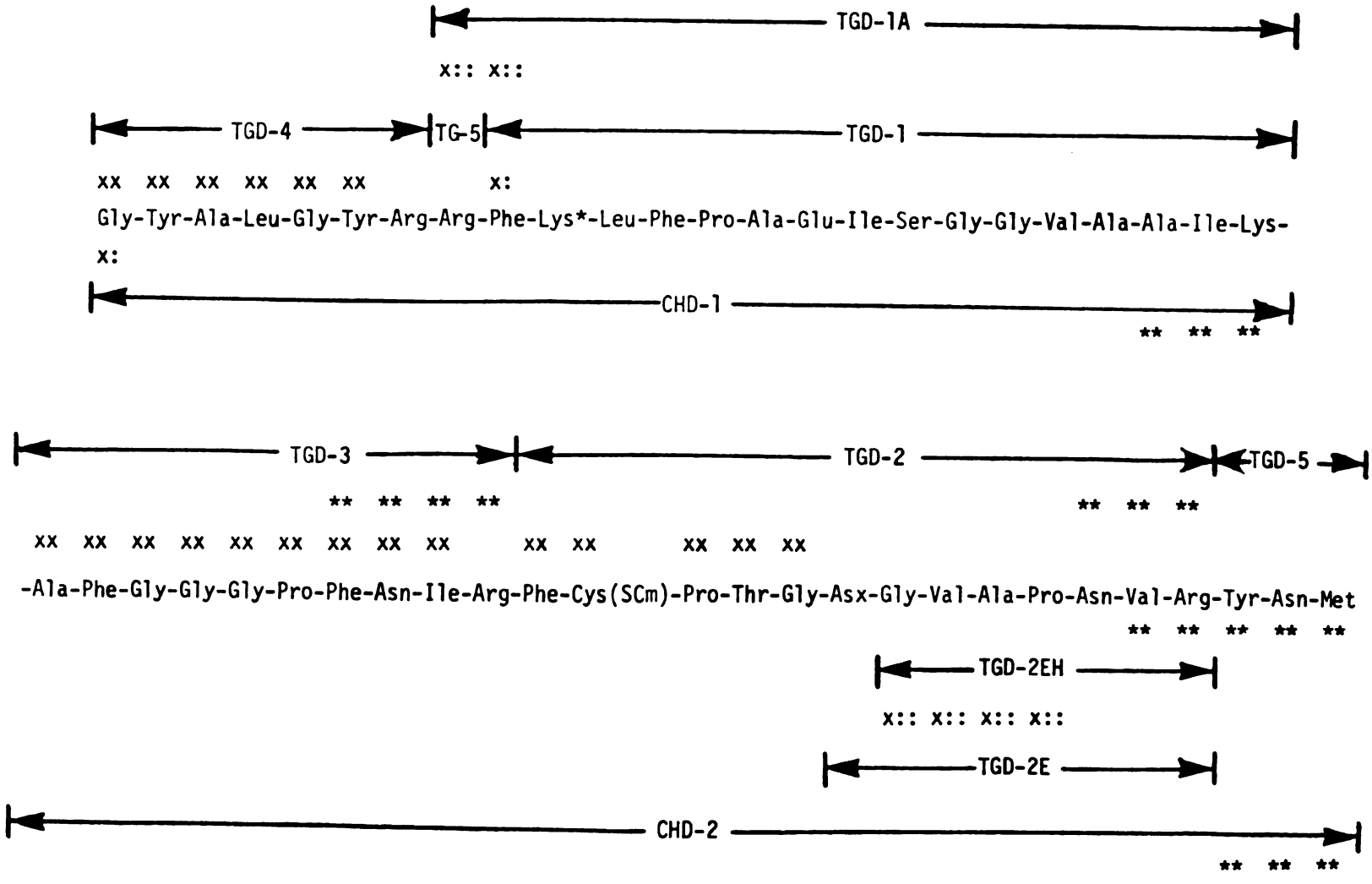
A 10 nmoles of CB-14GD peptide was digested with aminopeptidase M at an enzyme to substrate molar ratio of 1:100. The digestion was performed at 35°C for 12 hrs in 0.06 M potassium phosphate buffer, pH 7.6. The reaction was stopped by dilution with 0.2M citrate buffer, pH 2.0. The digest was examined on the amino acid analyzer for the free amino acids released. A blank which omitted the substrate was included. All values are expressed as nmoles per 10 nmoles of peptide. The molar ratio based on ε-N-(1-carboxyethyl)-L-lysine is also shown in the table.

Amino acid released		
	nmole	molar ratio
Glycine	26.14	2.97
Tyrosine	17.10	1.95
Alanine	21.49	2.45
Leucine	15.20	1.73
Arginine	19.86	2.26
Phenylalanine	14.48	1.65
ε-N-(1-carboxyethyl)-L-lysine	8.80	1.00
Glutamic	4.16	0.47
Isoleucine	7.16	0.81
Serine	4.85	0.55
Valine	5.15	0.59
Lysine	3.33	0.38

Figure 13. Summary of the Sequence Analysis of CB-14GD Peptide.

Various symbols are adopted to denote the methods employed to elucidate the sequences. They are as follows: xx denotes the residues determined by subtractive Edman degradation. x: denotes those residues elucidated by alkaline hydrolysis of their PTH derivatives. x:: denotes those residues identified by thin-layer chromatography of their PTH derivatives. ** indicates those residues released by carboxypeptidase A or a combination of carboxypeptidase A and B. Lys* is the azomethine-forming residue of KDPG aldolase and is identified as ε-N-(1-carboxylethyl)-L-lysine.

Figure 13.
SEQUENCE OF ACTIVE SITE PEPTIDE - KDPG ALDOLASE



DISCUSSION

The chromatogram of cyanogen bromide fragments of KDPG aldolase demonstrates that 85% of the initial radioactivity bound to the enzyme is located in a distinct peak area (Figure 2). This peak remained nearly intact in the subsequent purification processes from which the radioactive CB-14GD peptide was isolated (Figure 3 and 4). The minor radioactive peak, CB-14GDA as shown in Figure 4, is not regarded as having a distinct identity as already discussed in the Results section. The amino acid composition (Table 3) as well as the amino- and carboxy-terminal determinations indicate that the radioactive CB-14GD peptide is very unique and highly homogeneous. Also obviously shown is the fact that CB-14GD contained one equivalent of the azomethine-forming lysyl residue (Table 3). On the basis of all of these data, it is concluded that the CB-14GD peptide is the active site peptide of the KDPG aldolase, and that all subunits of the enzyme are identical. Furthermore, when the specific radioactivity of ^{14}C-pyruvate is used to calculate the amount of azomethine complex formed in a single KDPG aldolase molecule, it is found that one mole of enzyme (molecular weight of 72,000) binds 3.09 ± 0.14 (s.d.) moles of ^{14}C-pyruvate, a mean value obtained from 3 determinations. These data, in combination with the conclusion previously reached, constitute a good agreement with the proposed molecular model that KDPG aldolase contains 3 subunits (24-26), and that each subunit has an identical but independent substrate binding site, namely, the active lysyl residue (39).

Inspection of the primary structure of this active site peptide reveals certain anomalies of charge distribution (Figure 13). There are 8 basic side chains but only 1 acidic side chain. This inequality may contribute to the formation of an unusual microenvironment around the active site. It is also of interest that 2 consecutive arginyl residues are located just one residue away from the active lysyl residue. This is particularly attractive in view of the proposed catalytic mechanism of class I aldolases that an additional nucleophilic group of the enzyme, besides the Schiff base-forming lysyl residue, is presumably required to assist in the overall aldolization of the substrate (16,19). In the case of KDPG aldolase, this nucleophilic group is visualized as interacting with the C_4-hydroxyl group of KDPG in the direction of cleavage, or with the carbonyl group of glyceraldehyde-3-phosphate in the direction of condensation (19). The proximity of the agrinyl group to the active lysyl residue would facilitate their interaction with substrates without requiring excess folding of the peptide chain. This view may be better appreciated when considering the high stability of the KDPG aldolase toward severe pH change (24,42), in water (42), in response to freezing (4), and possibly toward high temperature treatment as demonstrated for a similar enzyme isolated from E. coli K 12 (13). However, positive identification of the involvement of an arginyl residue in the catalytic process will have to await further experimentation, such as chemical modification of the arginyl residues, or more directly, establishment of the 3-dimensional structure of the enzyme by X-ray crystallography as presently undertaken.

Of course, folding of the peptide chain may remove the arginyl residues from active participation in catalysis, and instead, folding may bring other nucleophiles into close proximity to the substrate. In this connection, Meloche (22) has proposed that a carboxylate group is the base involved in the aldolization process. Also, Barran and Wood (21) observed that destruction of histidyl residues of the KDPG aldolase during photooxidation is in parallel with inactivation of the cleavage reaction, suggesting a catalytic role for histidine. However, in their work, loss of structural integrity by photooxidation has not been eliminated.

Based on the established sequences and X-ray crystallographic structures of 15 proteins, Chou and Fasman (70,71) have recently deduced a set of rules to predict the secondary structures of peptides or proteins from their amino acid sequences. These generalized rules were followed in examining the structure of the current CB-14GD peptide. As depicted in Figure 14, based on the Chou and Fasman classification of residues, there is one α-helix region (7 residues), and three β-sheet segments (two with 6 residues and one with 5 residues). The remaining 26 residues are assumed to exist as random coil conformation. The azomethine-forming lysine, together with 12 other residues, is located in the random coil region. This is of interest because a random coil would provide the necessary flexibility for the active lysyl residue to interact with the substrate. The ultimate correlation between enzyme activity and conformation at active site region will have to await

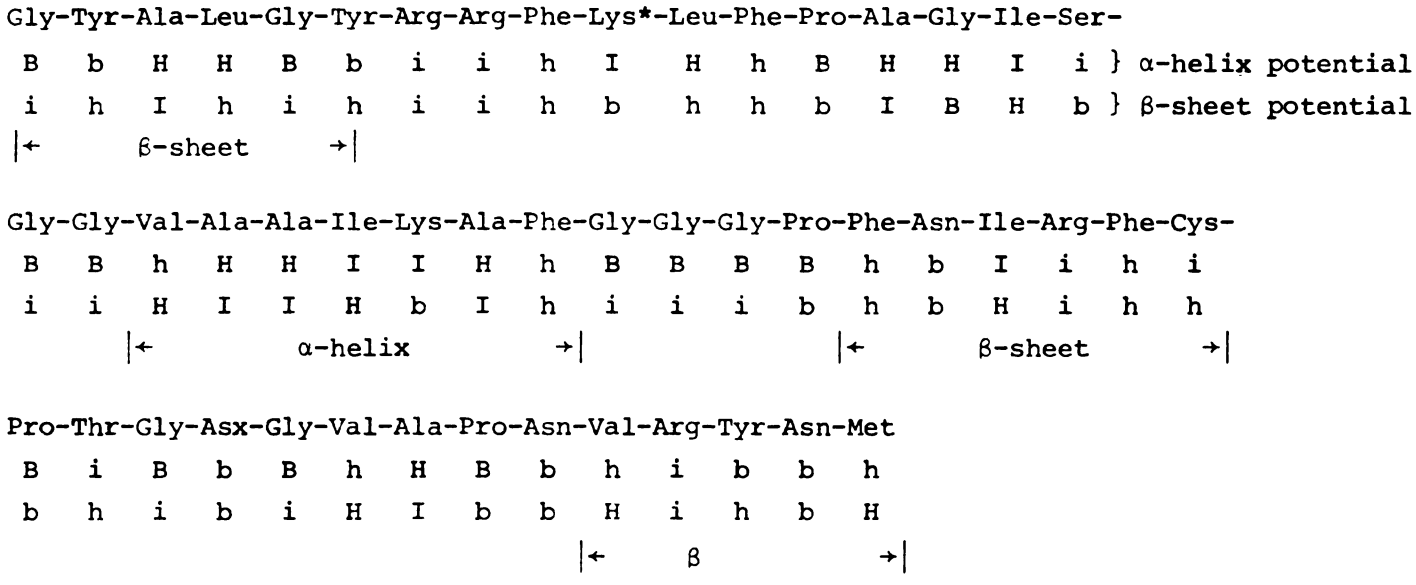


Figure 14. Preductive Analysis of α-helical, β-sheet, and Random Coil Regions in the CB-14GD Peptide.

The general rules of Chou and Fasman (70,71) were followed to assign the value of conformational potentiality for each amino acid residue as regard to α-helical and β-sheet formations. The H, h, I, i, B, and b represent the strong former, former, weak former, indifferent, strong breaker, and breaker, respectively, as defined by Chou and Fasman. The α-helical and β-sheet segments are as indicated in the figure, and the remaining segments are as the random coil conformation.

determination of the 3-dimensional structure by X-ray crystallography. The above analysis may have some usefulness in the interim.

In the past few years, studies on the primary structure of the active site of fructose-1,2-diphosphate (FDP) aldolase, another typical class I aldolase, have been accomplished with enzymes isolated from different species and tissues, including rabbit (72,73), ox (74,75), lobster (76), fish (76-78), and frog (79). In all cases, extensive homologies in the amino acid sequences at the active site region have been observed. Whereas KDPG aldolase resembles FDP aldolase in the catalytic mechanism, both enzymes are quite different in their amino acid sequences around the active site region. Another remarkable difference is that a cluster of hydrophobic amino acids is found near the active lysyl residue of FDP aldolase (72,73), while such a group of hydrophobic residues does not appear in the active site sequences of KDPG aldolase as established here. These observations as well as the basic difference in the quartery structures between KDPG aldolase and FDP aldolase, trimer vs. tetramer, may imply that a similar structure at the active site region is unnecessary for a common aldolization reaction. Consequently, it may not be surprising that the functional groups involved in assisting the overall aldolytic cleavage are different among the class I aldolases.

In the course of present sequence work, several alternative methodologies have been exploited to certain advantages. First, cyanoborohydride rather than borohydride is chosen to reduce the azomethine intermediate which is formed between the ^{14}C-pyruvate and

active lysyl residue of KDPG aldolase. It has been shown that at neutral or slightly acidic pH, reduction of the imminium group (i.e., azomethine intermediate) by cyanoborohydride is effective, while the reduction of ketones (i.e., pyruvate) is negligible (40). Therefore, by using this reagent, the amount of ^{14}C-pyruvate required is considerably decreased over that needed with borohydride. For example, an enzyme to ^{14}C-pyruvate molar ratio of 1:9 was used in the present experiment, whereas in the past a molar ratio of 1:100 was used with borohydride (24,39). Also, as a result of its efficiency, cyanoborohydride was used in the nearly stoichiometric quantity necessary to reduce the azomethine complexes.

Second, the Dowex-50 chromatographic procedure of Schroeder et al. (56) was modified as described in the Methods section, that is, packing the column with 4 M pyridine-acetate buffer (pH 5.3) and subsequently eluting the column with an ascending flow of buffer gradients. The column packed in such a way can usually be regenerated for the next use if the NaOH elution step is omitted. This modified procedure seems to improve peptide separation in some cases. The CB-14GDA, for example, is not separated from the CB-14GD peptide (Figure 4) if chromatographed on a descending column packed in 0.2 M pyridine-acetate buffer (pH 3.1).

Third, during the sequence studies on the TGD-2 peptide, Edman degradation failed to proceed any further after 5 cycles. It was reasoned that an anhydroaspartyl residue might exist as the next amino-terminal residue of the peptide (TGD-2E) because of the refractory

behavior of the peptide toward Edman degradation and because of the amino acid composition of the peptide as well as the portion remaining to be sequenced. Therefore, treatment with hydroxylamine was used to remove the amino-terminal anhydroaspartyl residue of TGD-2E peptide. The resultant peptide (TGD-2EH) was then degradable by the Edman procedure. This demonstrates that hydroxylamine can serve not only to fragment a large peptide or protein containing an anhydroaspartylglycyl linkage (61,66-68), but also to remove the amino-terminal anhydroaspartyl residue of a peptide which otherwise would be resistant to Edman degradation. This may be of considerable value in sequence work where an anhydroaspartyl residue is encountered.

The occurrence of an anhydroaspartyl residue in TGD-2 peptide is presumable due to cyclization of the original asparagyl (or aspartyl) residue during the peptide isolation processes. This is based on the observation that TGD-2 and TGD-2A are chromatographically distinct (Figure 7), but are identical in amino acid composition when acid hydrolysates of them are analyzed. Moreover, CB-14GD peptide (the parent peptide of TGD-2 and TGD-2A) is separated from CB-14GDA (Figure 4), nevertheless, both peptides are also identical in amino acid composition. In view of their elution behavior in cationic exchange chromatography, it suggests that CB-14GD and TGD-2 are more basic than CB-14GDA and TGD-2A, respectively. Furthermore, considering that after 5 cycles of Edman degradation, TGD-2 becomes fully resistant to further cleavage, it is reasonable to assume that the

asparagyl (or aspartyl) residue of TGD-2 exists as an imide, while it exists as the α- or β-aspartyl form in TGD-2A. The same supposition would be equally applicable to CB-14GD and CB-14GDA. The interconversion of both forms must then be in favor of imide form under the experimental conditions. It is uncertain whether cyclization of the original asparagyl (aspartyl) residue is catalyzed by Dowex resin, as has been suggested (69).

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