Pyrolysis of Alkaloid B. A vacuum distillation of 25 mg of alkaloid B was performed at 200° (20 μ). After 2 hr, the distillate (5.0 mg) was removed and identified as pleiocarpinine by tlc and its mass spectrum. The residue was separated by tlc into five fractions: (i) 10.5 mg, (ii) 2.1 mg, (iii) 2.1 mg, (iv) 1.3 mg, and (v) 0.5 mg. The mass spectrum of iv was that of pure pleiocarpinine. Fraction ii was identical with N_a-methyl-22-oxokopsane (XIV).[1c] Fraction iii contained 3-isopleiocarpinine, distinguished from pleiocarpinine by the marked intensity differences of peaks *m/e* 124 and 324 in the mass spectrum. These differences are reported to be characteristic of the C-3 epimers.[4] Also present in fraction iii was a dehydrogenated pleiocarpinine (V): mass spectrum *m/e* 350 (M^+), 291, 263, 122, 107. Fractions i and v did not give usable mass spectra, due to thermal decomposition; these compounds may be rearrangement products of the amine oxide IX.

Reaction of Alkaloid B with Phosphoric Acid-d_3. Alkaloid B (5 mg) was refluxed for 1 hr in 1 ml of 50% phosphoric acid-d_3 (from D_2O and P_2O_5). The solution was neutralized with sodium carbonate and extracted with chloroform. The product contained considerable amounts of starting material, together with a small amount of pleiocarpinine-d_2. The latter was isolated by tlc. Both deuterium atoms were in the aromatic nucleus;[1d] mass spectrum *m/e* 354 (M^+), 326, 323, 295, 267, 253, 231, 172, 124, 109.

Oxidation of Pleiocarpine (I) to Pleiocarpine N_b-Oxide (VIII). Pleiocarpine (I, 100 mg) in 0.5 ml of ethanol and 0.5 ml of hydrogen peroxide (31%) was stirred at room temperature for 24 hr. Water (10 ml) was added and the solution was extracted with three 10-ml portions of chloroform. After drying and evaporation of solvent, the product was crystallized from ethanol–ether to give 84 mg of pleiocarpine N_b-oxide (VIII), mp 245–250° dec; mmp 245° with alkaloid A; $[\alpha]^{26}D$ −137 ± 3° (*c* 1.985, $CHCl_3$); λ_{max}^{MeOH} 206 mμ (log ε 4.46), 244 (4.13), 280 (3.30), 287 (3.27); infrared and nmr spectra identical with spectra of alkaloid A, except for the lack of a peak at 3.33 ppm (CH_3OH). No analogous signal could be detected in the nmr spectrum for ethanol; the compound appears to be a hydrate. Upon recrystallization from methanol–ether, the product exhibited an nmr spectrum identical with that of alkaloid A.

Acknowledgment. We thank Dr. F. A. Hochstein (Chas. Pfizer & Co.) for the crude bark extract. This investigation was supported by a Public Health Service Fellowship (5-F1-GM-21,624-03) from the National Institute of General Medical Sciences (to D. W. T.) and a research grant from the National Science Foundation (GP-3734).

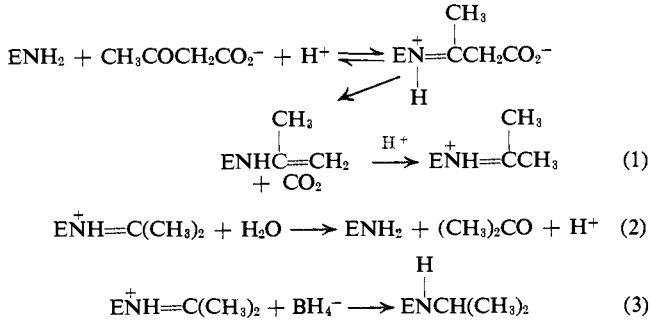
The Active Site of Acetoacetate Decarboxylase[1]

Richard A. Laursen[2] and F. H. Westheimer

Contribution from the James Bryant Conant Laboratory of Harvard University, Cambridge, Massachusetts. *Received February 3, 1966*

Abstract: Sodium borohydride reduces a mixture of acetoacetic acid, labeled in the 3 position with ^{14}C, and acetoacetate decarboxylase to incorporate radiocarbon into the enzyme. Hydrolysis of the resulting protein with trypsin has led to the formation of a single radioactive peptide, which contains ε-N-isopropyllysine. (This amino acid arises from reduction of a Schiff base between the enzyme and decarboxylated substrate.) Similarly, a single radioactive peptide could be isolated after digestion with chymotrypsin. Sequence analysis of these peptides shows that the active site of the enzyme has the structure -Glu-Leu-Ser-Ala-Tyr-Pro-Lys*-Lys-Leu-, where Schiff base formation and borohydride reduction occur at the starred lysine residue.

The decarboxylation of acetoacetic acid is catalyzed by a crystalline decarboxylase from *Clostridium acetobutylicum*.[3,4] The reaction proceeds by the formation of a Schiff base between the enzyme and acetoacetate, followed by decarboxylation to form an enamine; the enamine is in turn protonated to form the Schiff base salt of acetone, and then hydrolysis regenerates enzyme and liberates product.[5]



This mechanism (eq 1 and 2) was first postulated in 1959 on the basis of the discovery[6] that the enzymic decarboxylation involves the obligatory exchange of the carbonyl oxygen atom of acetoacetate with oxygen atoms of the solvent (water). It was confirmed by the observation[7] that the reduction of a solution containing enzyme and acetoacetate with sodium borohydride[8] leads to inactivation of the enzyme. When the reaction was carried out with acetoacetate labeled in the 3 position with ^{14}C, the resulting protein was radioactive, and on hydrolysis yielded a single radioactive amino acid. This amino acid has beeen identified as ε-N-isopropyllysine,[5,9] and it has been shown that it arises

(1) This work was supported by the National Institutes of Health under Grant GM-04712.

(2) National Institutes of Health Postdoctoral Fellow, 1964–1966, 5-F2-GM-17,608-02.

(3) G. Hamilton and F. H. Westheimer, *J. Am. Chem. Soc.*, 81, 2277 (1959).

(4) B. Zerner, S. M. Coutts, F. Lederer, H. H. Waters, and F. H. Westheimer, *Biochemistry*, **5**, 813 (1966).

(5) F. H. Westheimer, *Proc. Chem. Soc.*, 253 (1963).

(6) G. Hamilton and F. H. Westheimer, *J. Am. Chem. Soc.*, **81**, 6332 (1959).

(7) I. Fridovich and F. H. Westheimer, *ibid.*, **84**, 3208 (1962).

(8) See E. H. Fischer in "Structure and Activity of Enzymes," T. W. Goodwin, J. I. Harris, and B. S. Hartley, Ed., Academic Press Inc., New York, N. Y., 1964, p 111, for a review of borohydride reductions of enzymic systems.

(9) S. Warren, B. Zerner, and F. H. Westheimer, *Biochemistry*, **5**, 817 (1966).

from the sequence of reactions shown as eq 1 and 3 above.

The enzyme contains about 20 lysine residues in each active subunit[10,11] of molecular weight about 33,000, but only the particular lysine residue here identified is reactive in Schiff base formation and catalysis. The present paper reports the isolation of the peptide that contains the essential lysine.

Experimental Section

Materials. Trypsin (crystallized twice), chymotrypsin (crystallized twice), pepsin (crystallized twice), leucine amino peptidase, and carboxypeptidase B were obtained from Worthington Biochemical Corp. Acetoacetate decarboxylase, prepared by the procedure of Zerner, *et al.*,[4] was crystallized three times, and showed an OD_{280}/OD_{260} ratio of greater than 2.15; its specific activity in arbitrary units[4] was 34–40 units/mg.

ε-N-Isopropyllysine and ε-N-isopropyllysine methyl ester were prepared by methods previously published.[9,12] The borohydride reduction of the reaction mixture of enzyme and ^{14}C-labeled acetoacetic acid was carried out according to Warren, *et al.*[9] Their procedure leads to the formation of enzyme labeled with ^{14}C in the isopropyl group of a lysine residue, in accordance with the chemistry illustrated by eq 1 and 3. The labeled protein was isolated as previously[9] described, and hydrolyzed according to the procedures outlined in the section on methods below.

Pyridine was distilled from barium oxide and triethylamine from phthalic anhydride. Unless otherwise stated, other solvents and reagents were of the highest purity commercially available, and were used without further purification.

Methods. Edman Degradation. The procedure adopted was a modification of that used by Sarges and Witkop.[13] A solution containing 0.02–0.3 μmole of peptide was evaporated to dryness, and 0.1 ml of pyridine–triethylamine–phenyl isothiocyanate (100:3:1) was added. After 2.5 hr at 37°, 1.0 ml of methanol–water–triethylamine (50:50:2) was added, and the mixture was passed through a column (0.5 × 4 cm) of Dowex 1-X2 (chloride) which had previously been equilibrated with the methanol–water–triethylamine mixture. The column was washed with 1.0 ml of the same solvent, and then with 10 ml of methanol–water (1:1). The washings were discarded. The column was eluted with 6 ml of 1 *M* acetic acid in methanol–water (1:1). The effluent was evaporated to dryness and the residue was redissolved in 0.3 ml of anhydrous trifluoroacetic acid. The solution was allowed to stand for 1 hr at room temperature, and then 1 ml of methanol–water (1:1) was added. The solution was passed through a column (0.5 × 4 cm) of Dowex 50-X2 (hydrogen form) and the column was washed with 10 ml of methanol–water (1:1) to remove the phenylthiohydantoin, and then with 6 ml of 2 *M* ammonium hydroxide in methanol–water (1:1) to remove the residual peptide. The latter was then hydrolyzed in 6 *N* HCl for 22 hr at 110° for amino acid analysis.

The half-time for the hydrolysis of the phenylthiohydantoin of proline in 6 *N* HCl at 110° was found to be about 20 hr. The phenylthiohydantoin from the Edman degradation of TRY-1-chy-1 was hydrolyzed under these conditions for 36 hr, and the product was chromatographed using both the short and long columns of the amino acid analyzer. The composition of the product showed proline (1.0), plus isopropyllysine (0.2), plus lysine (0.2), as well as small (0.05–0.2) quantities of glutamic acid, glycine, and alanine. The analysis was run at the level of 0.005 μmole, and the contaminants were the usual ones.

Hydrazinolysis was conducted by the method of Spero, *et al.*[14]

Scintillation counting was carried out with a Nuclear Chicago Series 720 scintillation counter, using the scintillation mixtures and techniques previously described.[9] In some experiments, the effluent from a chromatographic column was passed through a 1-ml flow cell, containing an anthracene scintillator; counting efficiency was about 30%. Strip counting was carried out with a Vanguard Model 880 automatic chromatogram scanner, used with the kind permission of Professor Paul Doty.

Paper electrophoresis was performed with a Savant Instruments, Inc., high-voltage apparatus, using Whatman 3MM paper and a pyridine–acetic acid–water (1:10:289) buffer, pH 3.5. Peptides were eluted from the strips with a minimal amount of solvent, and the peptide solutions were evaporated to dryness in a vacuum desiccator. Evaporation proved more satisfactory than lyophilization, which left the peptide as a fluffy, easily lost residue.

Column chromatography was carried out with a Beckman Spinco Model 120B automatic amino acid analyzer, or with special columns. The latter were of two types: (a) columns modeled after those of the amino acid analyzer, where the temperature (usually 35°) was maintained in the jacketed column by circulation of thermostated water, and (b) columns of Sephadex G-25 (fine), where eluent was allowed to flow by gravity from a reservoir with a head of about 6–12 in.

Amino acid analyses were performed with the Beckman Spinco automatic amino acid analyzer, Model 120B, equipped for accelerated analysis. The recorder was supplied with a resistor that gave a full-scale deflection for 0.1 optical density units. In some analyses, a long path length cell was used which gave about a twofold increase in sensitivity. With this system, quantitative information can be obtained with 0.002 μmole of each amino acid except proline, and as little as 0.0005 μmole of each of these amino acids can be detected unambiguously. The levels required for the detection of proline are five times this high. Where the yield of proline was too low for integration of the peak by the method of counting "dots," an estimate of the peak area could be obtained by cutting out the peak and weighing the paper. Prior to analyses, peptides (usually 0.005–0.02 μmole) were hydrolyzed for 22 hr with 6 *N* HCl at 110° in sealed, evacuated ampoules. Tyrosine gave variable results, perhaps because of conversion to chlorotyrosine[15,16] by an oxidative process during hydrolysis. The color yield of isopropyllysine with ninhydrin was obtained by analysis of a hydrolyzed sample of ε-N-isopropyllysine methyl ester, prepared by the method of Schellenberg.[12]

ε-N-Isopropyllysine and histidine are not separated on the short column of the amino acid analyzer at pH 5.28; however, they can be separated by elution with pH 4.25 buffer, where ε-N-isopropyllysine is eluted at 100 ml, and histidine (and lysine) at 89 ml.

Alkaline hydrolysis and ninhydrin asssay of peptides were carried out according to standard procedures.[17]

Isolation[18] of TRY-1. A solution of 42.5 mg of ^{14}C-isopropylated acetoacetate decarboxylase in 0.05 *M* ammonium bicarbonate buffer was lyophilized, and the residual powder was dissolved in 4.0 ml of 0.1 *N* ammonium hydroxide. The solution was heated for about 5 min on a steam bath and then cooled to room temperature. The alkali was neutralized with Dry Ice and the pH was adjusted to 7–8 with solid ammonium carbonate; phenol red was added as an indicator. A solution of 0.85 mg of trypsin in 0.1 ml of 0.001 *M* HCl was added. In a few minutes the coagulated protein dissolved; the resulting solution was incubated at 37° for 8 hr. The solution was lyophilized and the residue dissolved in 2.0 ml of 0.1 *M* ammonium bicarbonate buffer, pH 7.8.

A portion (1.85 ml, 1.58 × 10^6 dpm, 0.71 μmole) of the peptide solution was applied to a column (1.6 × 190 cm) of Sephadex G-25 (fine). Elution was carried out with 0.05 *M* ammonium bicarbonate (pH 7.8), and approximately 3-ml samples were collected every 3 min. The optical density of fractions at 280 mμ was measured, and 10-μl aliquots were taken for scintillation counting (Figure 1). The fractions containing most of the radioactivity were pooled and lyophilized. The recovery of radioactivity was 1.51 × 10^6 dpm (96%). The residual powder was dissolved in 2.0 ml of 2% triethylamine solution.

A portion (1.93 ml, 1.44 × 10^6 dpm, 0.65 μmole) of the 2% triethylamine solution of the peptide was applied to a column (0.9 × 104 cm) of Dowex 1-X2 (acetate, 200–400 mesh) which had been equilibrated with an aqueous solution containing 1% 2-picoline and 1% 2,4-lutidine. Elution at 35° was conducted first with 5 ml of

(10) F. Lederer, S. M. Coutts, R. A. Laursen, and F. H. Westheimer, *Biochemistry*, **5**, 823 (1966).
(11) R. A. Laursen, unpublished.
(12) K. Schellenberg, *J. Org. Chem.*, **28**, 3259 (1963).
(13) R. Sarges and B. Witkop, *J. Am. Chem. Soc.*, **87**, 2011 (1965).
(14) L. Spero, D. Stefanye, P. I. Brecher, H. M. Jacoby, J. E. Dalidowicz, and E. J. Shantz, *Biochemistry*, **4**, 1024 (1965).
(15) R. L. Hill, *Advan. Protein Chem.*, **20**, 37 (1965).
(16) E. O. Thompson, *Biochim. Biophys. Acta*, **15**, 440 (1954).
(17) C. H. W. Hirs, S. Moore, and W. H. Stein, *J. Biol. Chem.*, **219**, 623 (1956).
(18) The following abbreviations are used: TRY-1 and CHY-1 are the radioactive peptides obtained by digesting the labeled decarboxylase with trypsin and chymotrypsin, respectively. The subsequent digestion of these peptides with trypsin, chymotrypsin, carboxypeptidase B, and pepsin yields peptides designated for example as TYR-1-chy-3 (the third peptide obtained from the chromatographic separation of the chymotryptic digest of TRY-1). ε-N-Isopropyllysine is abbreviated as Iprlys.

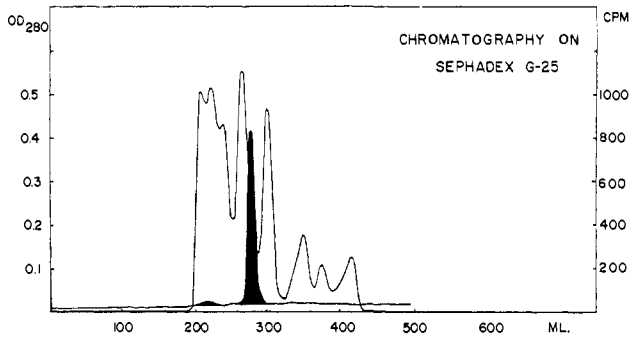


Figure 1. Chromatography of the tryptic digest of ^{14}C-labeled reduced acetoacetate decarboxylase on Sephadex G-25. Left-hand ordinate, optical density at 280 mμ; this registers tyrosine and tryptophan absorptions. Right-hand ordinate (for solid black peaks), counts per minute.

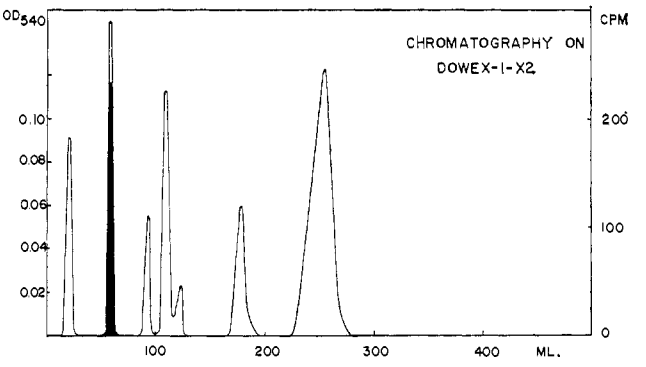


Figure 2. Chromatography of radioactive band from Sephadex separation on Dowex 1-X2. Left-hand ordinate, optical density of ninhydrin color obtained after alkaline hydrolysis of the various fractions. Right-hand ordinate (for solid black peak), counts per minute.

2% triethylamine, then with 20 ml of 1% 2-picoline–1% 2,4-lutidine mixture, and finally with an exponential gradient of 0.4 *M* acetic acid into 500 ml of the picoline–lutidine mixture. A flow rate of 0.7 ml/min was maintained with a Milton-Roy minipump, or with a New Brunswick peristaltic pump, and 2.5-ml fractions were collected. Radioactivity was measured automatically with the flow cell, or by counting 10-μl aliquots; small samples were removed for alkaline hydrolysis and analysis with ninhydrin[17] (Figure 2). The peak centered at 60 ml usually contained 65–75% of the radioactivity; the remaining activity has not been accounted for. The radioactive fractions were pooled and evaporated to dryness.

A small sample of the peptide was routinely tested by electrophoresis. Sometimes it showed more than one ninhydrin-positive spot; amino acid analysis suggested contamination by as much as 10% of a second peptide (Asp,Thr,Gly,Met,Ileu,Lys). When this proved to be the case, the entire sample was further purified by high voltage paper electrophoresis at pH 3.5. At this pH, the peptide had a mobility of 0.56 (lysine = 1.00). The radioactive peptide was located by radioautography, and the spot cut out and eluted to give pure TRY-1.

Isolation of CHY-1. A suspension of 25 mg of heat-denatured radiochemically labeled acetoacetate decarboxylase (8.98×10^5 dpm) in 12.5 ml of 0.1 *M* ammonium bicarbonate solution was prepared as described above for tryptic digestion, and 0.6 mg of chymotrypsin in 0.6 ml of 0.001 *M* HCl was added. The coagulated protein dissolved in a few minutes, and the solution was incubated at 37° for 16 hr, and then lyophilized; the residue was dissolved in 2.0 ml of 0.1 *M* ammonium hydroxide solution.

This solution was applied to a column (1.8 × 107 cm) of Sephadex G-25 equilibrated with 0.05 *M* ammonium bicarbonate (pH 7.8), and eluted with the same buffer. Fractions were collected and monitored as for the chromatographic treatment of the tryptic digest; fractions 66–71 (18.8 ml) were pooled and lyophilized. The recovery of radioactivity was 94%.

The lyophilized fractions were dissolved in 1.0 ml of a 1% aqueous solution of N-methylmorpholine, and the liquid was applied to a column (0.9 × 106 cm) of Dowex 1-X2 (acetate, 200–400 mesh) which had been equilibrated with a 1% 2-picoline–1% 2,4-lutidine buffer adjusted to pH 8.4 with acetic acid. Elution was carried out with an exponential gradient of 0.4 *M* acetic acid into 500 ml of the picoline–lutidine buffer at 35°. Fractions (3.2 ml) were collected every 4 min. Essentially all of the radioactivity was eluted with the solvent front, fractions 5–8. The total recovery of radioactivity was 74%. Fractions 5–8 were evaporated to dryness, the residue was subjected to electrophoresis at pH 3.5, and the major radioactive band eluted. Recovery was 5.98×10^5 dpm (96% yield) in the electrophoresis.

Specificity of Trypsin. Since trypsin hydrolyzes peptides of lysine and of arginine, and of various other amino acids with cationic side chains,[19] the question arose as to whether trypsin would attack peptides of ε-N-isopropyllysine. A test was conducted with lysine methyl ester and ε-N-isopropyllysine methyl ester under identical conditions (0.01 *M* sodium phosphate buffer, pH 8.02, 0.1 *M* sodium chloride, 15 μg/ml of trypsin, 25°). Although trypsin preferentially attacks esters and amides acylated on the α-amino group, the hydrolysis of lysine methyl ester itself proceeded smoothly to completion in about 15 min; the amount of acid generated from trypsin plus ε-N-isopropyllysine methyl ester did not exceed that of the control experiment with trypsin alone. Thus ε-N-isopropyllysine derivatives are presumably inert to trypsin, or hydrolyzed very slowly by it.

Tryptic and Chymotryptic Digests of Peptides. Peptide samples were dissolved in a minimal amount (0.05–0.2 ml) of 0.1 *M* ammonium bicarbonate (pH 7.8) and solutions of trypsin or chymotrypsin in 0.001 *M* HCl were added to give a 2–4% (w/w) ratio of enzyme to peptide. The solutions were incubated at 37° for 8–16 hr; since the proteolytic enzymes are somewhat unstable to self-digestion,[20] they were sometimes added in two separate portions at 8-hr intervals. For the hydrolysis of TRY-1 with chymotrypsin, up to 20% by weight of the enzyme had to be added to achieve hydrolysis of the tyrosyl–proline bond. After digestion, the reaction mixtures were separated by paper electrophoresis. Control experiments showed that the self-digestion products of chymotrypsin do not contaminate the resulting radioactive peptide.

Peptic digests were carried out in 0.01 *M* HCl (0.05 ml) at 37° for about 20 hr, using a 4% (w/w) ratio of enzyme to peptide. **Leucine amino peptidase** was activated according to Hill, *et al.*[21] Incubation at 37° was carried out in 0.022 *M* Tris buffer (pH 8.5), 0.005 *M* magnesium chloride, and 0.00011 *M* manganous chloride. The ratio of enzyme to peptide was 1% (w/w). At intervals, aliquots were removed and applied directly to the 50-cm column of the amino acid analyzer. **Carboxypeptidase B digestions** were performed with 33 μg of enzyme and about 0.08 μmole of peptide. The ratio of enzyme to peptide in the experiments was 1:1 (probably much higher than necessary). Periodically aliquots were removed and applied to the short column of the amino acid analyzer to measure the progress of hydrolysis.

Results

The Structure of TRY-1. The radioactive peptide TRY-1 was digested with chymotrypsin, as outlined in the Experimental Section. The various peptides, and TRY-1 itself, were hydrolyzed with HCl, and the products analyzed with the amino acid analyzer. The peptides had the following compositions:[22] (TRY-1)

(19) M. A. Rafferty and R. D. Cole, *Biochem. Biophys. Res. Commun.*, 10, 467 (1963); R. T. Jones, *Cold Spring Harbor Symp. Quant. Biol.*, 29, 297 (1964); K. Kitagawa and N. Izumiga, *J. Biochem.* (Tokyo), 46, 1159 (1959).

(20) R. E. Canfield and C. B. Anfinsen, *Proteins*, 1, 311 (1963).

(21) R. L. Hill, D. H. Spackman, D. M. Brown, and E. L. Smith, *Biochem. Prepn.*, 6, 35 (1958).

(22) Amino acid analyses were carried out on 0.0025–0.01 μmole of peptide hydrolysate per column of the amino acid analyzer. In some cases, where there was only a minimal amount of material (peptic peptides), the presence of lysine and isopropyllysine was not determined by amino acid analysis, but was inferred from the radioactivity and electrophoretic mobility of the peptides. In these cases, as well as when the proline peak was too small to integrate, the relative amounts of amino acids are given as integers with no other significant figures. The yields of serine and tyrosine have not been corrected for decomposition during hydrolysis.

$Ala_{0.98}$, $Glu_{0.83}$, $Iprlys_{0.90}$, $Leu_{1.00}$, $Lys_{1.18}$, $Pro_{1.26}$, $Ser_{0.74}$, $Tyr_{0.85}$ (this analysis was repeated several times with comparable results); (TRY-1-chy-1) $Iprlys_{1.00}$, $Lys_{1.21}$, $Pro_{0.82}$; (TRY-1-chy-2) $Ala_{1.0}$, $Iprlys_{0.8}$, $Lys_{1.2}$, Pro_1, $Ser_{0.7}$, $Tyr_{0.8}$ (proline was detected, but accurate integration was not possible).

As shown in the Experimental Section, derivatives of ε-N-isopropyllysine are not readily attacked by trypsin; so, in accordance with the specificity of trypsin, the C-terminal amino acid of TRY-1 is presumably lysine.

Edman degradation of TRY-1 gave a radioactive peptide that showed the following analyses for neutral amino acids: $Glu_{0.28}$, $Leu_{1.00}$, $Ser_{0.95}$, $Ala_{1.11}$, $Tyr_{0.74}$, $Pro_{1.25}$. No analyses were conducted on this Edman degradation product for the basic amino acids, but the presence of isopropyllysine is established by the radioactivity. These data show that glutamic acid (or glutamine) is the N-terminal amino acid of TRY-1.

Action of leucine amino peptidase on TRY-1 gave the data shown in Table I. The slow release of serine is in accord with the specificity of leucine amino peptidase.[23] The release of glutamic acid with this enzyme differentiates between that amino acid and glutamine.

Table I. Action of Leucine Amino Peptidase on TRY-1

Time, hr	μmole				
	Peptide (initial)	Glu	Leu	Ser	Other
1	0.018	0.002	~0.001	0	0
16	0.018	0.013	0.013	Trace	0

The C-terminal sequence of TRY-1 was established by the Edman degradation of TRY-1-chy-1 (identical with TRY-1-pep-1; see below). After a single stage Edman degradation of TRY-1-chy-1 the residual peptide showed on analysis $Pro_{0.0}$, $Iprlys_{1.00}$, $Lys_{0.81}$; repetition of the degradation on a different sample gave the composition $Pro_{0.36}$, $Iprlys_{1.00}$, $Lys_{0.94}$. These experiments establish proline as the N-terminal amino acid of this peptide. Hydrolysis of the phenylthiohydantoin obtained from the Edman degradation gave proline as the principal product, along with smaller amounts of other amino acids (see the Experimental Section).

Evidence consistent with the sequence of amino acids indicated above for TRY-1-chy-1 was obtained by digesting the peptide with carboxypeptidase, whereupon lysine was liberated nearly quantitatively. The residual peptide was chromatographed on a short column of Dowex 1. Although it was still impure, a 0.025-μmole sample was subjected to hydrazinolysis.[14] Essentially all the radioactivity remained in the aqueous layer after extraction of the various hydrazides with benzaldehyde. Analysis of the aqueous layer showed isopropyllysine as the major peak; although some of the other (contaminating) amino acids were still present, proline was not among them. The rather unusual nature of the cleavages that produce TRY-1-chy-1 and TRY-1-pep-1 are examined in the Discussion.

The results cited above have been supplemented by partial analysis of the peptides obtained from TRY-1 by digestion with pepsin. In these instances, as mentioned earlier,[22] the radioactive peptides obtained were analyzed only for the neutral and acidic amino acids. Although the analysis of TRY-1-pep-3 is poor, the list of amino acids indicated as present, as well as the electrophoretic mobility, show that this peptide is identical with TRY-1-chy-2, for which a reasonably good analysis is available. The compositions of the peptic peptides are as follows: (TRY-1-pep-1) $Pro_{1.0}$ (Iprlys, Lys); (TRY-1-pep-2) $Pro_{1.0}$, $Tyr_{0.85}$ (Iprlys, Lys); (TRY-1-pep-3) $Ala_{1.0}$, Pro_1, $Ser_{0.6}$, $Tyr_{0.4}$ (Iprlys, Lys).

The Structure of CHY-1. The radioactive peptide CHY-1 was subjected to digestion with trypsin, as outlined in the Experimental Section. The peptides were separated by electrophoresis much as outlined for the parallel peptides from TRY-1; the various peptides, and CHY-1 itself, were hydrolyzed with HCl, and the products analyzed. The peptides had the following composition: (CHY-1) $Ala_{1.05}$, $Iprlys_{0.90}$, $Leu_{1.00}$, $Lys_{1.24}$, $Pro_{0.70}$, $Ser_{1.12}$, $Tyr_{0.73}$; (CHY-1-try-1) $Ala_{1.00}$, $Iprlys_{0.90}$, $Lys_{1.11}$, $Pro_{0.76}$, $Ser_{0.80}$, $Tyr_{0.81}$; (CHY-1-try-2) $Leu_{1.00}$. The action of leucine amino peptidase on CHY-1 gave the data of Table II.

Table II. Action of Leucine Amino Peptidase on CHY-1

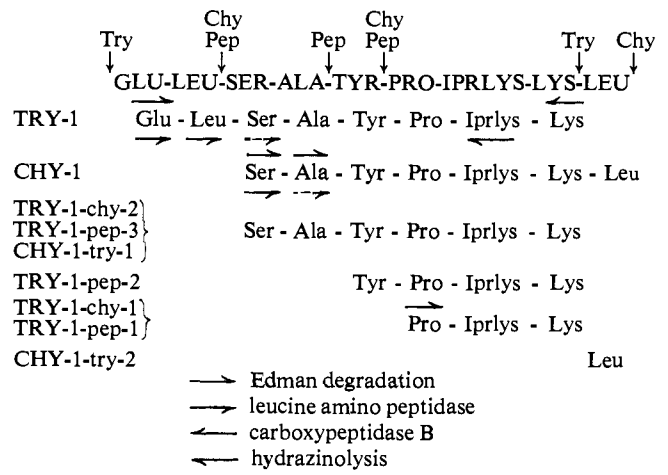
Time, hr	μmole				
	Peptide (initial)	Ser	Ala	Tyr	Others
5	0.014	Trace	0	0	0
24	0.014	0.003	Trace	Trace	0

These results were confirmed with a two-stage Edman degradation of CHY-1, which gave the following composition for the neutral and acidic amino acids in the radioactive peptides: (first stage) $Ser_{0.08}$, $Ala_{1.05}$, $Tyr_{0.78}$, $Pro_{0.95}$, $Leu_{1.00}$ (Iprlys, Lys); (second stage) $Ser_{0.07}$, $Ala_{0.29}$, $Tyr_{0.70}$, Pro_1, $Leu_{1.00}$ (Iprlys, Lys). As before, the presence of isopropyllysine was shown by radioactivity. These data show that serine and alanine, in that order, are the N-terminal amino acids of CHY-1.

Discussion

The data are summarized in Chart I. They establish the sequence at the active site of acetoacetate decarboxylase.

Chart I. Peptide at the Active Site of Acetoacetate Decarboxylase



(23) E. L. Smith and R. L. Hill, *Enzymes*, 4, 37 (1960).

The position of each of the amino acids has been established by more than one method. All the analyses (except that for CHY-1-try-2, which is simply leucine) have been conducted with radioactive peptides. Provided that the analysis of both TRY-1 and CHY-1 are sufficiently accurate to show that in each peptide only one molecule is present of each of the eight amino acids detected, then the compositions of the peptides can be established even with analyses of only moderate accuracy; the only decision to be made is between one residue of an amino acid, or none.

In detail, the C-terminal residue (leucine) of CHY-1 has been liberated with trypsin, indicating a lysine in the penultimate position. The composition of TRY-1-chy-1 and TRY-1-pep-1 indicate proline as well as isopropyllysine (by analysis and radioactivity) and lysine; the order from the carboxyl end of the peptide has been suggested by degradation with carboxypeptidase and by hydrazinolysis, and the order from the amino end established by Edman degradation. The composition of TRY-1-chy-2, TRY-1-pep-3, and CHY-1-try-1 show that serine, alanine and tyrosine are the next amino acids from the carboxyl end, and both Edman degradation and the action of leucine aminopeptidase place these amino acids in the order shown in Chart I. The position of tyrosine is confirmed by the composition of the neutral amino acids in TRY-1-pep-2. The N-terminal amino acids of TRY-1 have been found by Edman degradation and by the action of leucine aminopeptidase.

The hydrolysis of a tyrosyl–proline bond in TRY-1 by the action of either chymotrypsin or pepsin is unusual,[24] but similar cleavages have, on rare occasion, been reported. Thus, Leonis, *et al.*,[25] report the cleavage of a phenylalanyl–proline bond by chymotrypsin in α-corticotropin. Bell[26] reports the cleavage of the same type of bond by pepsin in ACTH, and Konigsberg and Hill[27] report two examples of such a cleavage by pepsin in the α-chain of hemoglobin. Other cleavages of peptide bonds to proline by pepsin have also been recorded.[28]

On the other hand, not only are most tyrosyl–prolyl and phenylalanyl–prolyl bonds resistant to the action of chymotrypsin and pepsin, but preliminary experiments in our laboratory showed no action of chymotrypsin on a synthetic sample (of uncertain optical purity, however) of N-acetyltyrosylproline methyl ester.[11]

Nevertheless, the amino acid analyses clearly establish that TRY-1-chy-1 and TRY-1-pep-1 contain proline, isopropyllysine, and lysine, and no other amino acids, and the Edman degradation leaves little doubt as to the sequence.

The complete sequence of amino acids at the active site of acetoacetate decarboxylase (Chart I) should be compared with those already published for other Schiff base enzymes. The partial sequence of 3-glycerophosphate aldolase[29] is: Ala-Leu-Ser-Asn-His-His-Ileu-Tyr-Leu-Gln-Gly-Thr-Leu-Leu-Lys*-Asn-Pro-Met-Val-Thr-Pro-Gly-His-Ala-Cys-Thr-Gln-Lys. It bears no obvious relationship to the active site of the decarboxylase. Further, the resemblances to our peptide of the published sequences at the active sites for glutamic–aspartic transaminase and of phosphorylase *b* are possibly misleading; probably our enzyme is the only one so far established with two adjacent lysine residues at the active site. Two versions have been published for the site for glutamic–aspartic transaminase after reduction with borohydride: -Ser-Thr-Glu-(Ala,Asp,Gly,Ileu,Lys,Pyridoxyllys)-Gly-Ser-Asp-Phe-[30] and -Lys-Pyridoxyllys-Ser-Asp-Phe-.[31] However, the sequence -Pyridoxyllys-Lys-Phe-, originally reported[30] for phosphorylase *b*, has since been corrected[32] to -Pyridoxyllys-Phe. The free lysine that is found on hydrolysis of this peptide apparently arises from the decomposition of pyridoxyllysine, and Fischer[33] has suggested that a similar decomposition may also account for the lysine found in the peptide from glutamic–aspartic transaminase. No such ambiguity in sequence obtains for acetoacetate decarboxylase. First, isopropyllysine is stable to hydrolysis in 6 *N* HCl. Second, quantitative data show that lysine and isopropyllysine are present in various peptides (*e.g.*, TRY-1) in approximately equal amounts. And, finally, the two amino acids were cleanly separated under mild conditions by the action of carboxypeptidase B on TRY-1.

If Fischer's suggestion proves to be correct, then the active site of acetoacetate decarboxylase does not resemble that of glutamic–aspartic transaminase or of phosphorylase *b* or of glycerophosphate aldolase, except in that each contains an active lysine residue. Of course, these enzymes come from quite different sources and have different functions. Nevertheless, the similarities in structure[34] noted among various esterases (admittedly from similar sources) is absent for the lysine enzymes.

It is tempting to speculate that the second lysine residue in acetoacetate decarboxylase is part of the positive binding site for the enzyme, a site demonstrated by observing the inhibition of the enzyme by anions.[35] However, the pK of the binding site for anions[35] is apparently only 5.8, and hence is more probably a histidine than a lysine residue. Further investigations of the active site of acetoacetate decarboxylase are in progress.

(24) R. G. Shepard, S. D. Willson, K. S. Howard, P. H. Bell, D. S. Davies, S. B. Davis, and N. E. Shakespeare, *J. Am. Chem. Soc.*, **78**, 5067 (1956); J. Goldstein, W. Konigsberg, and R. J. Hill, *J. Biol. Chem.*, **238**, 2016 (1963); W. Konigsberg, J. Goldstein and R. J. Hill, *ibid.*, **238**, 2028 (1963); B. S. Hartley, *Nature*, **201**, 1284 (1964).

(25) J. Leonis, C. H. Li, and D. Chung, *J. Am. Chem. Soc.*, **81**, 419 (1959).

(26) P. H. Bell, *ibid.*, **76**, 5565 (1954).

(27) W. Konigsberg and R. J. Hill, *J. Biol. Chem.*, **237**, 3157 (1962).

(28) F. A. Anderer, H. Uhlig, E. Weber, and G. Schramm, *Nature*, **186**, 922 (1960).

(29) C. Y. Lai, P. Hoffee, and D. Morse, *Federation Proc.*, **24**, 666 (1965); B. L. Horecker, private communication.

(30) R. C. Hughes, W. T. Jenkins, and E. H. Fischer, *Proc. Natl. Acad. Sci. U. S.*, **48**, 1615 (1962).

(31) O. L. Polianovsky and B. Keil, *Biokhimia*, **28**, 372 (1963).

(32) C. Nolan, W. B. Novoa, E. G. Krebs, and E. H. Fischer, *Biochemistry*, **3**, 542 (1964).

(33) E. H. Fischer, private communication, 1965.

(34) P. Desnuelle, *Enzymes*, **4**, 93 (1960); B. Keil, *Ann. Rev. Biochem.*, **34**, 175 (1965).

(35) I. Fridovich, *J. Biol. Chem.*, **238**, 592 (1963).