DETERMINATION OF THE C-TERMINAL STATUS OF RAT LIVER LACTATE DEHYDROGENASE BY AN IMPROVED SELECTIVE TRITIATION METHOD

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SUMMARY

Attempts to determine the C-terminal status of rat liver lactate dehydrogenase by the procedures of (a) hydrazinolysis, (b) controlled carboxypeptidase action, and (c) selective tritiation have led to inconclusive results. However, the use of a procedure which involves improved selective tritiation followed by short-term carboxypeptidase action has led to an unequivocal demonstration that phenylalanine is the C-terminal amino acid of rat liver lactate dehydrogenase. The procedure involves: (a) dissolution of the enzyme in tritiated water, (b) sequential addition of pyridine and acetic anhydride, (c) removal of excess reagents by dialysis, (d) short-term carboxypeptidase treatment, and (e) identification of the C-terminal amino acid by chromatography and radioassay.

For the determination of C-terminal amino acid residues of polypeptides or proteins, controlled carboxypeptidase treatment, hydrazinolysis, and selective tritiation have been used. These procedures, however, suffer from various limitations which have been extensively discussed (1,2,3,4). Attempts to determine the C-terminal amino acid residue(s) of rat liver lactate dehydrogenase (E.C. 1.1.1.27, L-Lactate: NAD Oxidoreductase) by these procedures, therefore, have led to inconclusive results.

This report describes the application of a procedure which involves improved selective tritiation of the C-terminal amino acid residue followed by short-term carboxypeptidase treatment. These steps have led to an unequivocal demonstration that phenylalanine is the C-terminal amino acid of rat liver lactate dehydrogenase.

EXPERIMENTAL PROCEDURE

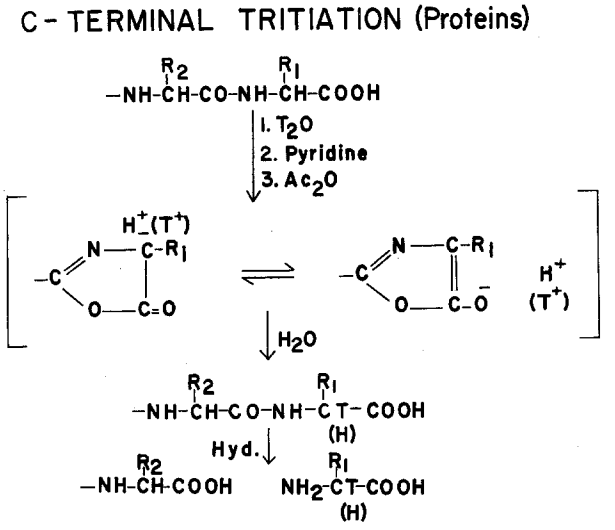
Materials

Diisopropyl phosphorofluoridated carboxypeptidases A and B were obtained from the Worthington Biochemical Corporation, Freehold, N. J., and the Mann Research Laboratories, New York, N. Y. Tritiated water was obtained from the New England Nuclear Corporation, Boston, Mass.

Methods

Rat liver lactate dehydrogenase was purified and assayed as previously described (5). For C-terminal amino acid studies, the purified enzyme was dialyzed against 0.05 M ammonium acetate and lyophilized. The lyophilized sample was stored in the presence of P_2O_5 under reduced pressure.

Selective Tritiation Method: A procedure modified from Matsuo et al. (6) and Holcomb et al. (4) was followed (Scheme 1). The sample, 4 mg, was dis-



Scheme 1.

solved in 0.5 ml of tritiated water (1 curie per gram). One milliliter of pyridine was added to the solution. The mixture was shaken vigorously for two minutes. The reaction was initiated by the addition of 0.5 ml of acetic anhydride. The mixture was incubated at 37° for four hours. The incubated

sample was dialyzed against one liter of water for four hours with changes of water every hour. The dialyzed preparation was taken to dryness under reduced pressure. The residue was hydrolyzed in 1 ml of constant boiling hydrochloric acid at 110° for 24 hours. The hydrolysate was dried under reduced pressure, and the residue was dissolved in 0.1 ml of water. The solution was subjected to two dimensional descending paper chromatography. Twenty microliters of the solution were spotted on Whatman No. 1 paper, 45 x 45 cm. The paper was developed by the following solvent systems: System 1; first dimension: n-butanol-acetic acid-water (4:1:5 v/v), second dimension: phenol-NH_4OH-water (75:4:21 v/v), System 2; first dimension: n-butanol-acetic acid-water (4:1:5 v/v), second dimension: isopropyl alcohol:formic acid:water (40:2:10 v/v), System 3; first dimension: isopropyl alcohol:formic acid:water (40:2:10 v/v), second dimension: t-butyl alcohol: methyl ethyl ketone: 0.88 M NH_4OH: water (50:30:10:10 v/v). The chromatography was developed by spraying with 0.3% ninhydrin and 3% acetic acid in n-butanol and by incubation at 80° for half an hour. The spots were cut from the paper and were immersed in 10 ml of Bray's solution (7) overnight. The radioactivity was measured with a Packard liquid scintillation spectrometer.

Selective Tritiation-Carboxypeptidase Method: The incubation of the sample protein in tritium water, pyridine and acetic anhydride was as described above. The incubation mixture was dialyzed against one liter of 0.01 M Tris-HCl, 0.01 M LiCl and 0.02 M NaCl, pH 8.2, buffer for four hours with changes of buffer every hour. The dialyzed mixture was treated with 0.1 ml of carboxypeptidase A and B solution (substrate : carboxypeptidase A : carboxypeptidase B, 10 : 1 : 1, mole ratio) and was incubated at 37°. Samples (0.5 ml) were taken at the specified times and incubated in boiling water for two minutes. The resulting suspensions were centrifuged to remove precipitate. The clear solutions were dried under reduced pressure, and the residues were dissolved in 0.1 ml of water. The solutions (50 µl) were subjected to chromatography using solvent system 1. The detection of amino acids and radioactivity was as described above.

RESULTS

Selective Tritiation Method: The results obtained from tritiation of the C-terminal amino acid are reported in Table 1. This experiment clearly demonstrates that phenylalanine is the C-terminal amino acid. It is not quite clear whether glutamic acid and aspartic acid are C-terminal amino acid residues. It has been observed that the tritiation of non-C-terminal glutamic acid and aspartic acid does occur in this system. A possible explanation

TABLE 1

Radioactivities of Tritiated Amino Acid Spots from Chromatography of Hydrolysate

Amino Acids	Solvent System 1	Solvent System 2	Solvent System 3
	cpm	cpm	cpm
Phenylalanine	18,660	19,580	20,560
Tyrosine	250	360	350
Leucine	350	380	420
Isoleucine	460	360	480
Methionine	650	780	720
Valine	250	310	280
Cystine	520	550	480
Alanine	360	520	450
Glycine	720	680	760
Proline	150	120	150
Serine	650	720	680
Threonine	240	280	270
Glutamic Acid	4,860	5,280	5,660
Aspartic Acid	3,200	3,100	3,600
Arginine	1,200	1,150	1,100
Histidine	1,150	1,250	1,300
Lysine	1,350	1,300	1,200
Origin	50	60	50
Background	50	40	100

for the incorporation of tritium by non-C-terminal glutamic acid and aspartic acid is that they form mixed anhydrides with acetic acid, and the resulting anhydrides undergo base catalyzed enolization. No obvious explanation can be made about the relatively smaller incorporation of tritium into other non-C-terminal amino acids. The amount of interior labeling was proportional to the incubation time and the amounts of protein. This anomaly was also observed (4).

Selective Tritiation and Carboxypeptidase Method: In order to eliminate the ambiguity which could be caused by the "interior labeling" of amino acids in the selective tritiation method, the tritiated sample was digested with carboxypeptidase A and B for a short period of time. It is hoped that by the combination of selective tritiation and carboxypeptidase digestion, the weaknesses of these two procedures could be minimized. The rate of appearance of free tritiated amino acid from a tritium labeled sample by digestion with carboxypeptidase A and B is illustrated in Figure 1. This experiment again clearly indicates that phenylalanine is the C-terminal amino acid. The possibility of glutamic acid, glutamine, aspartic acid and

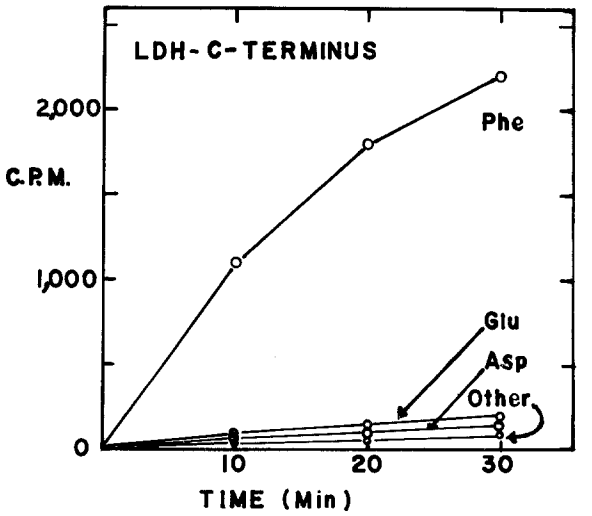


Figure 1. The rate of release of tritiated amino acids from tritiated rat liver lactate dehydrogenase with carboxypeptidase A and B.

asparagine as C-terminal amino acids could be ruled out by this experiment because no significant amount of tritiated forms of those amino acids could be identified at the early stage of carboxypeptidase digestion.

DISCUSSION

The use of carboxypeptidase as an analytical tool for the study of the C-terminal amino acid of rat liver lactate dehydrogenase is not experimentally feasible under the conditions used (1,2). This may be partially attributed to the limits imposed by the specificities of carboxypeptidase, by contamination with endopeptidases, and by differences in accessibility of certain amino acid residues. Since lactate dehydrogenase consists of four subunits, it is likely that there are at least four polypeptide chains. Thus, complication in the kinetics of the release of amino acids from complexes of polypeptide chains could be expected. Moreover, non-interpretable rates of release of amino acids could result if in a given sequence an amino acid that is released slowly by carboxypeptidase action is followed by one that is released very rapidly. It is also possible that a sequence degraded by carboxypeptidase will include more than one residue of a particular amino acid.

Difficulties by hydrazinolysis arise from the general fact that certain of the reaction products, the amino acid hydrazides, are not stable and undergo hydrolytic decomposition. The problems of general instability are minimized by quickly separating the free amino acids from the hydrazides. However, potential causes for error are numerous.

In selective tritiation procedures, one encounters extensive "interior labeling" of amino acid residues which makes it difficult to be certain that only one amino acid (or perhaps two or three) is primarily labeled as a result of its C-terminal position.

The combination of two procedures which have different limitations offers a demonstrated advantage. Thus, the procedure involving selective

tritiation and subsequent action of carboxypeptidases for a short period of time is probably the most convincing one for the qualitative identification of the C-terminal amino acid(s) of large proteins containing several polypeptide chains. It has not been possible to develop a quantitative method involving the selective tritiation reaction since oxazolone formation is readily reversed under the conditions used.

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