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Amino- and Carboxyl-terminal Analyses of Hepatoma Lactate Dehydrogenase Isozymes[1]

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SUMMARY

The M_4 isozyme of lactate dehydrogenase was purified to homogeneity from normal rat liver and from two Morris hepatomas (7777 and 7793). Amino-terminal analyses with fluorodinitrobenzene failed to detect the presence of free amino-terminal residues in each enzyme studied. Each enzyme contained between 3.7 and 4.1 moles of protein-bound acetyl groups per mole of enzyme. The amino-terminal peptide, characterized as *N*-acetylalanylalanine, was isolated from Pronase digests of each isozyme preparation, and quantitative recovery experiments indicated that all acetyl residues were bound at the amino termini. Carboxyl-terminal analyses demonstrated phenylalanine to be the carboxyl-terminal residue in each enzyme studied. These data indicate no differences in either amino- or carboxyl-terminal regions of the hepatoma M_4 isozymes compared to normal liver M_4 isozyme.

INTRODUCTION

Rees and Huggins (16) showed that a number of tumors possess a very high LDH[3] activity, and Huggins (12) suggested that the capacity to synthesize appreciable quantities of this enzyme is 1 definitive characteristic of cancerous tissue. Boxer and Devlin (2) proposed a plausible explanation for these observations with their report that glycerol-3-phosphate dehydrogenase and β-hydroxybutyrate dehydrogenase enzymes were missing in a number of tumorous tissues. They suggested that biochemical alterations in LDH synthesis might occur as a mechanism to promote regeneration of NAD^+ from the NADH formed during the anaerobic glycolysis of glucose.

More recent studies, particularly those carried out with the Morris hepatomas, suggest that elevated glycolytic rates are not characteristic of all malignant tissues. These studies indicate that respiration decreases moderately with a loss of differentiation in the tumor type. The well-differentiated hepatomas have low or negligible rates of glycolysis when compared to the high levels noted in the poorly differentiated hepatomas (22, 23).

Several investigators have reported the presence of specific LDH isozymes in malignant tissue with properties that differ from those of the same isozyme normally found in the tissue (7, 13, 15). These reports suggest the possibility that an altered or different LDH isozyme might be synthesized by some tumors. We have studied the amino- and carboxyl-terminal residues of M_4 isozymes of LDH isolated from normal rat liver, from a well-differentiated trabecular carcinoma of minimal metabolic deviation (Morris Hepatoma 7793), and from a poorly differentiated hepatoma expected to exhibit greater metabolic deviation (Morris Hepatoma 7777).

MATERIALS AND METHODS

The transplantable Morris hepatomas (7777 and 7793) were grown in the rear legs of young Buffalo male rats (150 to 200 g body weight). Hepatoma 7777 required about 1 month for development, while Hepatoma 7793 required approximately 4 months. The tumors were excised, freed from adhering tissue, frozen on solid CO_2, and stored at 20°.

The specific M_4 LDH isozymes studied were purified to homogeneity from normal rat liver and from Morris Hepatomas 7777 and 7793 using methods described previously (17). The proteins were homogeneous as judged by electrophoresis (starch gel, polyacrylamide gel, polyacrylamide-sodium dodecyl sulfate), immunochemical characterization (5), and specific catalytic activity (5). All enzymes had similar specific activities.

Amino-terminal analyses using fluorodinitrobenzene were carried out as described earlier for rat liver LDH (21). Carboxyl-terminal residues of the intact tumor LDH enzymes were determined by a modification of Method C of Holcomb *et al.* (10). The proteins (3.3 mg tumor 7777 enzyme, 4.3 mg tumor 7793 enzyme, and 4.8 mg normal liver enzyme) were each dissolved in 0.5 ml pyridine, 0.15 ml water, and 0.1 ml tritiated water (5 Ci/g; New England Nuclear, Boston Mass.) at room temperature. The reactions were initiated by the addition of 0.125 ml acetic anhydride followed by vigorous shaking. After 1.5 hr an additional 0.125 ml acetic anhydride was added to each, and the mixtures were allowed to stand overnight. The preparations were thoroughly dialyzed against water and the lyophilized materials were hydrolyzed (1 ml 6 N HCl, at 105°, for 72 hr). The hydrolysates were lyophilized and analyzed by simultaneous amino acid and radioactivity analysis as previously described (3).

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[3] The abbreviation used is: LDH, lactate dehydrogenase.

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The N-[^{14}C]-acetylalanylalanine and the N-acetyl derivatives of alanine, alanylalanine, and alanylalanylalanine were obtained as previously described (4). High-voltage electrophoresis was performed in 3.3% acetic acid titrated to pH 3.6 with pyridine (4). The N-acetylated peptides were detected by the method of Rydon and Smith (18). Acetyl group analyses were carried out by the microenzymic assay of Stegink (20).

The amino-terminal acetylated peptide was isolated from protease digests of each M_4 LDH isozyme using the basic technique first developed by Narita (14). Samples of heat-denatured LDH isozymes (220 mg tumor 7777 enzyme, 18 mg tumor 7793 enzyme, and 200 mg normal liver enzyme) containing tracer quantities of N-[^{14}C]-acetylalanylalanine were digested with 7 mg protease (39°, pH 7.9, 22 hr, 1 mM $CaCl_2$, 100 ml) previously chromatographed on Sephadex G-25 to remove free acetate. Each digest was acidified to pH 3.5, centrifuged to remove insoluble matter, passed over AG 50W-X2 (H^+) columns (200 to 400 mesh, 3.5 x 31 cm; Bio-Rad Laboratories, Richmond, Calif.) and the effluent (325 ml) was concentrated by lyophilization. The residue from each column was dissolved in a small volume of water, neutralized, and chromatographed on a Rexyn 201 (Cl^-) column (200 to 400 mesh, 1 x 58 cm; Fisher Scientific Co., Pittsburgh, Pa.) with a linear gradient of 140 ml water and 140 ml 0.02 N HCl. The effluent was collected in 2-ml fractions and pooled according to absorbance at 215 nm, and the individual pooled fractions were lyophilized. The residue was dissolved in a small amount of water in each case, assayed for the presence of N-acetyl groups, and analyzed for the amino acid content after hydrolysis for 15 hr in constant boiling HCl at 110°.

RESULTS

Previous studies in our laboratories have shown that phenylalanine is the carboxyl-terminal residue and N-acetylalanylalanine the amino-terminal peptide in normal M_4 rat liver LDH (11, 19). We wished to compare directly the normal M_4 isozyme with those isolated from hepatoma sources. Amino-terminal analyses using fluorodinitrobenzene failed to detect molar quantities of any amino-terminal amino acid, indicating the presence of a blocked amino-terminal residue in each isozyme studied, Acetyl group analysis of each preparation of M_4 isozyme yielded values of 3.7 to 4.1 moles of acetyl groups per mole of enzyme in each case.

The acetylated, amino-terminal peptide was isolated from protease digests of each of the M_4 LDH isozymes studied using the technique first developed by Narita (14). The protease digests were acidified, passed through an AG50W-X2 column, and chromatographed on Rexyn 201 (Cl^-) columns. In each case, chromatography resolved the mixture into 4 peaks as shown for the normal liver enzyme and the hepatoma 7777 enzyme in Chart 1. Fraction C was ninhydrin negative in each case, and it was the only fraction containing N-acetyl groups.

Amino acid and acetyl group analyses after acid hydrolysis of Fraction C isolated from each isozyme are shown in Table 1. These data show stoichiometric quantities of acetate and alanine in a 1:2 ratio, with only minor quantities of other amino acids except glutamate. The identity of the acetylated peptide as acetylalanylalanine was confirmed by high-voltage electrophoresis using N-acetylalanine, N-acetylalanylalanine, and N-acetylalanylalanylalanine as standards (4). In each case the peptide migrates with N-acetylalanylalanine and separates well from N-acetylalanine and N-acetylalanylalanylalanine.

Tracer quantities of N-[^{14}C]-acetylalanylalanine were added to each preparation during the Pronase digestion to determine what portion of acetyl groups present on the original enzyme was recovered as N-acetylalanylalanine and to correct for the loss of material during the isolation procedure. After correction for the loss of labeled internal standard peptide during the isolation procedure (16%), all of the acetyl groups present in each of the original LDH isozyme preparations could be accounted for as N-acetylalanylalanine.

Carboxyl-terminal selective tritiation analyses of the LDH isozymes studied are shown in Table 2. Each M_4 isozyme was shown to contain phenylalanine as the carboxyl-terminal amino acid.

DISCUSSION

A number of enzymes in the glycolytic pathway are present as tissue-specific enzyme variants in differentiated tis-

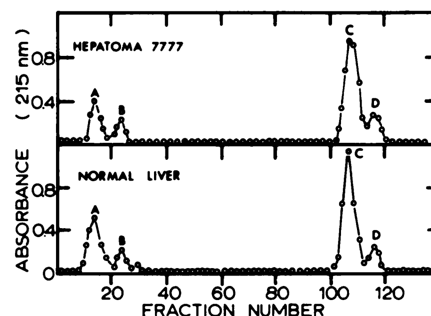

Chart 1. The elution pattern of peptides from Rexyn 201 (Cl^-) column, from normal rat liver M_4 LDH, and from Morris Hepatoma 7777 M_4 LDH using a water:0.02 N HCl gradient.

Table 1

Composition of peak C isolated by Rexyn 201 chromatography from protease digests of M_4 LDH isozymes isolated from normal rat liver and Morris Hepatomas 7777 and 7793

	Normal liver		Hepatoma 7777		Hepatoma 7793	
Compound	μmole	Amino acid: acetate	μmole	Amino acid: acetate	μmole	Amino acid: acetate
Acetate	0.118		0.350		0.134	
Aspartate	0.009	0.076	0.008	0.022	0.01	0.075
Threonine	0.023	0.195	0.00	0	0.005	0.037
Serine	0.023	0.195	0.025	0.071	0.017	0.127
Glutamate	0.025	0.211	0.110	0.314	0.043	0.320
Alanine	0.229	1.94	0.710	2.03	0.317	2.37
Valine	0.019	0.161	0.009	0.026	0.009	0.067

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Table 2
C-terminal tritium exchange analyses of intact M_4 LDH isozymes isolated from normal rat liver and Morris Hepatomas 7777 and 7793

	Counts/4 min[b]		
Amino acid[a]	Normal liver	Hepatoma 7777	Hepatoma 7793
Aspartate	5,895	4,301	6,133
Glutamate	4,936	4,532	3,425
Leucine	2,107	4,184	2,497
Phenylalanine	36,602	31,817	27,727
Histidine	2,611	4,074	1,950

[a] Only amino acids containing significant radioactivity.
[b] Not quench corrected.

sues, and a number of specific changes in these forms have been reported in tumor tissues. Such alterations have been reported for lactate dehydrogenase (7, 9, 13, 15), pyruvate kinase (6), and fructose diphosphate aldolases (8). Weinhouse *et al.* (22, 23) have reported alterations in the isozyme composition of a number of enzymes of the glycolytic pathway in liver tumors, noting a reversion to the prenatal form. Isozymes of glucose-ATP phosphotransferase, aldolase, and pyruvate kinase were altered in a manner suggesting a reactivation of genes in the tumor which had been repressed during embryonic development.

The data presented in the present study indicate that the M-type polypeptide chain of LDH synthesized by both the well-differentiated (7793) and the poorly differentiated hepatoma (7777) have identical amino- and carboxyl-terminal sequences as the normal liver chain. Thus the M chain synthesized appears similar to the usual vertebrate M-type chain (4) rather than that noted in the dogfish (Chondrichthyes) M-type chains (1) where *N*-acetylthreonylalanylleucine is found at the amino termini.

Our data, although dealing with only a limited portion of the polypeptide chain, support recent studies by Carlotti *et al.* (5). They noted no difference between the highly purified M_4 LDH from hepatomas 7777 and 7793 and normal rat liver using heat inactivation, oxamate inhibition, immunodiffusion and immunoelectrophoresis, enzyme inhibition by antiserum directed against liver M_4 LDH, starch gel electrophoresis, and polyacrylamide gel electrophoresis techniques.

Although the LDH isozyme patterns of tumor tissues generally contain increased proportions of M-type subunits (24), a number of cases have been reported in which an increased proportion of H-type subunits occurs (see review, Ref. 9). Although a number of investigators have reported extra or unique LDH isozymes in tumor tissues, our data indicate that the presence of such isozyme forms is not a requisite characteristic of neoplastic tissue. Many reports of unique isozymes are based on electrophoretic evidence. However, alterations in electrophoretic characteristics may be due to the complexing of the isozyme with other components, rather than being caused by an altered chemical structure. Recently, abnormal mobilities of serum LDH isozymes have been noted resulting from complex formation with immunoglobulins or with other serum proteins (see review, Ref. 9). Also, the occurrence of extra LDH isozymes is not a unique characteristic of neoplastic disease. Such alterations have been reported in both healthy individuals and those with nonmalignant diseases (see review, Ref. 9).

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