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CHARACTERIZATION OF MESSENGER RNA FOR ALDOLASE B IN ADULT AND FETAL HUMAN LIVER

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High molecular weight cellular RNA was isolated from adult and fetal human liver tissue by a procedure of ethanol precipitation in concentrated guanidine-HCl solutions. About 5 mg of RNA were obtained from one gram of liver. RNA was fractionated by sucrose gradient ultracentrifugation. Aldolase B neosynthesized in a reticulocyte lysate cell-free system under the direction of total or fractionated RNA was purified by immunoaffinity microchromatography. Messenger RNA specifying synthesis of aldolase B exhibited a sedimentation coefficient of 16 S both in adult and fetal liver. This enzyme represented 1.3 % of the total neosynthesized proteins in adult liver, 0.1 % in the liver of a 6-month-old fetus and less than 0.01 % in the liver of a 4.5 month-old fetus.

INTRODUCTION

Cell-free synthesis and detection of neosynthesized human enzymes are the first step for the preparation of specific messenger RNAs and characterization of the corresponding genomic DNA. It may also be a tool for studying mechanisms of some inherited metabolic disorders. Our group has developed methods for purification of total RNA from small amounts of human tissues. This has allowed us to obtain, for the first time to our knowledge, the cell-free synthesis of several human muscle enzymes (1) and of human liver pyruvate kinase (2).

The enzyme, aldolase, is present in the form of three genetically distinct isozymes (3), muscle type (A), liver type (B), brain type (C). Only the liver type is able to metabolize fructose : its deficiency is responsible for an inborn error of metabolism, Hereditary Fructose Intolerance (4, 5, 6).

Cell-free synthesis of aldolase A has been reported under the direction of chicken muscle RNA (7) and rat hepatoma RNA (8) since it is known that, in hepatoma, adult liver aldolase is replaced by "resurgent fetal isozymes" (9). Cell-free synthesis of aldolase B has been described on adult rat (10,11), but not yet on fetal mammals or in man. The present paper

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reports the synthesis of human aldolase B directed by human liver RNA in a cell-free rabbit reticulocyte system.

MATERIAL AND METHODS

. Material

Human adult liver was obtained after surgical partial hepatectomy. Human fetal liver was obtained after therapeutical abortion of 2 fetuses at 4.5 and 6 months of intra-uterine life.

Glutaraldehyde-activated "Ultrogel" was supplied by Industrie Biologique Française ; acrylamide, bisacrylamide, sodium dodecylsulfate, No Screen and Royal-X-Omatic R-1 films by Eastman Kodak ; some ^{14}C markers, ^{35}S methionine, Econofluor, Protosol, En^3 Hance autoradiography Enhancer by New England Nuclear ; methylmercuryhydroxide by Ventron-alpha Lab. Other reagents were of the highest purity grade and came from Sigma CC and from Merck.

. Methods

Preparation of aldolase and of specific antibodies

Human aldolase B was prepared from liver obtained at autopsy according to Penhoet and Rutter (12) with modifications. Isolation involved ethanol precipitation, followed by phosphocellulose chromatography and specific elution with 5 mM fructose-1-phosphate. The aldolase preparation showed only one band after sodium dodecylsulfate electrophoresis. Antibodies were produced by repeated intradermal injection into rabbits. They were monospecific and gave only one band against a liver extract on Ouchterlony plates. Specific antibodies were purified by chromatography on an aldolase B-linked agarose bead column (1).

Isolation of RNA

Total cellular RNA from human liver was isolated by a method of ethanol precipitation in guanidine HCl, slightly modified from Cox (13), Harding et al (14) and Deeley et al (15) and described in detail in (1) for isolation of human adult muscle RNA. For liver, some further modifications were brought to this technique : first ethanol precipitation was for 30 min at -20° C (instead of overnight) and 0.5 % (w/v) lauroyl sarcosine was added to all the solutions until the last two steps of washing in 66 % (v/v) ethanol.

Sucrose gradient sedimentation

250 µg of RNA were dissolved in 250 µl of a 20 mM borate buffer (pH 8) containing 20 mM methylmercuryhydroxide ; this solution was incubated at room temperature for 10 min, then diluted with 250 µl of a 10 mM Tris-HCl buffer (ph 7.5) containing 100 mM NaCl, 1 mM EDTA, 10 mM iodoacetate and 0.1 % (w/v) lauroylsarcosine. After centrifugation at 10 000 x g for 1 min, this sample was deposited at the top of 11 ml tubes containing a 5-20 % (w/v) sucrose gradient in the Tris-HCl buffer described above. The tubes were centrifuged at 20° C in a SW 41 Beckman rotor at 40 000 RPM for 5 h. 0.4 ml fractions were collected, RNA was precipitated with ethanol and used to direct cell free-synthesis.

Cell free translation and electrophoresis

Cell-free translation was performed in a nuclease-treated reticulocyte lysate according to Pelham and Jackson (16).

Neosynthesized aldolase B was purified by immunoaffinity microchromatography, as reported elsewhere (1), except that elution of the antigen was provoked by incubation of the immunoabsorbent in 50 µl of a 62.5 mM Tris-HCl buffer (ph 6.5) containing 2 % (w/v) sodium dodecyl sulfate, 5 % (v/v) β-mercaptoethanol, 10 % (v/v) glycerol and 2 mM diisopropylphosphofluoridate at 98° C for 3 min. Analysis was made by SDS polyacrylamide gel electrophoresis according to Laemmli (17), using ^{14}C labeled proteins as molecular weights standards (18). Gels were dried and the immunopurified polypeptides were revealed by autoradiography using Kodak NS films or by fluorography using preflashed XR-1 films (19). Specificity of the neosynthesized bands was checked by immunological competition with unlabeled aldolase (1).

RESULTS

The method of high molecular weight total cellular RNA isolation allowed to obtain, starting from one gram of liver, about 5 mg RNA. 260 : 280 absorbance ratio was about 2. Sucrose density gradient analysis of RNA showed sharp peaks of 18S and 28S ribosomal RNA in the ratio of 1:1.8 to 1:2. RNA prepared by the above procedure directed synthesis of numerous polypeptides, which could be analyzed by one dimensional SDS-polyacrylamide gradient gel electrophoresis. As already shown previously (1), a relatively high proportion of high molecular weight-chains (> 60 000 daltons) argues for the integrity of the RNA used.

Fig. 1 shows that it is possible, starting from a crude mixture of in vitro translation products whose synthesis was directed by human liver RNA, to purify by immunoaffinity microchromatography a neosynthesized polypeptide with similar molecular weight to that of aldolase B purified from human liver. For comparison, neosynthesized aldolase has been deposited on the electrophoresis slab gels between ^{14}C labeled (18) pure aldolase preparations, purified either by the usual procedure of specific elution from phospho-cellulose columns by fructose-1-phosphate or by immunoaffinity microchromatography starting from fresh, crude liver extract. Neosynthesized aldolase B proved to have exactly the same molecular weight as both ^{14}C aldolase markers.

Identification of the 38 000 band as neosynthesized aldolase B was confirmed by immunological competition as reported in ref. 1 and 2 : this polypeptide was not (or only slightly) retained by immunoabsorbent previously saturated by excess unlabeled aldolase, then was retained by a non-saturated immunoabsorbent column (not shown).

A further argument for the conclusion that aldolase was synthesized in the cell-free system with its definitive molecular weight comes from the fact that the addition of various antiproteolytic agents (1,2) did not modify the size of the aldolase subunit.

Since purified neosynthesized aldolase B appeared to be absolutely pure (fig. 1), the amount of enzyme synthesized under such conditions could be di-

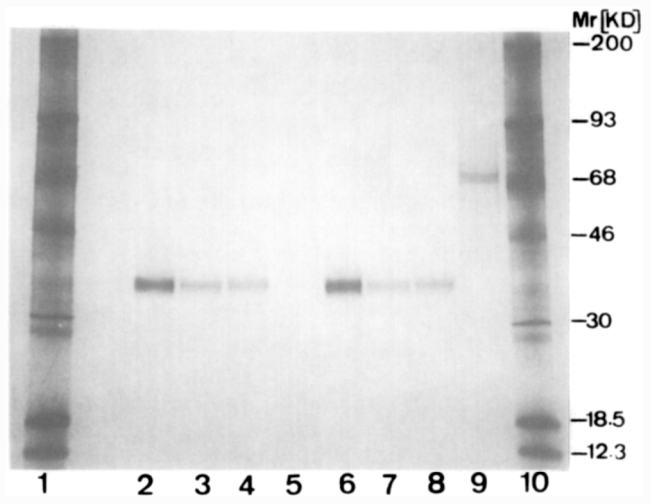

Figure 1. *SDS-polyacrylamide gel electrophoresis of ^{35}S labeled neosynthesized aldolase ; comparison with ^{14}C labeled enzyme purified from adult liver.* Channels 1 and 10 : ^{14}C labeled protein markers : Myosin heavy chain (Mr = 200 000) ; Muscle phosphorylase B (Mr = 93 000) ; Bovine serum albumin (Mr = 68 000) ; Ovalbumin (Mr = 46 000) ; Carbonic anhydrase (Mr = 30 000) ; Lactoglobulin A (Mr = 18 500) ; Cytochrome C (Mr = 12 300) ; channels 2 and 6 : Aldolase B purified from adult liver by affinity elution, from a phosphocellulose column, then ^{14}C labeled ; channels 3 and 7 : ^{35}S labeled neosynthesized aldolase B purified by immunoaffinity microchromatography (1,2) ; channels 4 and 8 : Aldolase B purified from liver crude extract by affinity microchromatography, then ^{14}C labeled ; channel 5 : Eluate from a microcolumn containing 10 µl of "ultrogel" coupled to non immune γ globulins, to which the medium containing the translation products has been applied ; channel 9 : Neosynthesized serum albumin. Cell-free synthesis was performed as described (1,2), in a total reaction mixture of 75 µl containing 22.5 µg total high molecular weight cellular RNA from adult liver and 1.5 m Ci [^{35}S] methionine (specific activity > 1000 Ci/mmol). Under these conditions, radioactivity incorporation into Trichloracetic precipitable material was about 150 000 CPM per µl of translation mixture, with a blank without addition of exogenous RNA of about 5000 to 6000 CPM/µl. Neosynthesized albumin represented 8 % and aldolase B 1.3 % of the total neosynthesized proteins. After electrophoresis according to Laemmli (17), the gel (10-20 %, w/v, acrylamide) was dried and exposed for 5 days to a "No Screen-1" Kodak film.

rectly measured from the TCA-precipitable radioactivity found in the eluate of the antibody-bound resin column. It represented 1.3 % of the proteins neosynthesized under the direction of adult liver RNA, 0.11 % using RNA from a 6-month-old fetus and less than 0.01 % using RNA from a 4.5-month-old fetus.

Ultracentrifugation of RNA in sucrose gradient after denaturation by high concentration of methylmercuryhydroxide enabled sedimentation coefficient of RNA for aldolase B to be determined : it was found to be 16 S for both adult and fetal RNA.

DISCUSSION

The present work demonstrates that it is possible, starting from total RNA extracted from fresh human liver tissue, to isolate in one step pure

neosynthesized liver aldolase subunits. They bind to specific immunoabsorbent microchromatography columns and compete with the authentic enzyme on columns saturated with unlabeled pure aldolase. No impurity is seen on autoradiographs prepared from electrophoretic gels of neosynthesized aldolase. The subunit molecular weight of 38 000 was somewhat smaller than the published values of 41 000 (20).

Several papers have been published about cell-free synthesis of liver enzymes in animals, but none in man ; it was, therefore, important to establish the possibility of such a work in man, and to evaluate the amount of liver tissue necessary to reach success. Oda and Omura (10) have shown that, contrary to serum albumin, aldolase is mainly synthesized on free ribosomes in rat livers. Aldolase is a cytosolic enzyme and, therefore, it is not astonishing that it is synthesized with its definitive molecular weight. The pure aldolase preparation used to elicit antibodies appears to have the same molecular weight as neosynthesized aldolase.

The high amount of neosynthesized aldolase may be of great practical importance in pathological studies. Our evaluation that aldolase represents more than 1 % of total protein synthesis in liver is in good agreement with the report of Oda and Omura (10) stating that about 0.5 % of total nascent peptides on free ribosomes from rat liver was aldolase. Given the high yield of liver RNA : 5 mg per gram of tissue, as compared to 100 µg per gram of adult muscle (1 and Munich et al, in preparation) and the high efficiency of translation, one may conclude that 10 mg of biopsy material would be sufficient to study the messenger RNA activity for aldolase. Work is in progress to apply these data to the molecular study of Hereditary Fructose Intolerance.

It has been established that fetal development was associated with a progressive disappearance in the liver of the fetal isozymes, aldolase A and C, correlated with a parallel increase of aldolase B (9). This work demonstrates that increase of liver aldolase B during development is due to increasing accumulation of translatable specific messenger RNA : a functional assay shows that aldolase B messenger RNA activity increases more than 10 fold between

4.5 and 6 months of intra-uterine life, then more than 10 fold between this latter fetal stage and the adult life.

The sedimentation coefficient found for aldolase B messenger RNA (16 S) is that expected for a 40 000 dalton protein, which signifies that this RNA does not contain extensive 3' or 5' untranslated sequences.

In conclusion, we have been able to isolate and characterize liver aldolase neosynthesized in a cell-free system under the direction of human liver total RNA. The yield of active aldolase messenger RNA in adult liver is such that it could be a good starting point for the preparation of a cDNA probe, and that it opens the possibility of analyzing needle biopsy material for the presence and action of mRNA in liver aldolase deficiencies. Increase of aldolase B synthesis during fetal development of human liver is shown to be due to increasing accumulation in the liver of translatable specific messenger RNA. Finally, messenger RNA specifying aldolase B synthesis was shown to sedimente in sucrose gradient as a 16 S species.

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