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RELATIONSHIPS AMONG THE MULTIPLE MOLECULAR FORMS OF RAT LIVER GLUCOSE 6-PHOSPHATE DEHYDROGENASE

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SUMMARY

1. An improved procedure is described for the purification of rat liver glucose 6-phosphate dehydrogenase(D-glucose 6-phosphate:NADP$^+$ oxidoreductase, EC 1.1.1.49).

2. The active enzyme is formed from inactive subunits of mol. wt. 64 000.

3. The purified enzyme exists in at least three different dimeric forms of mol. wt. 130 000 which account for 90% of the total activity. These dimers can then aggregate to form tetrameric and hexameric forms which account for almost all of the remaining activity.

4. Each of the dimeric forms of the enzyme shows immunological identity with the other dimeric forms and with the higher molecular weight aggregates of these dimers when using double diffusion plates and an antiserum against the purified rat liver enzyme.

INTRODUCTION

We are currently investigating the regulation of rat liver glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate:NADP$^+$ oxidoreductase, EC 1.1.1.49) levels *in vivo*. Recent estimates of the rate of synthesis and degradation of this enzyme[1] were based upon measurements of the total glucose 6-phosphate dehydrogenase activity in crude rat liver supernatant fractions. When subjected to electrophoresis on polyacrylamide gels, such fractions have been shown to contain several different bands staining for glucose 6-phosphate dehydrogenase activity. HORI AND MATSUI[2] have called these bands, B, D and F and shown that the activities in B and D are affected by Mg^{2+} and sex hormones, respectively. This suggests the possibility that there are several glucose 6-phosphate dehydrogenase isoenzymes (as broadly defined by MARKERT AND MØLLER[3]) for which the rates of synthesis and degradation are regulated independently. The research reported here was initiated to elucidate the relationships among the multiple forms of glucose 6-phosphate dehydrogenase found in the supernatant fraction of rat liver homogenates.

We have purified rat liver glucose 6-phosphate dehydrogenase by a modification of the procedure of MATSUDA AND YUGARI[4] and estimated the molecular weight of its subunits and polymeric forms. The majority of the purified enzyme is a dimer which shows 3–4 sub-bands after electrophoresis on polyacrylamide gels. All of the multiple molecular forms of glucose 6-phosphate dehydrogenase which separate on polyacrylamide disc gels show immunological identity. A preliminary report of these results has been published[5].

MATERIALS AND METHODS

Analytical methods

Rat liver supernatant fractions were prepared and assayed for glucose 6-phosphate dehydrogenase and protein as previously described[1]. The molecular weights of the multiple forms of glucose 6-phosphate dehydrogenase were estimated by the polyacrylamide disc gel procedure of HEDRICK AND SMITH[6] using 6 different gels ranging from 5–11 percent polyacrylamide. The molecular weight of the glucose 6-phosphate dehydrogenase subunit was determined by the procedure of WEBER AND OSBORN[7] using 10% polyacrylamide and 2/3 the amount of methylenebisacrylamide. Trypsin, pepsin and ovalbumin were purchased from Worthington; catalase, aldolase, lactic dehydrogenase (beef heart) and glucose 6-phosphate dehydrogenase (yeast) from Boehringer Mannheim; bovine serum albumin and ferritin from Calbiochem and ribulose diphosphate carboxylase was a gift from Dr. N. G. Pon. Polyacrylamide gels were stained for protein with Coomassie brilliant blue R-250[8]. Glucose 6-phosphate dehydrogenase activity on polyacrylamide gels or agar plates was detected by using a solution containing 150 μmoles glucose 6-phosphate, 15 μmoles $NADP^+$, 1 mmole $MgCl_2$, 5 mmoles Tris chloride, pH 8.0, 4.2 mg phenazine methosulfate and 24 mg nitro blue tetrazolium per 100 ml. All chemicals were commercial preparations and were used without further purification.

Purification procedure

Glucose 6-phosphate dehydrogenase from the soluble fraction of rat liver homogenates was purified by the following modifications of the procedure of MATSUDA AND YUGARI[4]. Liver supernatant fractions were prepared by homogenizing in 3 vol. of 0.15 M KCl and centrifuging at 70 000 $\times$ g for 60 min. The first DEAE-cellulose column was washed with 0.1 M potassium phosphate buffer (pH 7.0) (rather than with the 0.05 M buffer) prior to eluting the enzyme with the 0.2 M phosphate buffer. The carboxymethyl cellulose column was eluted with a linear gradient using 750 ml each of 0.02 M ammonium acetate (pH 5.5) and 0.03 M ammonium acetate (pH 6.5). Both buffers contained 10^{-4} M $NADP^+$. All other procedures were as described by MATSUDA AND YUGARI[4]. The purified enzyme had a specific activity of 210 enzyme units/mg protein and was obtained in approx. 15% yield.

Preparation of rabbit antiserum against purified glucose 6-phosphate dehydrogenase

Each New Zealand white rabbit was injected in the footpad with 1.1 mg of purified glucose 6-phosphate dehydrogenase (specific activity 210) in complete Freunds adjuvant on days 1 and 7. On day 10 each rabbit was injected subcutaneously with an additional 3 mg of the same enzyme preparation. By this procedure low titers

of antiserum (1 ml antiserum neutralizes 34 units of glucose 6-phosphate dehydrogenase) were produced 1 month after the initial injection.

RESULTS

In our initial attempts to purify rat liver glucose 6-phosphate dehydrogenase we were unable to specifically elute the enzyme from the carboxymethyl cellulose column with glucose 6-phosphate as described by MATSUDA AND YUGARI[4]. However, the modified procedure described in the MATERIALS AND METHODS section routinely yielded the enzyme in a near homogeneous state. In Fig. 1 polyacrylamide disc gels were stained for glucose 6-phosphate dehydrogenase activity or for protein. With either 5% (2 right gels) or 9% (2 left gels) polyacrylamide, there were no protein bands which did not coincide with an activity stain. In the 9% gel stained for glucose

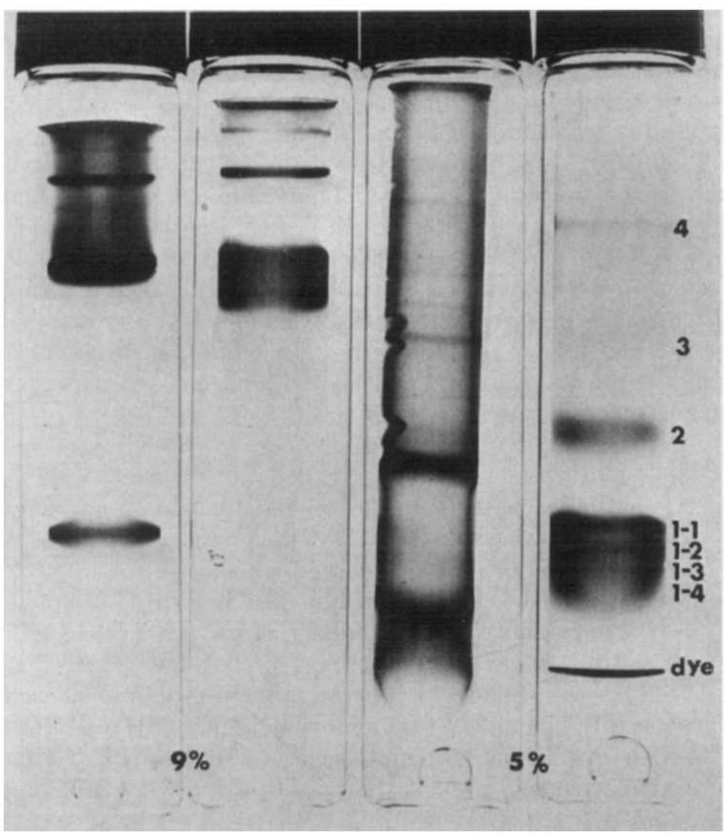

Fig. 1. Polyacrylamide disc gel electrophoresis of purified rat liver glucose 6-phosphate dehydrogenase using 5% and 9% gels. Gels were stained for both protein (right-hand gel of each pair) and activity (left-hand gel of each pair). Each gel was purposely overloaded with 57 μg of purified enzyme (specific activity 210) as a check for homogeneity. The major bands are numbered 1 through 4 and the sub-bands in Band 1 are numbered 1-1 through 1-4. The upper reservoir buffer contained 10^{-5} M $NADP^+$. In the activity stain of the 5% gel the dye has been cut off from the bottom in order to fit the gel into the vial. Some very faint bands between Bands 3 and 4 are visible on the activity stain but not the protein stain.

6-phosphate dehydrogenase activity the bottom band near the center of the gel is a dye marker. In this enzyme preparation (specific activity 210) a very minor activity band near the top of the 5% gel gave a disproportionately dark band when stained for protein. This may represent a minor contaminating protein or a form of the enzyme with a low specific activity.

The protein stain of the 5% gel shows that the most rapidly migrating major band (Band 1) has three to four distinct sub-bands. When less enzyme is used for these experiments (about 0.01 enzyme units per gel) an activity stain clearly demonstrates the sub-banding. In these experiments 10^{-5} M $NADP^+$ was included in the upper reservoir buffer to protect against the loss of activity during electrophoresis. The subbanding is still apparent however, if the $NADP^+$ is omitted. In this case the activity stains are lighter due to lability of the enzyme during electrophoresis in the absence of $NADP^+$. In our experiments approx. 0.06 unit of enzyme per gel gave an adequate activity stain in the absence of $NADP^+$ in the upper reservoir buffer.

Initial characterization of the relationships among the multiple forms of glucose 6-phosphate dehydrogenase involved a determination of the molecular weight of each major band by the procedure of HEDRICK AND SMITH[6]. These results are summarized in Fig. 2. Bands 1, 2 and 3 have molecular weights of 130 000, 280 000 and 370 000, respectively. Band 4 has a molecular weight slightly greater than 500 000. The sub-bands within Band 1 were all within experimental error of 130 000. In other preparations a faint activity band with a molecular weight of 210 000 was also observed between Bands 1 and 2.

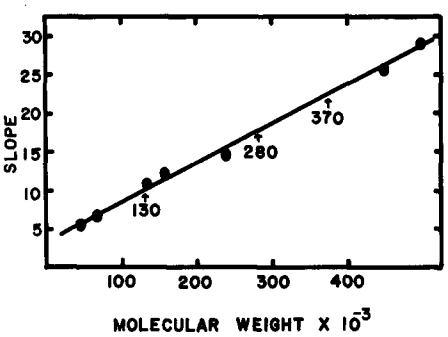

Fig. 2. Standard curve for calculation of the molecular weights of purified glucose 6-phosphate dehydrogenase. Six polyacrylamide disc gels containing between 5 and 11% acrylamide were used for each protein. Each gel was run with 0.06 unit of purified glucose 6-phosphate dehydrogenase (specific activity 216) and stained for activity. Protein standards (ovalbumin, pepsin, lactate dehydrogenase, aldolase, catalase, ferritin and ribulose diphosphate carboxylase) were run as described by HEDRICK AND SMITH[6] and stained for protein. Molecular weights ($\pm$ standard error) calculated from 4 separate determinations were 130 000 $\pm$ 2000, 280 000 $\pm$ 5000 and 370 000 $\pm$ 10 000 for Bands 1, 2 and 3, respectively. Sub-bands in Band 1 were all within experimental error of 130 000. A band routinely found between Bands 1 and 2 in this preparation had a molecular weight of 210 000 $\pm$ 6000. The upper reservoir buffer contained no $NADP^+$ in these experiments. However, in two separate experiments, identical results were found using 10^{-5} M $NADP^+$ in the upper reservoir buffer.

Disc gel electrophoresis of purified glucose 6-phosphate dehydrogenase in the presence of sodium dodecyl sulfate by the procedure of WEBER AND OSBORN[7] showed a major protein band with a molecular weight of 64 000 and a second, very faint band

with a molecular weight of 75 000 (Fig. 3). These molecular weight determinations suggest that glucose 6-phosphate dehydrogenase Bands 1, 2 and 3 on polyacrylamide disc gels are dimers, tetramers, and hexamers respectively of subunits of molecular weight 64 000. Since we have never observed an active glucose 6-phosphate dehydrogenase with a molecular weight less than 130 000 the subunit is apparently inactive.

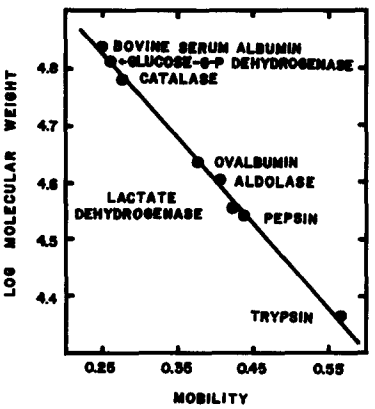

Fig. 3. Standard curve used to determine the molecular weight of the glucose 6-phosphate dehydrogenase subunit. The indicated standard proteins were used according to the procedure of WEBER AND OSBORN[7]. Each gel had 15 μg of purified glucose 6-phosphate dehydrogenase (specific activity 216) and, in two separate runs gave mean molecular weights of 64 000 for the subunit.

The intensity of the activity and protein stains of polyacrylamide disc gels in Fig. 1 suggest that most of the enzyme exists as a dimer with decreasing amounts of the tetramer and hexamer, respectively. We have estimated the relative concentrations of these forms of glucose 6-phosphate dehydrogenase after chromatography on Sephadex G-200 (Fig. 4). Two peaks of activity were observed. A large, symmetrical peak eluted with a V_e/V_0 ratio equal to 1.54 and a smaller peak which accounted for 10 percent of the total activity eluted with a V_e/V_0 ratio of 1.18. The molecular weights of these enzymes were calculated with the equation proposed by DETERMANN[9] [$\log M = 6.698 - 0.987\ (V_e/V_0)$] as 150 000 and 340 000, respectively, for the major and minor peaks. Polyacrylamide disc gel electrophoresis of chromatography tubes No. 50 and 65 showed that the major peak corresponded to Band 1 and the minor peak was a mixture of Bands 2 and 3. It was concluded that about 90% of the purified glucose 6-phosphate dehydrogenase was in the form of dimers and the other 10% was a mixture of higher molecular weight aggregates of these dimers.

We next attempted to obtain additional evidence that the higher molecular weight forms of glucose 6-phosphate dehydrogenase had resulted from an aggregation of glucose 6-phosphate dehydrogenase dimers. Consequently, we prepared an antiserum against purified rat liver glucose 6-phosphate dehydrogenase (see MATERIALS AND METHODS). This antiserum could precipitate all of the glucose 6-phosphate dehydrogenase activity from either a purified preparation or the crude supernatant fraction from a fresh liver homogenate.

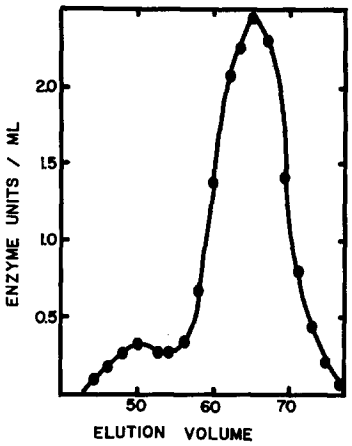

Fig. 4. Separation of glucose 6-phosphate dehydrogenase dimers from higher molecular weight forms on a Sephadex G-200 column (1.5 cm × 80 cm). The Sephadex was equilibrated with 5 mM Tris chloride–38 mM glycine (pH 8.3)–10^{-5} M $NADP^+$ and had a V_0 of 42.5 ml measured using blue dextran. 32 units of purified glucose 6-phosphate dehydrogenase (specific activity 160) in a volume of 1 ml was dialyzed *versus* the column buffer prior to being put on the column. Two peaks of glucose 6-phosphate dehydrogenase activity eluted at 50 ml (minor peak) and 65 ml (major peak).

10 units of purified glucose 6-phosphate dehydrogenase were subjected to electrophoresis on two 5% polyacrylamide disc gels as in Fig. 1 and stained for glucose 6-phosphate dehydrogenase activity. All discernible activity bands were dissected out and placed into holes cut into agar plates as in Fig. 5. Rabbit antiserum

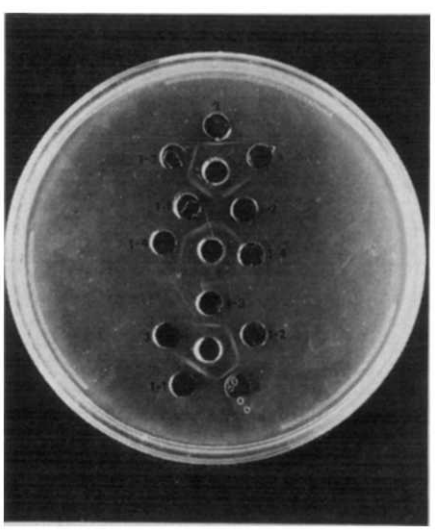

Fig. 5. Immunochemical identity among glucose 6-phosphate dehydrogenase bands dissected from 5% polyacrylamide disc gels. Purified glucose 6-phosphate dehydrogenase (57 μg protein, specific activity 210) was put on top of each of 2 gels and subjected to electrophoresis as described in MATERIALS AND METHODS. Gels were then stained for glucose 6-phosphate dehydrogenase activity and the bands were dissected out and placed in the appropriate holes cut into an agar plate. Each center hole contained 0.1 ml of rabbit antiserum raised against purified rat liver glucose 6-phosphate dehydrogenase. The precipitin lines were allowed to develop for 24 h.

prepared against purified rat liver glucose 6-phosphate dehydrogenase was placed into the three center holes in Fig. 5 so that precipitin lines could form by the double diffusion method of OUCHTERLONY[10]. In this experiment dimers 1-1, 1-2, 1-3 and a very faint dimer 1-4, as well as the higher molecular weight forms, 2 and 3, were dissected out and placed in the indicated positions in Fig. 5. This photograph was taken after allowing 24 h for formation of the precipitin lines. Fig. 5 shows that there is immunological identity between all bands of glucose 6-phosphate dehydrogenase. Band 3 gave no precipitin line probably because the enzyme concentration was too low.

Band 4 was absent from this enzyme preparation and was not tested against the antiserum. After the photograph in Fig. 5 had been taken, the plate was stained for glucose 6-phosphate dehydrogenase activity (see legend to Fig. 6). All precipitin lines contained an active glucose 6-phosphate dehydrogenase, thus insuring that these precipitin lines had derived from a precipitation of the enzyme rather than some other protein. These results indicate a close structural similarity between each of the multiple forms of glucose 6-phosphate dehydrogenase.

MATSUDA AND YUGARI[4] had previously reported that the rat liver and erythrocyte glucose 6-phosphate dehydrogenase showed an immunochemical reaction of non-identity. Some of our antiserum preparations raised against impure glucose 6-phosphate dehydrogenase formed precipitin lines against proteins other than glu-

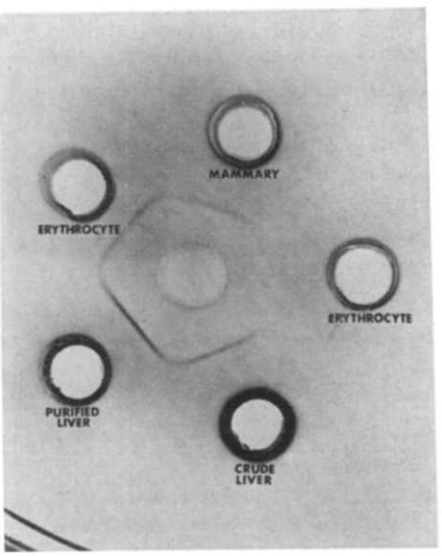

Fig. 6. Immunochemical identity among rat liver, mammary gland and erythrocyte glucose 6-phosphate dehydrogenases. Mammary glands from a nursing rat were homogenized in 0.15 M KCl and centrifuged at 66 000 × *g* for 60 min. Erythrocytes from fresh, heparinized, rat blood were washed twice with 0.15 M KCl, allowed to lyse in an equal volume of water for 30 min and centrifuged at 30 000 × *g* for 10 min. The crude supernatant fractions from the erythrocyte, mammary gland and liver were placed in the appropriate holes in an agar plate and allowed to react with the antiserum raised against the purified liver enzyme. The purified liver enzyme was included for comparison. After the precipitin lines had formed the agar plate was washed several times with 0.15 M KCl over a period of about 8 h. After washing, the precipitin lines were stained for glucose 6-phosphate dehydrogenase activity by using the activity stain described in MATERIALS AND METHODS.

cose 6-phosphate dehydrogenase. Since such precipitin lines could lead to incorrect conclusions about immunological identity or non-identity, Fig. 6 is a photograph of an Ouchterlony plate where the precipitin lines have been stained for glucose 6-phosphate dehydrogenase activity. Thus, Fig. 6 provides evidence for immunological identity among glucose 6-phosphate dehydrogenases present in rat liver, erythrocyte, and mammary gland. In a similar experiment yeast glucose 6-phosphate dehydrogenase did not react with antiserum raised against the liver enzyme.

DISCUSSION

Fig. 1 demonstrates that even when large amounts of purified liver glucose 6-phosphate dehydrogenase are subjected to polyacrylamide disc gel electrophoresis, there is complete coincidence between protein and activitity stains. After treatment with sodium dodecyl sulfate only one major protein band is present. Although difficult to quantitate, this preparation (specific activity 210) appears to be at least 95% pure. The purified enzyme is very unstable unless protected by $NADP^+$. In our experience, elution of the enzyme from the carboxymethyl cellulose column with glucose 6-phosphate[4] results in a poor recovery and purification probably because $NADP^+$ present in the buffer to protect the enzyme is reduced when glucose 6-phosphate is also added to this buffer in the presence of the enzyme. The pH gradient we have employed avoids this problem and the enzyme elutes at approx. pH 5.8. It is important that the next step, concentration on a small DEAE column, be carried out immediately since the carboxymethyl cellulose eluate is unstable. We have routinely washed the final small DEAE column with 0.05 M phosphate buffer (pH 7.0) (about 3 column volumes) prior to elution of the final enzyme preparation with 0.2 M phosphate buffer (pH 7.0). This purification procedure gives very reproducible results yielding a final specific activity between 210 and 220.

Our molecular weight estimates are in excellent agreement with those of NEVALDINE AND LEVY[11] for the rat mammary gland enzyme (130 000) and subunit (63 300) and those of RATTAZZI[12] for the rat erythrocyte enzyme (131 000). Our results also agree reasonably well with those of SCHMUKLER[13] for crude rat liver glucose 6-phosphate dehydrogenase (118 000, 242 000 and 350 000 respectively for Bands 1, 2 and 3). MATSUDA AND YUGARI[4] calculated a molecular weight of 110 000 for the purified rat liver enzyme which, in our preparations is about 90% dimer. However, the latter author's estimates[14] of the molecular weight of the active enzyme (57 000–60 000) and inactive subunit (28 000) based upon elution from Sephadex G-200 columns differ considerably from our results.

Evidence presented in Fig. 5 demonstrates an immunological identity among each of the multiple forms of glucose 6-phosphate dehydrogenase and suggests that these all share structural similarity. The simplest mechanism which would account for this would be if each of the multiple forms of this enzyme had derived from an original form of glucose 6-phosphate dehydrogenase. Thus, an original dimer of glucose 6-phosphate dehydrogenase might undergo alteration of one or more sulfhydryl groups or another component and give rise to the three or four multiple forms of that dimer which we have observed. This possibility is suggested by the demonstration by TAKETA AND WATANABE[15] that the multiple forms of the rat liver glucose

6-phosphate dehydrogenase dimer may be interconverted by treatment with β-mercaptoethanol or $HgCl_2$.

The molecular weight determinations of the higher molecular weight forms of this enzyme suggest that Bands 2 and 3 represent aggregates of these dimers. Furthermore, these aggregates are immunologically indentical with the dimers and are formed from subunits of the same molecular weight as the dimer subunit. The dimers in Band 1 may be dissected from polyacrylamide gels and then extracted at pH 5.5 in the presence of $NADP^+$. Under these conditions we have shown that these dimers aggregate and generate Bands 2, 3 and 4 upon re-electrophoresis on polyacrylamide disc gels (M. FROLICH AND D. HOLTEN, unpublished observations). Thus, there can be little doubt that the higher molecular weight forms of glucose 6-phosphate dehydrogenase are formed by aggregation of the dimers.

Our present data do not allow us to decide whether active glucose 6-phosphate dehydrogenase is formed from identical subunits. Although these subunits have identical molecular weights a decision regarding their identity must await chemical evidence.

The problems involved in studying the regulation of the synthesis of an enzyme with as many multiple forms as rat liver glucose 6-phosphate dehydrogenase are decreased by the data presented here and by others[13,15] demonstrating that these multiple forms may arise from alterations introduced after translation. Such an organization encourages the continued use of this enzyme in detailed investigations regarding the regulation of the levels of lipogenic enzymes in liver.

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