Intracellular Localization of Fructose 1,6-Bisphosphate Aldolase*

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SUMMARY

Submission of a rat liver homogenate made in 250 mm sucrose-1 mM EDTA to centrifugation between 9,500 × g for 10 min and 105,000 × g for 60 min results in the sedimentation of 60 to 70% of the total cellular fructose 1,6-bisphosphate aldolase (EC 4.1.2.13). Under these conditions only about one-quarter of the total triose phosphate dehydrogenase and phosphoglycerate kinase appears in the microsomal fraction. Ultrastructural immunologic localization techniques have demonstrated that the aldolase is associated with the endoplasmic reticulum, in situ. The binding of this enzyme to the membrane is sensitive to changes in pH with an optimum at 6.0, and to increasing concentrations of NaCl and fructose 1,6-bisphosphate, being about 100-fold more sensitive to the ester than to the inorganic salt.

Traditionally, the glycolytic enzymes have been considered to be located in the soluble portion of the cell (cf. Ref. 1) although there are data which suggest that particular enzymes of this sequence are associated with specific subcellular particulate elements (cf. Ref. 2).

Green et al. (2) reported that when bovine erythrocytes were treated with 250 mm sucrose and then lysed in distilled water, most of the glycolytic enzymes remained at least partially associated with the membranous fraction. Hexokinase, fructose-1,6-P2 aldolase, and triose phosphate isomerase were recovered in 50% or greater yield by sedimentation at 60,000 × g. However, in the presence of 150 mm NaCl, these enzymes dissociated from the membranes which sedimented at 60,000 × g and remained in the supernatant solution. Green and colleagues suggested that all glycolytic enzymes were associated with membranes in the bovine erythrocyte. These workers obtained supporting data for this concept with Saccharomyces cerevisiae.

Hernandez and Crane (3) reported the sedimentation of 80 to 90% of the hexokinase activity when muscle from pig heart was homogenized in 300 mm sucrose and submitted to centrifugation at 105,000 × g. Addition of 400 mm KCl solubilized about one-third of the activity, while increasing the pH from 5 to 9 solubilized nearly all of the enzyme. The enzyme reassembled with the particulate fraction after removal of the salt by dialysis. The solubilization could also be effected by 0.5 mm glucose-6-P, the enzyme's product. The authors postulated an equilibrium dependent upon salt and pH between the soluble and particulate states of the enzyme with glucose-6-P as a modulator of this equilibrium.

Rose and Warm (4) localized hexokinase in the mitochondria of cells in an ascites tumor. Solubilization was accomplished by exposure to 1 mM NaCl or 0.1 mm glucose-6-P. Again reassociation could be achieved after dialysis. Li and Ch'ien (5) suggested that this equilibrium might play a regulatory role since the Km for ATP was about 3 times greater for the soluble than for the bound enzyme.

Roodyn (6, 7) found that when nuclei from rat hepatocytes were ultrasonicated in 250 mm sucrose-0.018 mm CaCl2, only 3% of the total fructose-P2 aldolase was released into solution. However, addition of 150 mm NaCl to the sucrose-CaCl2 mixture caused the solubilization of more than 90% of the enzyme without ultrasonication. Clarke et al. (8) found that 20 to 30% of the total fructose-P2 aldolase sedimented when a homogenate of rat brain or muscle in 250 mm sucrose-10 mm Tris-HCl, pH 7.4, was centrifuged at 100,000 × g. A reduction of the pH from 7.4 to 5.5 with lactic acid, but not with acetic acid, resulted in sedimentation of 60% of the brain enzyme but only 10% of the muscle enzyme. These workers proposed that this effect was a function of the differential effect of lactic acid on the binding of the two isozymes of this aldolase in these tissues.

Arnold and Pette (9, 10) found that fructose-P2 aldolase and triose phosphate dehydrogenase from rabbit muscle could be reversibly bound in vitro to F-actin, one of the structural proteins of the muscle contractile apparatus. The binding of the aldolase was sensitive to alkaline pH, to ionic strengths greater than 100 mm, and to the presence of metabolites such as fructose-1,6-P2, glucose-1,6-P2, dihydroxyacetone-P, and 2,3-P-glycerate. The Km of the bound enzyme was nearly 10 times greater than the Km for the soluble enzyme while the Vmax was twice as great. Histochernical staining confirmed the relevance of this observation, in situ. Staining for fructose-P2 aldolase at the light microscopic...
level indicated that in sections of rabbit muscle, the enzyme was located at the site of the actin filaments.

Data in the present report show that in rat liver, fructose-1,6-P₂ aldolase is associated with the endoplasmic reticulum. By differential centrifugation of a homogenate of this tissue made in 250 mm sucrose-1 mm EDTA, 60 to 70% of the total cellular aldolase sedimented in the microsomal fraction. Ultrastructural immunologic localization techniques showed specific labeling for fructose-1,6-P₂ aldolase on and around the endoplasmic reticulum. The binding is sensitive to changes in pH, and to variations in the concentration and identity of salts and metabolite intermediates.

**METHODS AND MATERIALS**

All work was done with adult male rats of the CFN strain (Carp-rsworth Farms) from a randomly inbred colony maintained in this laboratory.

Subfractionation of Rat Liver Homogenate—Microsomal fractions were prepared from a 10% (w/v) homogenate in 250 mm sucrose-1 mm EDTA as described by Dallner (11). Rat liver homogenate was fractionated into nuclear, mitochondrial, microsomal, and soluble fractions by a slight modification of the method of Schachter (12).

Enzymatic Assays—The conversion of fructose-1,6-P₂ to 1,3-P₃-glycerate was measured by the conversion in 10 min at 37° of [³²P]phosphate in the presence of 1 mm ADP, 1 mm ATP, 1 mm NAD⁺, 1.7 mm MgCl₂, 1 mm fructose-1,6-P₂, 38 mm Tris-HCl, pH 7.6, 1.8 mm sodium 32P₂, and homogenate or extract (0.1 to 1.0 mg of protein) in a final volume of 3.0 ml. After addition of 1 ml of 10% NH₄OH containing 300 mm P₂, all radioactive P₂ was precipitated at 0° by the addition of 1.0 ml of magnesium mixture (cf. Ref. 13) leaving organically bound ³²P, primarily ATP, in solution. Radioactivity was measured by monitoring Cerenkov radiation (14) at the normal setting for tritium in a Packard Tri-Carb 3375 liquid scintillation counter, corrected for quench by the channel’s ratio method (15).

Fruuctose-1,6-P₂ aldolase was assayed either spectrophotometrically (16) or colorimetrically (17); horseradish peroxidase was assayed as described by Madsby and Chance (18), and glucose 6-phosphatase was assayed by the method of Swanson (19) as modified by Hübner and West (20).

Chemical Analysis—Protein was determined by the method of Lowry (21). DNA was extracted from cell subfractions by the method of Schneider (22), and analyzed by the diphenylamine method of Dounce (23). Calf thymus DNA was used as a standard.

Purification of Fruuctose-1,6-P₂ aldolase (EC 4.1.2.13) from Rat Liver Microsomes for Preparation of Anti-Aldolase—A microsomal fraction was obtained from a homogenate of rat liver, made in 250 mm sucrose-1 mm EDTA, by ultracentrifugation between 7,500 × g for 10 min at 4° (the post-mitochondrial fraction) and 70,000 × g for 105 min at 4°. Fruuctose-1,6-P₂ aldolase was then precipitated by dialysis against saturated (NH₄)₂SO₄. The precipitate was resuspended in 1 mm EDTA-20 mm Tris-HCl, pH 7.6, to a final concentration of 20 mg per ml, dialyzed against 2.77% formaldehyde-0.2% picric acid-250 mm sucrose-l mM EDTA as described by Oryce et al. (24). Elution of the second column with fructose-1,6-P₂ resulted in one peak with coincidence of protein and aldolase activity which exhibited only one band of protein by disc gel electrophoresis (25) on 7.7% polyacrylamide in 380 mM Tris-glycine at pH 8.3. The final recovery of enzyme, based upon the level of activity observed in the homogenate, was 10% at a purification of 63-fold with a specific activity of 6.03 units per mg of protein assayed (16) at 37°. These characteristics agreed well with values obtained for a crystalline preparation of this enzyme by Matsuo et al. (26).

Preparation of Rabbit IgG Anti-Aldolase—Antiserum to purified fructose-1,6-P₂ aldolase was obtained from New Zealand white rabbits which had received, at 10-day intervals, three series of injections (0.1 ml in each foot pad and 0.1 ml intradermally) of an emulsified equal volume mixture of the aldolase in physiological saline (0.9%, NaCl solution) (2 mg per ml) and Freund’s complete adjuvant. Beginning 2 weeks after the last injection, bleedings from the ear vein were done once each 2 weeks for 2 months. The IgG fraction was isolated from antiserum by precipitation with Na₂SO₄ and purification on DEAE-cellulose (27). The purity and specificity of the antibody was examined against the supernatant fraction of a homogenate (5% w/v) of rat liver in 250 mm sucrose-1 mm EDTA-10 mm NaCl centrifuged at 100,000 × g for 60 min. When the antiserum and anti-aldolase IgG were tested by immunodiffusion, a single precipitin band with no spur was observed. Control rabbit serum and IgG obtained from the same rabbits prior to immunization showed no precipitin bands. Immunoelectrophoresis (28) indicated a single band when tested against the rat liver extract. Anti-aldolase IgG precipitated 95% of the aldolase from the extract when incubated at 4° for 4 days and centrifuged at 100,000 × g for 30 min at 4°. Control rabbit serum had no such effect.

Purification of Peroxidase-labeled Goat IgG (Anti-Rabbit-IgG)—Peroxidase was conjugated to goat (anti-rabbit-IgG) IgG using p,p'-diphenyl-dinitrophenylmethyl as the coupling reagent according to the procedure described by Nakane and Pierce (29). The conjugate was further purified by adsorption on activated charcoal, and rat liver acetone powderr was added as the buffer to remove the last traces of fixative. The light yellow supernatant solution (about 0.7 ml) which finally resulted was dialyzed overnight against 100 mm Tris-HCl, pH 7.4 and used immediately.

Tissue Fixation—Liver was excised, diced into pieces of about 2 mm³ while in 2.7% formaldehyde-0.2% picric acid-250 mm sucrose-l mM EDTA as described by Kraehenbuhl et al. (31). A tissue section was exposed to the “acetone powder” procedure twice more with the addition each time of 10 mg of activated charcoal. The light yellow supernatant solution (about 0.7 ml) which finally resulted was dialyzed overnight against 100 mm Tris-HCl, pH 7.4 and used immediately.

Cytochemical Procedure—Ultrastructural immunologic localization of fructose-1,6-P₂ aldolase on and around the endoplasmic reticulum. The binding is sensitive to changes in pH, and to variations in the concentration and identity of salts and metabolite intermediates.
var and carbon. The mounted sections were poststained in a
drop of cacodylate-buffered 0.04 for 10 min at room temperature
and washed with distilled water. The specimens were examined
by an AEI Corinth 275 electron microscope.

Reagents—Reagents were obtained from the following sources.
Fructose-1,6-P$_2$ heptahydrate was from Wessex Biochemicals,
adrenaline nucleotides and 3,3'-diaminobenzidine from Sigma Che-
marial Co., NADH from Boehringer Mannheim Corp., ultrapure
(NH$_4$)$_2$SO$_4$ from Schwarz-Mann, phosphocellulose from Mann Bio-
chemicals, horseradish peroxidase (type II) from Sigma Chemical
Co., goat (anti-rabbit-IgG) IgG from Pentex, Freund's complete
adjuvant from Difco Laboratories, agar and p,p'-difluoro-m,m'-
dinitrodiphenylsulfone from General Biochemicals, activated
charcoal from Atlas Power Co., OsO$_4$ from Merck and Co., p-for-
maldehyde from Mallinckrodt Chemical Works, and glutaralde-
hyde (as 70% solution in sealed ampules) was from Ladd Research
Industries.

RESULTS

In a preliminary effort to delineate the extent to which cer-
tain of the glycolytic enzymes (fructose-P$_2$ aldolase, triose phos-
tate dehydrogenase, and phosphoglycerate kinase) are present in the
microsomal fraction from rat liver, the conversion of $^{32}$P$_1$ to or-
ganic $^{32}$P$_2$, dependent upon added fructose-1,6-P$_2$, was measured.
When assaying for the level of each of the three enzymes, excesses
of the other two enzymes, as commercial
preparations, were
added. The microsomal fraction contained 65% of the aldolase,
but only 3% of the dehydrogenase, and 10% of the kinase which
were present in the post-mitochondrial supernatant solution.

| Fraction              | Aldolase | Protein | Relative specific activity | Glucose-6-phosphate | DNA
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<td>1.0</td>
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<tr>
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<td>19</td>
<td>9</td>
<td>2.1</td>
<td>2</td>
<td>91</td>
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<tr>
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<td>0.1</td>
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<td>30</td>
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| FIG. 1. Effect of NaCl on the subcellular localization of fruc-
tose-P$_2$ aldolase activity. Subfractions of rat liver homogenates
were prepared as described under "Methods and Materials" ex-
cept that NaCl was added to the crude homogenate before sub-
fractionation to make the final concentrations indicated. All
buffers used in further subfractionation contained these NaCl
concentrations.

FIG. 2. Effect of added NaCl and fructose-1,6-P$_2$ on the ap-
parent subcellular localization of fructose-P$_2$ aldolase. A 10% (w/v)

FIG. 3. Effect of pH on the binding of fructose-P$_2$ aldolase to
the microsomal fraction. Technical details were as described for
Fig. 2 except that the pH of 10-ml aliquots of the post-mitochon-
drial fraction was adjusted by addition of 100 mM HCl or NaOH
to the desired values (volume addition less than 0.02 ml).
total cellular aldolase reattached to the microsomes. The data in Fig. 1 indicate that not only was the enzyme almost completely solubilized from the particulate fractions by addition of 40 mM NaCl but that the total recovered activity exceeded 100% of that present in the homogenate in the absence of salt. While it took 5 mM NaCl to solubilize 50% of the microsomal aldolase, as little as 0.05 mM fructose-1,6-P₂ produced the same effect (Fig. 2). The binding of aldolase to the microsomes was also found to be sensitive to changes in pH being maximal at pH 6.0 and 20% at pH 8.0 (Fig. 3).

Since the enzyme can be reversibly solubilized by changing the pH or the concentrations of various salts, the binding might be an artifact and not exist in vivo. The best available proof for the physiological relevance of the binding would be the immunological ultrastructural localization of the enzyme in tissue sections. Fig. 4A demonstrates by this technique that the enzyme is associated with the endoplasmic reticulum. Control IgG produced no labeling (Fig. 4B). Although by biochemical fractionation, the nucleus appeared to contain aldolase, no nuclear labeling was seen (Fig. 4C) by the immunological technique. Fig. 4C shows that the enzyme is associated with both the smooth and rough endoplasmic reticular membranes. When the im-

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**Fig. 4.** Electron microscopic demonstration of fructose-P₂ aldolase specific staining in the endoplasmic reticulum of fixed liver tissue. A and C, anti-aldolase IgG; B, control IgG. Post-stained in 2% OsO₄. Magnification: A and B, X 30,000; C, X 12,000.
DISCUSSION

Although there are published data, obtained in vitro, which suggest that certain of the glycolytic enzymes are localized in an organelle or on a cellular structural element, most of these data could also be interpreted as representing an artifactual binding which occurred during the experimental procedure employed. The most compelling data favoring particular localizations of these enzymes, in situ, would make use of ultrastructural immunological localization techniques. The present study utilized such techniques to demonstrate the association of fructose-1,2 aldolase with the endoplasmic reticulum, in situ. Rabbit anti-fructose-1,2 aldolase IgG was shown by electron microscopy to localize on or in close apposition to these membranes in tissue sections of rat liver. The specificity of the anti-aldolase IgG was shown by its ability to precipitate all of the aldolase from a crude extract of rat liver which had been made in the presence of 150 mM NaCl; the purity was shown by the formation of only one precipitin band when tested by immunoelectrophoresis against the extract. Although it is not presently possible to quantitate cytoimmunochemically the percentage of the total aldolase which is present on the endoplasmic reticulum, biochemical fractionation showed that at least 60% of the enzyme is associated with these membranes.

The increase in total cellular enzymatic activity seen when all of the fructose-1,2 aldolase was solubilized (Fig. 1) could be explained by an increase in the $V_{\text{max}}$ of the enzyme as was found for fructose-1,2 aldolase in rabbit muscle (10). Further investigation of this point is in progress.

Recent studies in this laboratory\(^3\) have demonstrated an increase of at least 350% in the total activity of microsomal fructose-1,2 aldolase when 80 mM NaCl was added to a preparation of microsomes which had been isolated by centrifugation from a rat liver homogenate and resuspended in 250 mM sucrose-1 mM EDTA. Simply adding 80 mM NaCl to a soluble dialyzed preparation of the aldolase does not result in an increase of activity. These observations and the finding that the association of fructose-1,2 aldolase with the endoplasmic reticular membranes is sensitive to relatively small changes in the substrate suggest that reversible association of the enzyme with the membranes may be a mechanism for controlling the rate of glycolysis in rat liver. Similar results have been reported in rat brain where the addition of 2 mM fructose-1,6-P\(_2\) completely solubilizes fructose-1,2 aldolase from microsomes isolated in sucrose-EDTA, whereas about 150 mM NaCl is necessary to accomplish the same effect (32).

Arnold and Pette (10) found that the binding of aldolase to F-actin was sensitive to low concentrations of fructose-1,6-P\(_2\), dihydroxyacetone-P, 2,3-P\(_2\)-glycerate, and glucose-1,6-P\(_2\) but much less sensitive or insensitive to other glycolytic intermediates. Support for the proposal that modulation of the activity of fructose-1,2 aldolase by interaction with the endoplasmic reticulum represents a regulatory mechanism for the glycolytic pathway would include evidence that (a) the enzyme can reversibly bind to the membranes, (b) the percentage of the enzyme bound to the membranes or free in the cytosol is affected by biochemical or physiological parameters; (c) the effective activity of soluble aldolase is different from bound aldolase; and (d) physiologically, this enzyme limits the rate of glycolysis in either direction by

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*\(^3\) T. Weiss, Department of Environmental and Industrial Health, The University of Michigan, personal communication.*
virtue of its transit between the particulate and soluble phases of the cell.

The reversible association of fructose-P\textsubscript{2} aldolase with the membranous cellular fraction has now been documented for cells in the brain and muscle (8) as well as in the liver of the rat; in the muscle of the rabbit (9, 10), in the bovine erythrocyte (2), and in yeast (2), in vitro, and for muscle actin in the rabbit (10) and the endoplasmic reticulum in the hepatocyte of the rat, in situ. In rat brain, one of two isozymes of aldolase is preferentially bound to the microsomes while the other is preferentially located in the cytosol (8). This binding has been shown to be sensitive to lactate acid in the case of the brain of the rat (8), to some glycolytic intermediates in the muscle of the rabbit (10), and to fructose-1,6-P\textsubscript{2} in the liver of the rat. Increases in total cellular activity of the enzyme upon solubilization have been demonstrated in the latter two systems. That the reversible dissociation of fructose-P\textsubscript{2} aldolase from the membranes can be an allosteric control mechanism is suggested by the observation that upon adsorption on phosphocellulose, the enzyme exhibits the sigmoidal kinetics of allosteric enzymes when enzymatic activity is plotted against the concentration of fructose-1,6-P\textsubscript{2} (33).

However, the crucial demonstration that the increase in activity, resulting from solubilization, is associated with an alteration of the physiological rate of glycolysis, has not as yet been achieved.

REFERENCES

Intracellular localization of fructose 1,6-bisphosphate aldolase.
R S Foemmel, R H Gray and I A Bernstein


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