THE INTRACELLULAR LOCATION OF HEPATIC FRUCTOSE 1,6-BIPHOSPHATE ALDOLASE

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Received December 2,1975

SUMMARY: Foemmel et al. (J. Biol. Chem. 250, 1892-1897, 1975) have presented evidence that rat liver aldolase exists in situ as a specific complex with the endoplasmic reticulum. In the present study we have examined the alternative possibility that binding of enzyme to the particulate element represents an artifact of the dilution of the ionic constituents of the cytoplasmic milieu. To this end, procedures were developed for homogenization and subfractionation which effected less than a 3-fold dilution of the intracellular content. Using these procedures, virtually all of the liver aldolase was recovered in the soluble supernatant fraction. We conclude that aldolase is not associated with the endoplasmic reticulum in situ.

The present communication considers the question of the intracellular locus of rat liver fructose 1,6-biphosphate aldolase (EC 4.1.2.13). Foemmel, Gray and Bernstein (1) recently concluded that rat liver aldolase is, for the most part, associated with the elements of the endoplasmic reticulum in situ. As evidence in support of this conclusion, they presented data from studies of the subcellular distribution of aldolase activity following subfractionation of liver homogenates and the results of cytoimmunological experiments with fixed liver preparations.

On reviewing many of the studies concerned with the intracellular localization of the glycolytic enzymes (1-7), we noted that, in general, the subfractionation of tissue homogenates or cell lysates were performed in low-salt media (viz. 0.25 M sucrose or distilled water). Moreover, in most instances the bound enzymes readily dissociated from the particulate component upon addition of moderately dilute silutions of NaCl. These general observations suggested the alternative, but equally plausible, explanation that binding of the glycolytic enzymes to membranous elements may arise artifactually during subfractionation procedures as the consequence of diluting the ionic con-

stituents of the cytoplasmic milieu. To test this postulate, we have employed a procedure whereby tissue homogenization and subfractionation could be accomplished with much less dilution of the intracellular constituents than is usually obtained. Analyses of the distribution of adolase activity within the particulate and soluble subfractions obtained by this procedure revealed the virtual absence of association of this glycolytic enzyme with the membranous elements of the rat liver cell.

MATERIALS AND METHODS

[^{14}C]dextran (M.W. = 17,000 daltons) was purchased from the International Chemical and Nuclear Corporation (Irvine, Calif.) and further purified by gel chromotography. All other biochemicals or reagents were obtained from the Sigma Chemical Co. (St. Louis, Mo.) and were the highest purity available. Cacodylic acid was purified as described elsewhere (8).

Experimental animals and preparations-Male, Long-Evans rats, weighing from 150 to 175 g were obtained from Blue Spruce Farms, Altamont, N.Y. The rats were maintained on Charles River laboratory chow and water, ad libitum, for two weeks and then fasted overnight prior to use. On the day of the experiment, the rats were anesthetized with diethyl ether, and a midventral incision made to expose the liver, femoral artery, portal vein and inferior vena cava. Sodium heparin (100 units in 0.1 ml) was injected into the femoral artery, and blood was removed from the liver by perfusion in situ with approximately 40 ml of isotonic saline followed by 10 to 20 ml of 0.25 M sucrose.The livers were excised, blotted, weighed and supplemented with exactly 0.5 ml of 0.25 M sucrose for each gram of wet tissue. Homogenization was carried out for 90 sec at 0° in a Potter-Elvehjem mortar equipped with a motor-driven Teflon pestle operating at 600 rpm. The resulting homogenate, herein termed the "concentrated homogenate", was supplemented with 0.01 volumes of [^{14}C]dextran (30 mg/ml; 4 X 10^5cpm/ml) and treated as described below to obtain the "concentrated supernatant" fraction.

A standard 10% homogenate was prepared by supplementing an aliquot of the concentrated homogenate with 0.25 M sucrose to yield 10 ml of suspension per gram of original liver. A particle-free supernatant (i.e. the "standard supernatant") was obtained from the 10% homogenate by standard procedures (9). The concentrated supernatant was obtained by subjecting the concentrated homogenate to a sequence of centrifugations at 30,000 X g for 6 min in the Beckman Type 30 rotor (to sediment cellular debris, nuclei and mitochondria) and 160,000 X g for 50 min in the Beckman Type 50Ti rotor (to sediment the microsomal fraction).

In the case of the concentrated preparations, the pellets of particulate material occupied a substantial fraction of the total homogenate volume with the consequence that a rather large fraction of the total supernatant fluid was occluded within the interstices of the sedimented particles. Therefore, the volume of supernatant fluid actually recovered for analysis represented only about one-third of the total supernatant volume. The dextran marker (see above) was used to obtain estimates of the total supernatant volumes. For example, in a typical preparation the concentration of labelled dextran in the concentrated supernatant exceeded by a factor of 1.82 that in the

homogenate, which indicated that 55.1% of the homogenate volume was accessible to the high molecular weight dextran. The dextran accessible space was taken as the total supernatant volume. The validity of using the dextran marker to estimate supernatant volumes was supported by the results of analyses of the supernatant protein concentrations. It was found that the products obtained by multiplying the concentration of protein (mg/ml) in the recovered supernatants by the dextran accessible space were similar for the standard and concentrated preparations (56.8 and 57.9 mg of supernatant protein per gram liver, respectively, as the means for 3 experiments). For a typical experiment with the standard 10% homogenate, the dextran accessible space was 94.5% of the total homogenate volume compared with 93.0% obtained by direct measurements of the volume of decanted supernatant. Since any potential error introduced by using the dextran accessible space is clearly minimal in the case of the standard preparations, it follows that the close agreement between the values for supernatant protein concentration per g liver in the two systems necessarily indicates that the cytosolic proteins and the dextran marker occupied essentially the same spaces.

Assays- Fructose biphosphate aldolase was assayed spectrophotometrically as described by Rajkumar, Woodfin and Rutter (10), except the assay media and tissue preparations were equilibrated with carbon monoxide to reduce the rather substantial rates of NADH reduction observed with homogenates in the absence of fructose 1,6-diphosphate. Glucose 6-phosphatase was assayed in media containing 20 mM glucose 6-phosphate, pH 6.5, as described previously (11). A unit of activity is defined as that amount of enzyme necessary to catalyze the cleavage of one μmole of either fructose biphosphate or glucose 6-phosphate in one minute. Protein was assayed by the biuret procedure (12) using crystalline bovine plasma albumin as the reference standard.

RESULTS AND DISCUSSION

The distribution of liver aldolase within the particulate and soluble subfractions of the standard 10% homogenate were compared with values for the corresponding fractions of the concentrated homogenate (Table I). Our results with the standard homogenate agree with the findings of Foemmel et al. (1) in that the major fraction of aldolase activity (60% in our studies) was not recoverable in the standard postmicrosomal supernatant. In sharp contrast to these observations with the more dilute tissue preparations, we observed that 97% of the total homogenate activity was recovered in the concentrated supernatants. The results of a simultaneous assessment of the distribution of microsomal glucose 6-phosphatase (Table I) demonstrated that the postmicrosomal supernatants obtained from standard or concentrated homogenates were devoid of significant contamination with microsomes. Therefore the high level of aldolase activity in the concentrated supernatant cannot be attributed to the presence of enzyme bound to unsedimented microsomal vesicles.

In the present studies we did not add EDTA to the homogenization medium, whereas Foemmel et al. (1) supplemented the isotonic sucrose with 1 mM EDTA. However, the results of supplementary experiments demonstrated that the presence of EDTA did not alter the distribution of aldolase between soluble and particulate fractions.

It is important to note, that either by conscious design or happenstance, the specific conditions used to prepare the tissue fractions were essentially those reported (1) to favor maximal binding of aldolase to microsomes. First, the acidity of the concentrated homogenates and supernatants were found to be within the narrow range between pH 6.5 and 6.6. Foemmel et al. (1) found that the binding of aldolase to microsomes was maximal between pH 6 and 6.5. Secondly, the procedure for preparing concentrated homogenates was designed to minimize the introduction of exogenous electrolytes into the final preparation. The liver was perfused in situ with isotonic saline to eliminate blood cell aldolase from contributing to the total liver activity. It has been estimated (13) that 22% of the rat

TABLE I: SUBCELLULAR DISTRIBUTION OF ALDOLASE ACTIVITY

RAT LIVER PREPARATION	ENZYMIC ACTIVITY*			
	ALDOLASE		GLUCOSE 6-PHOSPHATASE	
	units/g liver	% recovered in supernatant	units/g liver	% recovered in supernatant
HOMOGENATES:				
standard	3.24	---	12.0	---
concentrated	3.37	---	12.2	---
SUPERNATANTS:				
standard	1.29	40	0.11	0.9
concentrated	3.27	97	0.21	1.7

* Tabulated values are the means from three experiments.

liver is extracellular space. From this figure it is easily calculated that the concentrated homogenate would be about 20 mM NaCl if the extracellular space were equilibrated with 0.15 M NaCl. Since Foemmel et al. (1) found that concentrations of NaCl as low as 5 mM would solubilize 50% of the particulate aldolase, it was clearly necessary to eliminate the solubilizing potential of the extracellular salt. This was accomplished by a second perfusion with 0.25 M sucrose. Therefore, the recovery of aldolase in the soluble fraction reflects solely the ability of intracellular constituents (presumably ionic in nature) to maintain the enzyme in the particle-free state.

We are able to provide an estimate of the extent to which the constituents of the cytoplasm are diluted in the course of preparing the concentrated homogenate. A reasonable estimate of the cytosolic space is 0.25 ml per gram wet liver (A. J. Lange and W. J. Arion, unpublished experiments). Since the homogenate volume per gram liver was 1.5 ml and 0.83 ml of this could be attributed to the supernatant fraction (see "Materials and Methods"), one can estimate that the constituents of the cytosolic milieu were diluted by a factor of 2.8. Despite this dilution, sufficient amounts were present to maintain aldolase in an unassociated state. From these considerations, we conclude that liver aldolase is not associated with the endoplasmic reticulum in situ. The binding of the enzyme to microsomes would appear to represent an artifact of the dilution of the cytoplasmic milieu that occurs during preparation of the standard 10% homogenate.

Foemmel et al. (1) have argued that the most compelling support for the particulate localization of aldolase in situ is provided by the findings from ultrastructural immunochemical studies. In our opinion, the electron micrographs presented in support of this conclusion are of insufficient quality to permit the assessment of whether aldolase exists in a free or bound state in the fixed liver cell. Moreover, in view of the marked pro-

pensity of aldolase to associate with microsomes *in vitro*, caution must be invoked in interpreting the cytochemical findings. Fixation *per se* could alter aldolase or the membrane so as to enhance binding. Alternatively, fixation in buffered sucrose may lead to a loss of the small ionic constituents from the tissue, which in effect would induce binding. We believe the present technique of employing highly concentrated tissue preparations represents a more definitive approach to the problem of the intracellular localization of aldolase.

The present results with liver aldolase may be offered as a challenge to the validity of the conclusions regarding the particulate locations of other enzymes of the glycolytic pathway in other tissues or cells (2-7). Moreover, the concept (2) that the glycolytic enzymes exist *in situ* as multi-enzyme complexes with membranous elements of cells needs to be carefully scrutinized.

ACKNOWLEDGEMENT: This investigation was supported, in part, by New York State funds appropriated to the New York State College of Veterinary Medicine.

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