Changes in activity of fructose-1,6-bisphosphate aldolase in livers of fasted rabbits and accumulation of crossreacting immune material

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ABSTRACT The activity of fructose-1,6-bisphosphate aldolase (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) in livers of fasted rabbits decreases to less than one-half the value found in livers of fed rabbits. However, the concentration of aldolase protein in the liver extracts, measured with a specific antibody, remains unchanged. More than twice as much antibody is required to neutralize the aldolase activity in liver extracts from fasted compared with fed rabbits. The results suggest that modification of liver aldolase occurs during fasting, resulting in loss of catalytic activity without loss of immunoreactivity.

We have previously observed (1) that rabbit liver aldolase tends to copurify with fructose-1,6-bisphosphatase when the latter enzyme is isolated from livers of fed rabbits but not when the enzyme is prepared from livers of fasted rabbits. In an effort to understand the molecular basis for this observation, we have examined the changes in properties of aldolase from livers of fasted rabbits. We have found that during fasting the enzyme activity decreases, in agreement with the earlier report by Adelman et al. (2). However, the total amount of immunoreactive aldolase protein remains unchanged, and the decrease in aldolase activity is attributed to the accumulation of a crossreacting material (CRM) that retains the electrophoretic mobility and immunological reactivity of the native enzyme. The results are reminiscent of the changes in liver aldolase reported by Gershon and Gershon (3) in aging mice.

MATERIALS AND METHODS

Materials. Adult brown rabbits, weighing 2.5–2.8 kg, were purchased from a local market (Genoa) and fed ad lib on a diet of Altromin MS/K (Reipol Baglan, Italy) for 1 week before the experiments were begun. Fructose-1,6-bisphosphate (Fru-P₂) aldolase (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) was isolated from the livers as described (4). Triose-phosphate isomerase, glyceral-3-phosphate dehydrogenase, NADH, and Fru-P₂ were purchased from Sigma. Sepharose 4B was obtained from Pharmacia. Phosphocellulose P-11 was purchased from Whatman Biochemicals, Maidstone, England. Ammonium, pH 3.5–10, was from LKB.

Methods. Fru-P₂ aldolase activity was assayed as described by Gracy et al. (5). Antiserum against rabbit liver aldolase prepared in the guinea pig was a gift of Charles Isaacs (Roche Institute of Molecular Biology) and was coupled to CNBr-activated Sepharose 4B (6). The Sepharose-coupled antibody was washed with 0.1 M NaHCO₃ (pH 9.0) and suspended in 0.15 M NaCl containing 20 mM Na phosphate pH 7.0 buffer. It was then packed into glass columns (1×6 cm) and washed extensively with the same NaCl/phosphate buffer solution before and after each use.

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FIG. 1. Change in specific activity of aldolase purified from crude liver extracts by adsorption and elution from Sepharose-coupled antibody columns. The specific activities were calculated from the activity measured in the crude extracts and the amount of protein eluted with 4 M MgCl₂ from the antibody-Sepharose columns (described in Table 1) and defined as amol of Fru-P₂ cleaved/min per mg of protein. The bars show the range of values obtained from individual animals; two to four animals were sacrificed for each time point.

Protein concentration for purified aldolase was calculated from the absorbance at 280 nm (5). For crude extracts or for aldolase eluted from the antibody columns, the method of Lowry et al. (7) was used with bovine serum albumin (Sigma) as standard.

Electrofocusing was carried out with the LKB 2117 Multiphor system at 12°C in 4.7% polyacrylamide gel slabs, containing 8 M urea and 2% Ampholine in the pH range 3.5–10. Development of the gels was for 2.5 hr at 16 W. Standard slab gel electrophoresis in 4% polyacrylamide gels and slab gel electrophoresis in sodium dodecyl sulfate/10% polyacrylamide were performed as described by Maizel (8). RESULTS

The activity of Fru-P₂ aldolase in extracts of rabbit liver during a 90-hr fast decreased to nearly one-third of the values found in similar extracts prepared from livers of fed rabbits (Table 1). However the proportion of immunoreactive protein, measured by adsorption to and elution from Sepharose-bound anti-rabbit liver aldolase, remained essentially unchanged. The increase in CRM during fasting was reflected by the decrease

Abbreviations: CRM, crossreacting material; Fru-P₂, fructose 1,6-bisphosphate.
Table 1. Aldolase activities in crude extracts prepared from livers of fed and fasted rabbits

<table>
<thead>
<tr>
<th>Condition</th>
<th>Animal</th>
<th>Liver weight, g/kg</th>
<th>Protein, mg/ml</th>
<th>Aldolase activity, Units/ml</th>
<th>Immunoreactive aldolase protein,* mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed</td>
<td>1</td>
<td>42</td>
<td>22.6</td>
<td>0.58</td>
<td>0.026</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>45</td>
<td>24.7</td>
<td>0.54</td>
<td>0.022</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>43</td>
<td>24.0</td>
<td>0.54</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>40</td>
<td>23.7</td>
<td>0.55</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td>Av.</td>
<td></td>
<td></td>
<td>0.55</td>
<td>0.023</td>
</tr>
<tr>
<td>Fasted, 24 hr</td>
<td>1</td>
<td>40</td>
<td>24.1</td>
<td>0.48</td>
<td>0.020</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>37</td>
<td>25.7</td>
<td>0.45</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>Av.</td>
<td></td>
<td></td>
<td>0.46</td>
<td>0.019</td>
</tr>
<tr>
<td>Fasted, 36 hr</td>
<td>1</td>
<td>35</td>
<td>24.0</td>
<td>0.29</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>34</td>
<td>26.8</td>
<td>0.31</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>Av.</td>
<td></td>
<td></td>
<td>0.30</td>
<td>0.012</td>
</tr>
<tr>
<td>Fasted, 60 hr</td>
<td>1</td>
<td>32</td>
<td>26.7</td>
<td>0.23</td>
<td>0.0086</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>32</td>
<td>25.4</td>
<td>0.25</td>
<td>0.0098</td>
</tr>
<tr>
<td></td>
<td>Av.</td>
<td></td>
<td></td>
<td>0.24</td>
<td>0.0092</td>
</tr>
<tr>
<td>Fasted, 96 hr</td>
<td>1</td>
<td>30</td>
<td>27.4</td>
<td>0.21</td>
<td>0.0076</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>29</td>
<td>25.9</td>
<td>0.18</td>
<td>0.0069</td>
</tr>
<tr>
<td></td>
<td>Av.</td>
<td></td>
<td></td>
<td>0.20</td>
<td>0.0072</td>
</tr>
</tbody>
</table>

Rabbits were killed by cervical dislocation, and the livers were collected immediately, weighed, and chilled on ice. Crude extracts were prepared with a Potter-Elvehjem homogenizer by using 10 g of fresh liver and 45 ml of 0.25 M sucrose/1 mM EDTA, pH 7.0. The homogenates were centrifuged for 30 min at 15,000 × g and the supernatant solutions were assayed.

* For determination of total immunoreactive aldolase protein, 5 ml of each crude extract was passed through a column of Sepharose-coupled antibody and after washing with the NaCl/phosphate buffer the adsorbed proteins were eluted with 4.0 M MgCl₂. The eluates were dialyzed and assayed for protein by the method of Lowry et al. (7). The values are expressed as mg of protein eluted per ml of crude extract applied.

in the specific activity of the enzyme purified by adsorption and elution from the antibody column, from an average of 1.70 to 0.60 μmol/min per mg (Fig. 1). The value of 1.70 calculated for the specific activity of the enzyme recovered from the extracts of livers from fed animals compares favorably with that reported for liver aldolase purified by conventional methods (4, 5).

The accumulation of inactive or less active CRM was also demonstrated by titration of the crude extracts with the Sepharose-bound antibody (Fig. 2). There was very little difference between the titration curves for purified enzyme and extracts from livers of fed rabbits. On the other hand, nearly twice as much antibody was required to neutralize the same amount of enzyme activity in the extracts of livers from fasted rabbits.

Despite the decrease in specific activity of the enzyme purified from livers of fasted rabbits by adsorption to the specific antibody columns, no change in the apparent molecular weight of the subunits was detected when the samples were analyzed by sodium dodecyl sulfate/polyacrylamide electrophoresis (Fig. 3). Similarly, the enzyme isolated from livers of fed or fasted rabbits by adsorption to Sepharose-coupled antibody showed the same behavior as the native enzyme protein in isoelectric focusing; in each case, multiple forms with pK values of approximately 8.4, 8.2, 7.9, and 7.6 were detected, similar to those reported (9) for the crystalline enzymes from human liver (data not shown).

The possibility that the modification may have occurred in vitro during preparation of the extracts was tested by adding purified aldolase to aldolase-depleted extracts. Extracts prepared from livers of rabbits fasted for 60 hr were treated with an excess of specific anti-aldolase bound to Sepharose, which was then removed by centrifugation. No aldolase activity could be detected in the supernatant solutions. Native rabbit liver aldolase, purified from livers of fed rabbits, was added to the depleted extracts and the mixture was incubated for 2 hr at room temperature. No change in the aldolase activity was observed. Preliminary results (data not shown) of an investigation of the effects of a lysosomal enzyme preparation on purified rabbit liver aldolase suggest that this preparation causes changes in the properties of aldolase similar to those observed during fasting.

**DISCUSSION**

Fasting has been shown to induce changes in the levels of activity of a number of liver enzymes. In general, enzymes required for glycolysis, such as gluconokinase, phosphofructokinase, and pyruvate kinase, show decreased activity (10, 11) whereas enzymes involved in gluconeogenesis, such as fructose-1,6-bisphosphatase (11) and phosphoenolpyruvate carboxykinase (12), increase in activity. The changes in enzyme activity levels are generally considered to be related to changes in the rates of synthesis and degradation, which appear to be regulated in such a manner as to maintain the level of enzyme activity appropriate to the physiological state of the animal. In the case of pyruvate kinase, the activity of the enzyme also appears to be regulated by phosphorylation/dephosphorylation (13) under the influence of glucagon and insulin (14).

Whether the decreased activity is the result of covalent modification or limited proteolysis has not been established. Either would be consistent with the fact that the molecular weight of the modified enzyme appears to be unchanged, as is the total amount of immunoreactive protein detected by binding to the antibody-Sepharose columns. It is of interest that an inactive crossreacting form of aldolase has also been shown
During gluconeogenesis in liver, aldolase is required for the conversion of triose phosphates to Fru-P2, which is in turn converted to fructose-6-phosphate by the action of fructose-1,6-bisphosphatase. Fructose-1,6-bisphosphatase is inhibited by excess Fru-P2 (15), which also activates the opposing glycolytic enzyme, phosphofructokinase (16). The conversion of aldolase to a less-active form may be designed to maintain the steady-state concentration of Fru-P2 at low levels that would favor the metabolic flux in the direction of gluconeogenesis rather than in the direction of glycolysis.

The change in properties of aldolase in livers of fasted rabbits may explain our earlier observations on the copurification of this enzyme with fructose-1,6-bisphosphatase from fed, but not fasted, rabbit livers (1). We have reported other evidence for an interaction and possible molecular association of liver fructose-1,6-bisphosphatase and liver aldolase (4); this tendency of the two native enzymes to associate appears to be lost when aldolase is modified by subtilisin and may account for the differences in its behavior during purification of fructose-bisphosphatase from livers of fed and fasted animals.

Note Added in Proof. After this paper was submitted, Petell and Lebherz (17) reported evidence that the defective aldolase molecules observed in extracts of livers of aged mice are not produced in vivo but are formed during isolation of the enzyme. We have carried out additional experiments to exclude the possibility that the cross-reacting material detected in the livers of fasted rabbits is the result of proteolytic modification during the isolation procedure. The details of these experiments will be reported elsewhere.

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FIG. 2. Immunotitration of liver aldolase activity. The aldolase was purified from livers of fed rabbits as described in Materials and Methods. Crude liver extracts were prepared from livers of fed or fasted rabbits as described in Table 1. Aliquots were diluted in 0.2 ml of 0.15 M NaCl/20 mM Na phosphate, pH 7.0. The indicated amounts of Sepharose-coupled antibody were added and the suspensions were gently stirred for 1 hr at 4°C. The Sepharose-coupled antibody was then removed by centrifugation at 2000 g for 10 min, and aldolase activity in the supernatant was determined. Aldolase activity is expressed as total units; 1 unit is the amount that cleaves 1 µmol of Fru-P2 per min in the standard assay. □, extracts from livers of fed rabbits; ■, extracts from livers of rabbits fasted 60 hr.

FIG. 3. Sodium dodecyl sulfate/10% g/l electrophoresis of aldolase purified by immunoadsorption from livers of fed or fasted rabbits. Purified aldolase (25 µg) (lane 1) from livers of fed rabbits (ref. 4; see Materials and Methods) was compared with immunoreactive aldolase purified by adsorption to Sepharose-coupled antibody from livers of fed (lane 2) or 60-hr fasted (lane 3) rabbits. Gels were stained with Coomassie blue in 25% ethanol and destained in 25% ethanol/10% acetic acid.

to accumulate in livers of aged animals (3). We have obtained preliminary evidence indicating that this modification may be due to the action of a lysosomal protease(s).