Involvement of Cytosol Proteins in Oleate Activation of Rabbit Liver Fructose-1,6-diphosphatase*

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SUMMARY

Dialyzed rabbit liver cytosol was specifically freed of endogenous fructose-1,6-diphosphatase by immunoadsorption on a column of Sepharose-immobilized anti-fructose-1,6-diphosphatase. This material increased the specific activity of homogeneous enzyme to the maximal rate observed with EDTA and shifted the pH optimum from 8.4 to 7.4. With oleate or other fatty acids as activators, the hydrolysis of fructose-1,6-diphosphatase by enzyme, at neutral pH, showed nonlinear initial rates dropping to lower linear rates. Cytosol activator acted synergistically with oleate both to increase neutral enzyme activity and to maintain the high initial catalytic rates. After sucrose density centrifugation or gel filtration, the cytosol had no effect by itself, but still potentiated oleate activation. The factor was destroyed by treatment with subtilisin or trypsin, but all attempts to identify a unique protein component in cytosol were unsuccessful. The presence of Na dodecyl-S04, deoxycholate, or urea did not improve the resolution of the factor, but these compounds did lower the Kₘ for activation by cytosol. Since fatty acids are the only unique compounds which have been isolated from cytosol which activated fructose-1,6-diphosphatase, it appears that soluble proteins can act as natural carriers for the fatty acids. This was supported by the fact that both dialyzed rabbit α-globulins and muscle phosphofructokinase also acted synergistically with oleate in a manner similar to cytosol. Phosphatidic acid and phosphatidylserine activated fructose-1,6-diphosphatase, and their action was synergistic with oleate. Glutathione (1 mM) activated the enzyme 5-fold at pH 7.3 and its effects were additive with oleate and cytosol or α-globulins.

We have identified fatty acids, particularly oleate, as unique components in rabbit liver pH 6 precipitate which stimulate the activity of fructose-1,6-diphosphatase in the neutral pH range (1-3). What role specific proteins or other macromolecules in this fraction might play in increasing the catalytic rate was left unresolved. It was clear that fatty acids alone were not sufficient for full activation of homogeneous enzyme, since the rate of fructose-1,6-diphosphatase hydrolysis at neutral pH rapidly dropped even in the presence of excess oleate (3).

As one approach to this question, dialyzed liver cytosol was specifically and completely freed of fructose-1,6-diphosphatase by passage through a column of immobilized goat anti-rabbit liver enzyme. This initial procedure greatly simplified further investigation of the nature of the natural factor. Such preparations increased the specific activity of homogeneous enzyme to the maximal rate observed with EDTA and shifted the pH optimum from the alkaline to neutral region in the absence of other added effectors. Details of the synergistic action of proteins from this fraction with oleate and the probable general nature of the proteins involved are described in the present report.

EXPERIMENTAL PROCEDURE

Materials

Sodium salts of fructose-1,6-P₂, TPN⁺, oleate, ATP, and AMP, reduced glutathione, ovalbumin, muscle F₁-fructosekinase, yeast glucose-6-P isomerase (630 units/mg of protein, 16 mg/ml), and glucose-6-P dehydrogenase (from bakers' yeast, 390 units/mg of protein, 9 mg/ml) were purchased from Sigma. The latter two enzymes were diluted 1/10 and 1/25, respectively, in water and dialyzed twice for 2 to 4 hours against 200 volumes of 0.5 M ammonium sulfate (pH 7). [1-¹⁴C]Oleic acid (60 mCi/mmol) was purchased from Amer sham/Seal ele. Horse liver alcohol dehydrogenase and 3-P-glycerate were from Boehringer Mannheim; bovine liver catalase was from Calbiochem; subtilisin was from Novo Industries, Copenhagen, Denmark; DNase II and trypsin were from Worthington. Dioleoyl phosphatidic acid, phosphatidylserine (bovine), dioleoyl lecithin, and cardiolipin were from Applied Science Laboratories. A natural mixture of phosphatidic acids was obtained from Pierce Chemicals. Urea (ultrapure) and succrose (enzyme grade) were purchased from Schwarz/Mann. Purified asloectin (4) was a gift from A. Martonosi, Saint Louis University, St. Louis, Mo. Purified rabbit and human serum globulin fractions were from Miles. CM-cellulose (CM52) and DEAE-cellulose (DE52) were from Whatman, Bio-Gel A-0.5m from Bio-Rad, and Sephadexes and CNBr-activated Sepharose 4B from Pharmacia. All other chemicals used were of analytical grade. Homogeneous rabbit liver fructose-1,6-diphosphatase was prepared as previously described (3, 5). Distilled deionized water was used throughout.
Since this effector activates fructose-1,6-diphosphatase at neutral pH, further protein was removed, as measured by A280, Damp gel tase, in order to ensure even distribution of the protein on the gel, fasted overnight, was killed by cervical dislocation and exsan-1ginated. The liver was quickly removed, homogenized for 3 min to eliminate ambiguity in assaying and fractionating the cytosol. The concentration of homogeneous enzyme was determined assuming an absorbance of 0.880 at 380 nm for a solution of 1 mg per ml (6).

Pure fructose-1,6-diphosphatase was diluted from concentrated stocks (5 to 12 mg/ml) into a solution containing 50 mM Tris-HCl (pH 7.2), 10 mM MgSO4 or 0.1 mM MnCl2 and 0.5 mM KC1 before assay. It has been repeatedly observed that different dilutions of fructose-1,6-diphosphatase from concentrated stocks, prior to assay, resulted in variable specific activities at neutral pH. We have found that these variations can be minimized and maximal specific activity attained by keeping the protein concentrations as high as practicable and by including high salt (0.5 mM KC1) in the dilution buffer. In general, the specific activity obtained was 10 to 40% lower than that observed with 0.5 mM KC1 in the enzyme range of 4 to 10 &ml. This salt effect on initial dilution was nonspecific, and either NaCl or NH4Cl gave qualitatively the same result. The inclusion of EDTA in dilutions of enzyme also yielded high specific activities, but had to be avoided, since this effector activates fructose-1,6-diphosphatase at neutral pH (7) and prevents inactivation by ATP (8). These effects took place in the initial dilution and were observed independent of the salt concentration in the assay. KC1 in excess of 0.15 mM in the assay did not increase the observed activity. In addition to affecting enzyme activity, increasing concentrations of homogeneous, unproteolyzed enzyme protein or KC1 also significantly reduced the extent of inactivation by either ATP or P2,2'-methylene ATP (8). No evidence of gross change in molecular size was found under any of these conditions.

**Methods**

**Preparation of Dialyzed Rabbit Liver Cytosol**—A normal rabbit, fasted overnight, was killed by cervical dislocation and exsan-1ginated. The liver was quickly removed, homogenized for 3 min in a Waring Blender in 4 volumes of 0.15 M KC1/0.01 M NaHCO3/0.01 M MgSO4 per g of tissue, and centrifuged for 1 hour at 40,000 rpm (114,000 × g) in a Spinco 60 Ti rotor. The supernatant under the lipid layer was collected and dialyzed overnight against two changes of 0.01 M NaHCO3/0.01 M MgSO4; the precipitate formed was removed by centrifugation and the cytosol was stored at −20° or −65°.

**Preparation of Immobilized Proteins**—CNBr-activated Sephrose 4B gel was used to prepare several immobilized proteins. The procedures given here, based on that in Pharmacia literature, was typical. The efficiency of coupling varied with the proteins used and the ratio of protein to gel. All operations were carried out at 4°.

Two grams of CNBr-activated Sepharose 4B gel were placed on a sintered glass filter (15 ml, type C) and washed with 400 ml of 1 mM HCl over a 15-min period. Regenerated gel was transferred quantitatively to a screw-cap tube (20 × 150 mm) with 0.5 ml of coupling solution (0.1 mM NaHCO3/0.5 mM NaCl) and 0.5 ml of partially purified goat anti-rabbit liver fructose-1,6-diphosphatase (A12,000 = 40) added. The capped tube was mixed immediately by inversion to ensure even distribution of the protein on the gel, and then rotated slowly end-over-end for 2 hours on a multipurpose rotator (Scientific Industries, Inc.). The gel was collected on a sintered glass filter and washed with coupling buffer until no further protein was removed, as measured by A280. Damp gel was transferred to a screw-cap tube (20 × 150 mm) with 1.0 ml of coupling solution (0.1 mM NaHCO3/0.5 mM NaCl), 0.5 ml of partially purified goat anti-rabbit liver fructose-1,6-diphosphatase (A12,000 = 40) added. The capped tube was mixed immediately by inversion to ensure even distribution of the protein on the gel, and then rotated slowly end-over-end for 2 hours on a multipurpose rotator (Scientific Industries, Inc.). The gel was collected on a sintered glass filter and washed with coupling buffer until no further protein was removed, as measured by A280. Damp gel

**RESULTS AND DISCUSSION**

**Activation by Fructose-1,6-diphosphatase-free Cytosol**—In order to eliminate ambiguity in assaying and fractionating the cytosol activator, it was desirable first to remove the endogenous fructose-1,6-diphosphatase. An immunological procedure proved ideal for this purpose and offered the advantages of high specificity as well as no requirement for fractionation of the starting material. The use of antibodies coupled to an insoluble matrix alleviated problems of nonspecific adsorption to the antigen-antibody precipitate and contamination of the liver extract with serum protein. As is illustrated in Fig. 1, goat anti-rabbit liver fructose-1,6-diphosphatase coupled to Sepharose 4B (1 ml of gel, see “Methods”) was capable of quantitatively removing the fructose-1,6-diphosphatase from 2 ml of dialyzed rabbit liver cytosol.

Such crude rabbit liver preparations were potent activators of pure fructose-1,6-diphosphatase at neutral pH. The data shown in Fig. 2a are tracings of assays run with increasing amounts of deFDPase-cytosol.1 In addition to increasing the activity of

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1 The abbreviation used is: deFDPase-cytosol, dialyzed rabbit liver supernatant freed of fructose-1,6-diphosphatase by passage over immobilized goat anti-rabbit liver fructose-1,6-diphosphatase.
In the presence of 5 mM Mg2+, cytosol factor (15 ~1) increased the rate and linearity of enzyme catalysis in the presence of oleate (3). The late reaction rates, which remained essentially linear in the presence of factor, have been plotted. The difference between these two curves (Fig. 3, inset) indicated a maximum degree of activation at pH 7.4.

**Removal of Fatty Acids from Cytosol**—The identification of fatty acids as activators of fructose-1,6-diphosphatase in rabbit liver pH 6 precipitate left unanswered the question of the possible involvement of macromolecules in the action of the natural factor. One possible explanation was that the preparations above contained loosely bound fatty acids. As one approach to this problem, we attempted to remove as much fatty acid as possible from cytosol and to see whether activation still occurred. Bovine serum albumin was treated with acid-charcoal as described by Chen (9) to remove bound free fatty acids and then coupled to Sepharose (see “Methods”).

The ability of the immobilized bovine serum albumin to bind free fatty acids was tested by passing a sample of dialyzed liver supernatant, which had been incubated with tracer amounts of [14C]oleate, over a column of the material. When the total fatty acid present was below the binding capacity of the bovine serum albumin, the efficiency of removal of added oleate was 97% compared to a control Sepharose column where only 25% of the radioactivity was retained. After treatment with immobilized bovine serum albumin, dialyzed liver supernatant still gave the upward curvature in plots of enzyme rates versus concentrations of oleate (7) and deFDPased-cytosol still fully activated fructose-1,6-diphosphatase. Although these results could still be explained by the presence of trace amounts of nonexchangeable, bound fatty acids, the involvement of some other component than free fatty acids was clearly indicated.

**Synergism in Activation by Oleate and deFDPased-cytosol**—Attempts to fractionate the cytosol factor by either sucrose density gradient centrifugation or gel filtration resulted in loss of activator when assayed in the absence of added fatty acid. However, the protein fractions still potentiased the action of oleate (Fig. 4). The starting material retained its activity when stored under comparable conditions. It may also be noted that no significant purification was achieved. These fractions increased both the rate and the linearity of enzyme catalysis in the presence of oleate (Fig. 5), suggesting that they might be functioning in some manner as carrier molecules for fatty acids. Several other examples of the synergistic action of oleate and various preparations from deFDPased-cytosol are also shown in Fig. 5.

The addition of oleate reduced the amount of deFDPased-cytosol required to achieve 50% of maximal activation; however, deFDPased-cytosol, in general, increased the $K_{so}$ for oleate activation, possibly because of nonspecific binding in the crude fraction. This latter observation may also explain both the higher $K_{so}$ (oleate) and the sigmoidal response to increasing concentration of the fatty acid observed with crude enzyme (2).

**Protein Nature of deFDPased-cytosol Activator**—As previously found in studies with rabbit liver pH 6 precipitate (7), the non-
subtilisin equal to that added in these assays had no effect on the activation by oleate and deFDPased-cytosol.

The effect of deFDPased-cytosol was lost when assayed with or without added oleate. An amount of dialyzable activator component was destroyed by both subtilisin and 50 ml of each fraction without (O—O) or with (□—□) the addition of 40 μM oleate. Absorbance at 280 nm was determined on a 1/17 dilution of each fraction. The basal rate and rates without and with 40 μM oleate and 10 μl of deFDPased-cytosol are shown on the left ordinate.

![Fig. 4. Distribution of cytosol activator in a sucrose density gradient. DeFDPased-cytosol (0.2 ml) was layered on 4.6 ml of a 5 to 20% sucrose gradient containing 50 mM Tris-HCl, pH 6.5, and 10 mM MgSO₄, with the use of hand-forming caps. Centrifugation was performed in an SW 50.1 rotor at 40,000 rpm for 17 hours at 5°C. Fractions (0.2 ml) were collected from the top of the gradient using a Buchler Auto Densi-Flow. Initial reaction rates were determined in the standard assay system with 0.32 μg/ml of homogeneous enzyme and 50 μl of each fraction without (O—O) or with (□—□) the addition of 40 μM oleate. Absorbance at 280 nm was determined on a 1/17 dilution of each fraction. The basal rate and rates without and with 40 μM oleate and 10 μl of deFDPased-cytosol are shown on the left ordinate.

![Fig. 5. Synergistic effects of various deFDPased-cytosol preparations and oleate. All assays were under standard conditions using 0.32 μg/ml of homogeneous enzyme. A, 50 μl of Fraction 7 from a sucrose density gradient (see Fig. 4); B, 4 μl of a preparation of deFDPased-cytosol; C, 50 μl of rabbit liver dialyzed pH 6 supernatant freed of fructose-1,6-diphosphatase (diluted 7.7-fold); D, 60 μl of a deFDPased-cytosol fraction eluted from DEAE-cellulose (10) by 0.1 M KCl.

dialyzable activator component was destroyed by both subtilisin and higher concentrations of trypsin (protein ratio of 1/46 to 1/8 (w/w)). Subtilisin was much more effective in terms of both the rate of destruction and the degree to which the activator was destroyed (Table I). The effect of deFDPased-cytosol was lost when assayed with or without added oleate. An amount of subtilisin equal to that added in these assays had no effect on the activation by oleate and deFDPased-cytosol.

Attempts to Purify the Activator from deFDPased-cytosol—We have tried numerous and varied types of purification techniques to identify a unique protein component in the cytosol which activates fructose-1,6-diphosphatase, but all of these approaches have proved fruitless. On the basis of the ratio of activation to absorption at 280 nm, none of the procedures that follow gave better than a 2- to 3-fold purification: sucrose density gradient centrifugation (Fig. 4), gel filtration on Sephadex G-200 or agarose (Bio-Gel A-0.5m), or ion exchange chromatography on CM- or DEAE-cellulose. The activator usually was present to some extent in all fractions and required oleate to be detected. The small purification achieved by gel filtration was primarily due to removal of heavier, excluded material which had no effect on enzyme activity. Upon chromatography on DEAE-cellulose in 20 mM triethanolamine, pH 8.5, with stepwise elution between 0 and 1.0 M KCl, all of the fractions containing protein were activators. The material which did not bind to the column was about one-half as effective as the starting material, while all of the other fractions were roughly twice as effective. Salt fractionation with ammonium sulfate gave no purification. The pH 5 precipitate of deFDPased-cytosol contained 10 to 20% of the protein and all of the detectable activator when assayed without added oleate; however, when assayed with oleate, the pH 5 supernatant was equal to or better than the precipitate as an activator. Some specificity of the protein factor is suggested by the fact that three purified proteins, spleen DNase (0.2 mg/ml), liver alcohol dehydrogenase (0.2 mg/ml), and liver catalase (0.24 mg/ml), did not increase the fructose-1,6-diphosphatase rate in the presence of 10 μM oleate.

In addition to albumin, two other proteins have been purified from rat liver cytosol, as well as a number of other tissues that bind free fatty acids very tightly (11). We purified the “Z fractions” from both rat and rabbit liver and tested these proteins as activators in the presence and absence of oleate. Z fraction produced no activation and, in fact, inhibited the fructose-1,6-diphosphatase rate in the presence of oleate. Other Sephadex G-75 fractions from the gel filtration of rat liver cytosol did activate fructose-1,6-diphosphatase with oleate addition.

The possibility that the activator might be concentrated in a glycoprotein fraction was eliminated by the following experiments: (a) the factor was precipitated by 0.6 M perchloric acid,
The following experiments supported this hypothesis: (a) When deFDPased-cytosol was placed in boiling water for 2 min, the remaining supernatant no longer acted synergistically with oleate. However, addition of 0.5% Na dodecyl-SO₄ to the precipitate extracted activator. II 0.5% Na dodecyl-SO₄ was added before boiling, the supernatant obtained increased the enzyme rate in the presence of oleate equivalently to an unheated control. (b) The residue after chloroform/methanol extraction (14) of deFDPased-cytosol was not an activator. However, it was possible to extract material with 1% Na dodecyl-SO₄ which, when assayed in the presence of 0.004% Na dodecyl-SO₄, acted synergistically with oleate. (c) Ovalbumin (0.5 mg/ml) did not activate fructose-1,6-diphosphatase nor increase the rate with added oleate. However, addition of 0.5% Na dodecyl-SO₄ to the precipitate extracted activator. If 0.5% Na dodecyl-SO₄ was added before boiling, the supernatant obtained increased the enzyme rate in the presence of oleate equivalently to an unheated control. (b) The residue after chloroform/methanol extraction (14) of deFDPased-cytosol was not an activator. However, it was possible to extract material with 1% Na dodecyl-SO₄ which, when assayed in the presence of 0.004% Na dodecyl-SO₄, acted synergistically with oleate. (c) Ovalbumin (0.5 mg/ml) did not activate fructose-1,6-diphosphatase nor increase the rate with added oleate. However, in the presence of 0.5% Na dodecyl-SO₄, ovalbumin increased the base-line 2- to 3-fold in the absence and 5-fold in the presence of 0.004% Na dodecyl-SO₄. A similar but smaller effect was seen with spleen DNase.

α-Globulins—Rabbit serum proteins were dissolved as much as possible in 0.9% saline and centrifuged, and protein concentrations were determined assuming an absorbance of 1 at 280 nm for a 0.1% solution. All assays were run in the standard manner at pH 7.3 (30°) with 0.16 µg/ml (Experiment 1), 0.1 µg/ml (Experiment 2), and 0.32 µg/ml (Experiment 3) of homogeneous fructose-1,6-diphosphatase. In Experiments 2 and 3, protein solutions were first dialyzed against saline. The fold-activation is indicated in parentheses.

### Table II

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Addition</th>
<th>(ΔA/min) X 10³ (late rates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 None</td>
<td>EDTA (0.1 mm)</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Oleate (10 µM)</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Oleate, Na dodecyl-SO₄ (0.004%)</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Oleate, deFDPased-cytosol (10 µl)</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Oleate, deFDPased-cytosol (2 µl), Na dodecyl-SO₄</td>
<td>51</td>
</tr>
</tbody>
</table>

- The Table II and deFDPased-cytosol were mixed for 10 min at room temperature before addition to assays.

### Table III

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Fraction</th>
<th>Concentration</th>
<th>(ΔA/min) X 10³</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 None</td>
<td>EDTA (0.1 mm)</td>
<td>32</td>
<td>6</td>
</tr>
<tr>
<td>α-Globulins</td>
<td>5</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>β-Globulins</td>
<td>23</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>γ-Globulins</td>
<td>25</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Albumin</td>
<td>126</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>91</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

- Late rates.
- (Calculated rate: 16).

The only reasonable explanation we can conclude from these results is that specific regions in liver proteins for the binding of fatty acids (a combination of hydrophobic regions in proper juxtaposition to an anionic binding site (13)) are exposed by detergents and urea and serve as good "carriers" for fatty acids. The following experiments supported this hypothesis: (a) When deFDPased-cytosol was placed in boiling water for 2 min, the remaining supernatant no longer acted synergistically with oleate. However, addition of 0.5% Na dodecyl-SO₄ to the precipitate extracted activator. II 0.5% Na dodecyl-SO₄ was added before boiling, the supernatant obtained increased the enzyme rate in the presence of oleate equivalently to an unheated control. (b) The residue after chloroform/methanol extraction (14) of deFDPased-cytosol was not an activator. However, it was possible to extract material with 1% Na dodecyl-SO₄ which, when assayed in the presence of 0.004% Na dodecyl-SO₄, acted synergistically with oleate. (c) Ovalbumin (0.5 mg/ml) did not activate fructose-1,6-diphosphatase nor increase the rate with added oleate. However, in the presence of 0.5% Na dodecyl-SO₄, ovalbumin increased the base-line 2- to 3-fold in the absence and 5-fold in the presence of 0.004% Na dodecyl-SO₄. A similar but smaller effect was seen with spleen DNase.
activated synergistically with oleate (Table III). No fractionation of the factor in the α-globulins was achieved by Sephadex G-200 gel filtration or equilibrium centrifugation in CsCl (12). Human α-globulin Fractions IV, IV-1, and IV-4 also increased fructose-1,6-diphosphatase activity in the presence of oleate, but for their maximal effect, the required protein concentration was about 10 to 15 times that of rabbit α-globulins.

It is possible that these serum proteins are involved in fructose-1,6-diphosphatase activation in liver. However, deDFPased-cytosol after removal of serum proteins was still equally effective as an activator in the presence of oleate. Passage of deDFPased-cytosol through immobilized goat anti-rabbit serum-Sepharose completely removed rabbit serum proteins, as checked by the Ouchterlony double immunodiffusion technique.

Effect of Phospholipids—Allen and Blair (10) have reported the activation of rabbit liver fructose-1,6-diphosphatase and prevention and reversal of AMP inhibition by muscle P-fructokinase (7). Part of this effect can be explained by the role of P-fructokinase as a protein carrier for oleate. Crystalline rabbit muscle P-fructokinase (33 μg/ml; dialyzed extensively against prevention and reversal of AMP inhibition by muscle P-fructokinase (as measured by double immunodiffusion) removed rabbit serum proteins, as checked by the Ouchterlony double immunodiffusion technique.

Eject of Phosphofructokinase—We have previously reported the activation of rabbit liver fructose-1,6-diphosphatase and prevention and reversal of AMP inhibition by muscle P-fructokinase (7). Part of this effect can be explained by the role of P-fructokinase as a protein carrier for oleate. Crystalline rabbit muscle P-fructokinase (33 μg/ml; dialyzed extensively against 0.2 M ammonium sulfate, pH 7.2) gave 80% of the maximal fructose-1,6-diphosphatase rate (EDTA, pH 7.3) with 20 μM oleate and 92% with 30 μM oleate. However, deDFPased-cytosol, from which liver P-fructokinase had been completely removed by passage over Sepharose-immobilized goat anti-muscle P-fructokinase (as measured by double immunodiffusion) still fully activated fructose-1,6-diphosphatase in the presence of oleate. Thus, there was no obvious specificity for liver P-fructokinase as a carrier of fatty acids.

Effects of Phospholipids—Allen and Blair (10) have reported that several phospholipids carrying a net negative charge were activators of pure rabbit liver fructose-1,6-diphosphatase. They suggested that these phospholipids bound to protein might serve as natural activators and presented evidence that a liver protein fraction obtained by DEAE-cellulose chromatography activated the enzyme and also contained diphosphonositide. Another protein fraction inhibited fructose-1,6-diphosphatase. All of the protein fractions that we obtained by DEAE-cellulose chromatography of deDFPased-cytosol were activators of fructose-1,6-diphosphatase. The fraction eluted with 0.1 M KCl was extracted with chloroform/methanol, but this extract was found to be ineffective as an activator of fructose-1,6-diphosphatase. We have confirmed their observations that phosphatic acid (8 μM) and higher concentrations of phosphatidylethanolamine (92 μM) both activated fructose-1,6-diphosphatase; in addition, we found that these phospholipids acted synergistically with oleate. However, these effects were found to be quite variable. Part of the problem was the requirement for sonication before testing these compounds and the possibility of variable liberation of fatty acids. Neither a natural mixture of phospholipids (purified asolectin), lecithin, nor cardiolipin increased fructose-1,6-diphosphatase activity in the presence or absence of added oleate. Furthermore, lecithin (160 μg/ml) completely inhibited activation by a mixture of 10 μM oleate and phosphatic acid (6 μM). It thus seems probable that the predominance of lecithin and phosphatidylethanolamine in natural phospholipid mixtures explains the failure of crude liver phospholipids (2, 3) or asolectin to activate the enzyme. The high concentration of liver activator in the pH 6 precipitate, which contains the bulk of the phospholipids (15), does suggest a possible role of phospholipid-containing proteins.

Subcellular Localization of Liver Activator—Rabbit liver microsomal and postmicrosomal fractions (16, 17) were found to contain about 20 to 30% of the total activator. However, removal of these particles in 0.19 M KCl or 0.1 M Tris-HCl, or storage at 4°C, resulted in complete loss of activator. Also, after isopycnic centrifugation (18) of a postmitochondrial supernatant layered over a 60% (w/w) sucrose cushion and under a 20 to 50% (w/w) sucrose gradient (5°, 63 hours, 40,000 rpm, SW 40 rotor), the clearly visible upper particle fractions were very poor activators, whereas the cytosol fraction at the bottom still activated fructose-1,6-diphosphatase the same as deDFPased-cytosol. It would thus appear that any activator associated with these membrane preparations, which are known to bind fatty acids, was very easily removed.

Glutathione—Sulfhydryl groups are known to activate fructose-1,6-diphosphatase (7, 19), and another possible natural activator is GSH, which occurs at relatively high concentration in liver (3 to 6 μmol/g wet weight). Activation of homogeneous enzyme by GSH (K<sub>50</sub> = 0.2 mM) gave enzyme rates approaching those found with EDTA. GSH activation was also additive with oleate-deDFPased-cytosol.

Effects of Fructose-1,6-P<sub>2</sub> and Methyl Fructoseide-1,6-P<sub>2</sub> on Initial Enzyme Rates—The initial burst of activity upon substrate addition followed by a lower rate suggested that an equilibrium of at least two enzyme species (i.e. "low" and "high" activity forms) exists which is altered by substrate. We have found that this initial burst can be eliminated by incubation in the presence of 1 μM methyl fructoseide-1,6-P<sub>2</sub> (Fig. 6) or with substrate in the absence of Mg<sup>2+</sup>. Following such a treatment, linear rates are observed in the absence or presence of oleate. The enzyme catalytic rate remains essentially linear in the presence of EDTA or an excess of other activators. Substrate and methyl fructoseide-1,6-P<sub>2</sub> are known to induce a significant conformational change in fructose-1,6-diphosphatase based on both physical and kinetic studies (8, 21-23). One plausible explanation for these observations is that the binding of substrate changes the enzyme completely to the low activity form. The presence of activators can either prevent or reverse this conversion with the formation of high activity enzyme. To explain our results with oleate, we would have to assume that oleate activated both forms of the enzyme, but in the absence of protein "carrier" could not maintain fructose-1,6-diphosphatase in the high activity form.
CONCLUDING COMMENT

We would like to stress the fact that in testing the activation of fructose-1,6-diphosphatase by fatty acids, it is important to observe one of the following conditions during assay: (a) Use relatively high concentrations of fructose-1,6-diphosphatase (with our preparation, at least 0.4 μg/0.5 ml) and measure the initial rates within 1 min after adding substrate. As illustrated in Fig. 1 of Ref. 3, good activation by oleate was observed. Much lower activation by oleate was found with lower levels of enzyme (0.16 μg/0.5 ml) in the absence of added protein factor, as may be seen in Fig. 5. We still do not understand the reason for this peculiar behavior of the enzyme. (b) Include either deFDPase-digested cytosol or dialyzed rabbit α-globulin in the assays with appropriate controls.

The question still left unanswered is how naturally occurring effectors of liver fructose-1,6-diphosphatase interact in vivo to regulate neutral enzyme activity. During assays in the absence of added effectors, we have found that the neutral enzyme activity remains linearly proportional to increasing concentrations of fructose-1,6-diphosphatase (at the nonactivated rate). Several cytosol protein fractions as well as reduced glutathione have the ability to increase this neutral activity markedly and fatty acids enhance the effect. Moreover, inactivation by ATP with corresponding changes in sensitivity to AMP inhibition (8) is specifically prevented and reversed by 3-P-glycerate and GSH, as well as protein carrier-oleate. Studies of changes in fructose-1,6-diphosphatase activity under conditions more closely approaching those found in vivo will be required to resolve this problem. In this regard, our recent findings of high gluconeogenic rates in chick embryo liver homogenates (24) may provide a suitable model system for such studies.

REFERENCES

Involvement of cytosol proteins in oleate activation of rabbit liver fructose-1,6-diphosphatase.
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