Characterization of the Fatty Acid-Sensitive Glucose 6-Phosphate Dehydrogenase from *Pseudomonas cepacia*

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The adenosine 5'-triphosphate-insensitive glucose 6-phosphate dehydrogenase from *Pseudomonas cepacia* has been found to be strongly inhibited by long-chain fatty acids and their acyl coenzyme A esters, suggesting that an important role of this isoenzyme might be to provide reduced nicotinamide adenine dinucleotide phosphate for reductive steps in fatty acid synthesis. The enzyme, which has been redesignated the fatty acid-sensitive glucose 6-phosphate dehydrogenase, has been purified to homogeneity using affinity chromatography with nicotinamide adenine dinucleotide phosphate-substituted Sepharose as a key step in the purification. The purified preparations were used to study the immunological properties and subunit composition of the enzyme and its relationship to the adenosine 5'-triphosphate-sensitive glucose 6-phosphate dehydrogenase present in extracts of *P. cepacia*. Although both enzymes were found to be composed of similar size subunits of about 60,000 daltons, immunological studies failed to demonstrate any antigenic similarity between them. Studies of the sedimentation behavior of the fatty acid-sensitive enzyme in sucrose gradients indicated that its apparent molecular weight is increased in the presence of glucose 6-phosphate and suggest that it may exist in an aggregated state in vivo. Palmitoyl coenzyme A, which strongly inhibited the enzyme, failed to influence its sedimentation behavior.

The catabolically versatile bacterium *Pseudomonas cepacia* (*P. multivorans*) possesses two species of glucose 6-phosphate dehydrogenase, which differ in molecular weight, pyridine nucleotide specificity, and sensitivity to inhibition by adenosine 5'-triphosphate (ATP) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) (7). One of the isoenzymes, which is active primarily with NAD and subject to inhibition by ATP, was purified and shown to be a tetramer composed of 60,000-molecular-weight subunits (14). Inhibition of this enzyme by ATP presumably serves to conserve hexose phosphate whenever there is an alternate source of NADH for ATP synthesis. The second glucose 6-phosphate dehydrogenase which is active primarily with NAD, was reported to have a molecular weight of about 120,000 and to be insensitive to ATP inhibition (7). We report here that the enzyme is strongly inhibited by long-chain fatty acids and their acyl coenzyme A (CoA) esters, suggesting its primary role might be to provide NADPH for reductive steps in fatty acid synthesis.

Several lines of evidence suggested that the two glucose 6-phosphate dehydrogenase species might be different oligomeric forms of the same protein, a possibility consistent with their molecular weights. First, the two enzymes appeared to be lost jointly in mutant strains of *P. cepacia* (T. G. Lessie and H. A. Shuman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, P123, p. 165). Second, as is described here, the fatty acid-sensitive enzyme can be made to increase in apparent molecular weight in the presence of glucose 6-phosphate to values close to that of the ATP-sensitive enzyme. Third, at high concentrations of NAD, the fatty acid-sensitive enzyme has significant ATP-sensitive NAD-linked activity. At first it seemed reasonable that such activity might represent conversion of a fraction of the fatty acid-sensitive enzyme to the ATP-sensitive species.

To further examine the relationship between the two isoenzymes, we have purified the fatty acid-sensitive glucose 6-phosphate dehydrogenase and compared its subunit composition and antigenic properties with those of the ATP-sensitive enzyme. The results indicate that the two isoenzymes are immunologically unrelated and lead to the conclusion that they are products of different genes that serve distinct physiological roles.
MATERIALS AND METHODS

Growth of bacteria. P. cepacia strain 249 (ATCC 17616) was grown at 37°C in 24-litre batches of medium containing 5 × 10⁻² M phosphate buffer, pH 6.5 (3.3 × 10⁻² M KH₂PO₄ and 1.7 × 10⁻² M Na₂HPO₄), 10⁻³ M MgSO₄, 10⁻¹ M CaCl₂, 10⁻³ M FeSO₄, and 0.2% (wt/vol) (NH₄)₂SO₄, with 0.5% (wt/vol) glucose as the carbon source. Cultures (24 litres) were aerated vigorously in a 28-litre Micro- ferment fermentor (New Brunswick Scientific Co., Inc., New Brunswick, N.J.). The bacteria were harvested by centrifugation with an RC2B centrifuge equipped with an SZ-14 continuous-flow rotor (Ivan Sorvall Inc., Norwalk, Conn.), and the cell paste was frozen at −20°C until use.

Assay of glucose 6-phosphate dehydrogenase. Enzyme activity was determined spectrophotometrically by monitoring the formation of NADPH or NADH at 24°C in assay mixtures containing 2 × 10⁻² M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 8.5), 5 × 10⁻¹ M NAD or NADP, and 10⁻² M d-glucose 6-phosphate as described earlier (7). Determination of protein for calculation of enzyme specific activities was by the method of Lowry et al. (8).

Purification of the fatty acid-sensitive glucose 6-phosphate dehydrogenase. Approximately 150 g (wet weight) of frozen bacteria was thawed and suspended in 600 ml of 2 × 10⁻² M phosphate buffer, pH 6.8, containing 10⁻¹ M sodium azide, lysozyme (2 mg/ml), and deoxyribonuclease II (10 μg/ml). The cell suspension was incubated with stirring at 37°C for 30 to 60 min to convert the cells to spheroplasts. The bacteria were then disrupted by exposure to high pressure (1,000 to 1,200 lb/in²) in a Parr bomb (Parr Instrument Co., Moline, Ill.) followed by rapid decompression to atmospheric pressure. The disrupted cell suspension was centrifuged at 15,000 × g for 15 min to remove cell debris and unbroken cells. The supernatant fraction was treated with an equal volume of 2% (wt/vol) streptomycin sulfate in 2 × 10⁻² M phosphate buffer, pH 6.8, containing 10⁻¹ M sodium azide and 10⁻² M 2-mercaptoethanol (standard phosphate buffer) and centrifuged to remove precipitated nucleic acids. In this and subsequent steps, the preparations were maintained at between 0 and 5°C. The supernatant fraction was treated with ammonium sulfate in three successive precipitation steps, using, respectively, 22, 6, and 10 g of solid ammonium sulfate per 100 ml of supernatant fluid. The precipitated material was collected by centrifugation after each addition of ammonium sulfate and suspended in standard phosphate buffer. The fraction obtained after the second addition of ammonium sulfate contained the bulk of the glucose 6-phosphate dehydrogenase activity (Table 1, step 2). This fraction, which contained both species of glucose 6-phosphate dehydrogenase, was dialyzed against standard phosphate buffer to remove ammonium sulfate and applied to a column (2.5 by 45 cm) of diethylaminoethyl-cellulose equilibrated with the same buffer. Elution of the two glucose 6-phosphate dehydrogenase species was accomplished by pumping 1 liter of a linear 0.02 to 0.2 M gradient of phosphate buffer, pH 6.8, containing 10⁻² M sodium azide and 10⁻² M 2-mercaptoethanol through the column. Fractions were collected and assayed for glucose 6-phosphate dehydrogenase activity. The fatty acid-sensitive enzyme eluted first at a phosphate concentration of 8 × 10⁻² M, followed by the ATP-sensitive dehydrogenase at a phosphate concentration of 1.3 × 10⁻¹ M. The two isoenzymes were completely separated in this step. The fractions containing ATP-sensitive glucose 6-phosphate dehydrogenase activity were pooled and stored at 0°C for use in experiments comparing the antigenic and kinetic properties of the two isoenzymes. The fractions containing fatty acid-sensitive enzyme were pooled and concentrated by ultrafiltration under N₂ (25 lb/in²) to a volume of 2 ml in an Amicon model 50 pressure cell fitted with a PM-10 membrane (Amicon Co., Lexington, Mass.). The concentrated material was dialyzed against standard phosphate buffer and then applied to a column (1 by 15 cm) containing NADP-Sepharose, which was equilibrated with the same buffer. NADP-Sepharose was prepared according to the procedure of Larsson and Mosbach (5). The column was washed with 50 ml of equilibration buffer to remove proteins that failed to bind to the NADP-Sepharose. No glucose 6-phosphate dehydrogenase eluted from the column in this step. Finally, equilibration buffer containing 5 × 10⁻¹ M NADP was pumped through the column to elute the fatty acid-sensitive glucose 6-phosphate dehydrogenase. Fractions containing glucose 6-phosphate dehydrogenase activity were pooled and stored at 0°C for subsequent use in studies of the subunit composition of the enzyme and for preparation of specific antisera. The steps of the purification procedure are summarized in Table 1 and discussed in Results.

Acrylamide gel electrophoresis. Preparation of gels and buffers for examination of the homogeneity of...
and molecular weight of the purified fatty acid-sensitive glucose 6-phosphate dehydrogenase was essentially as described by Davis (2). Protein resolved on the gels was stained with 0.01% (wt/vol) amido black in 7% (vol/vol) acetic acid. The position of glucose 6-phosphate dehydrogenase was confirmed by incubating the gels in assay mixtures supplemented with phenazine methosulfate (50 μg/ml) and 2,3,5-triphenyl tetrazolium chloride (μg/ml) as described earlier (7). The molecular weight of the holoenzyme was estimated by comparing its electrophoretic mobility on gels of different acrylamide concentration with those of reference proteins of known molecular weight, as detailed earlier (4, 7).

The subunit composition of the fatty acid-sensitive glucose 6-phosphate dehydrogenase was determined according to the method of Talbot and Yphantis (12). In this procedure proteins are dansylated before resolution to permit fluorescent monitoring of the subunits during acrylamide gel electrophoresis. Preparations of the purified enzyme and reference proteins were dansylated in 1-ml reaction mixtures containing 10−1 M Tris-acetate buffer (pH 8.2), 5% (wt/vol) SDS, and 20 μl of dansyl (1-dimethylamino-5-naphthalenesulfonyl)-chloride (0.1% in acetone). The mixture was mixed vigorously and placed in a boiling-water bath for 5 min. 2-Mercaptoethanol was added to a final concentration of 1% (vol/vol), and the samples were maintained at 100°C for 1 additional min. The mixtures were cooled and either dialyzed against 10−3 M Tris-acetate buffer (pH 8.2) or passed through a column (1 by 15 cm) of Bio-Gel P-30 (Bio-Rad Laboratories, Richmond, Calif.) equilibrated with the same buffer to remove unreacted dansyl chloride and other low-molecular-weight reaction products. Samples (0.02 to 0.05 ml) of the dansylated proteins containing bromophenol blue as a tracking dye were resolved electrophoretically on 7.5% (wt/vol) acrylamide gels containing 10−1 M Tris-acetate buffer (pH 8.2) and 0.1% (wt/vol) SDS with the same buffer in the reservoirs. The migration of the protein bands during electrophoresis was followed by monitoring their orange fluorescence under short-wave (254-nm) ultraviolet light. The gels were fixed in 7% acetic acid, and SDS was removed electrophoretically, using a Canaco gel destainer (Canaco Co., Rockville, Md.). The gels were permanently stained by overnight incubation in 0.25% (wt/vol) Coomassie brilliant blue in 7% acetic acid and electrophoretically destained. The molecular weight of the subunits was estimated by comparing their migration with that of subunits of reference proteins as detailed earlier (14).

Preparation of antiserum for use in the enzyme inhibition and immunodiffusion experiments. Samples of fatty acid-sensitive glucose 6-phosphate dehydrogenase equivalent to step 4 of Table 1 containing 1 mg of protein were emulsified in Freund incomplete adjuvant and injected subcutaneously into New Zealand rabbits. A booster injection consisting of a similar preparation was administered 5 weeks after the initial injection, and blood samples were obtained by cardiac puncture 1 week later. The serum fraction was supplemented with 10−2 M sodium azide and stored at 5°C.

Enzyme inhibition tests were carried out by preincubating appropriately diluted antiserum and enzyme for 5 min in assay mixtures from which pyridine nucleotide and glucose 6-phosphate were omitted. The enzyme reactions were initiated by addition of glucose 6-phosphate and NAD or NADP. Immunodiffusion experiments were carried out with micro-Ouchterlony plates prepared as described earlier (6). The wells of the immunodiffusion plates were charged with 1:2-diluted antiserum and with 5 × 10−2 U of fatty acid-sensitive glucose 6-phosphate dehydrogenase or 2 × 10−2 U of ATP-sensitive isoenzyme obtained from step 3 of Table 1.

Sedimentation properties of the fatty acid-sensitive glucose 6-phosphate dehydrogenase. Samples (0.5 ml) of fatty acid-sensitive enzyme from a preparation equivalent to step 3 of Table 1 were supplemented with 1 mg of bovine hemoglobin and applied to 30-ml linear sucrose gradients in polyallomer tubes appropriate to the SB 210 rotor of a Spinco ultracentrifuge (International Instruments Co., Needham, Mass.). The gradients contained 2 × 10−2 M phosphate buffer (pH 6.8), 10−2 M sodium azide, 10−2 M 2-mercaptoethanol, and the indicated amounts of glucose 6-phosphate. The gradient tubes were centrifuged at 5°C for 15 to 40 h at a speed of 24,000 rpm. The bottoms of the tubes were punctured, and between 25 and 30 fractions of equal size were collected. Portions of each fraction were assayed for glucose 6-phosphate dehydrogenase activity. The position of hemoglobin was determined by measuring the absorbance of the fractions at 405 nm. The apparent molecular weight of the glucose 6-phosphate dehydrogenase was estimated using the relationship of Martin and Ames (9), with hemoglobin (molecular weight, 64,500) as the reference protein.

Chemicals. Solutions of dansyl chloride were purchased from Pierce Chemical Co., Rockford, Ill. All other chemicals were obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Purification of the fatty acid-sensitive glucose 6-phosphate dehydrogenase from P. cepacia. Our initial studies of the two P. cepacia glucose 6-phosphate dehydrogenases indicated that the isoenzymes could be separated without loss of activity of either species (7). However, efforts to extensively purify the enzymes were successful only in the case of the ATP-sensitive dehydrogenase (14). The ATP-insensitive species, which we have redesignated the fatty acid-sensitive enzyme on the basis of experiments described later in this paper, proved to be highly labile in the final stages of purification. More recently, we have tried to obtain homogeneous preparations of the fatty acid-sensitive enzyme by a number of novel procedures. As is described below, the fatty acid-
sensitive enzyme sediments much more rapidly in sucrose gradients containing glucose 6-phosphate than in its absence. Thus, it seemed reasonable that the enzyme might first be resolved from high-molecular-weight and then from low-molecular-weight proteins by successive centrifugation steps in the absence and presence of glucose 6-phosphate. When this possibility was examined, enzyme recovery and specific activity were high in a few experiments, but the results were not always reproducible, and it was not possible to obtain sufficient quantities of homogeneous enzyme for further characterization. The use of affinity chromatography with blue Sepharose 6B was also explored. Cibacron blue F3GA dye coupled to Sepharose has been reported to bind tightly to the dinucleotide fold present in a variety of dehydrogenases and kinases (3, 12). The fatty acid-sensitive glucose 6-phosphate dehydrogenase appeared to bind tightly to columns of this material; however, we were unable to recover active enzyme, even after eluting the columns with buffer solutions containing up to $10^{-2}$ M NADP or 1 M phosphate, procedures that have proven effective with other dehydrogenases (3). In view of the previously reported lability of the fatty acid-sensitive dehydrogenase, it is likely that the enzyme was inactivated under these conditions.

A reexamination of affinity chromatography using columns of NADP-substituted Sepharose proved more successful. The fatty acid-sensitive glucose 6-phosphate dehydrogenase bound tightly to NADP-Sepharose and could be recovered by eluting the column with buffer containing NADP. The interaction of the fatty acid-sensitive enzyme with NADP-Sepharose was relatively specific, since the enzyme failed to bind columns of NAD-Sepharose. The procedure we finally adopted for purification of the fatty acid-sensitive enzyme is summarized in Table 1. It includes treatment of bacterial extracts with streptomycin and ammonium sulfate to remove nucleic acids and concentrate the enzyme, chromatographic separation of the two glucose 6-phosphate dehydrogenase isoenzymes on diethylaminoethyl-cellulose columns, and, finally, elution of the fatty acid-sensitive enzyme from NADP-Sepharose columns with buffer containing $5 \times 10^{-1}$ M NADP.

The overall increase in specific activity compared to that of the fatty acid-sensitive enzyme estimated to be present in extracts of the bacteria was about 600-fold. The total recovery of the enzyme was about 9%. The final preparation maintained full activity for at least 2 weeks when stored at 0°C.

**Homogeneity of the fatty acid-sensitive enzyme and molecular weight of the holoenzyme as determined by acrylamide gel electrophoresis.** The homogeneity of the preparations was examined by resolving samples containing 10 μg of protein on 4.5, 6.0, and 7.5% acrylamide gels as outlined earlier (14). In each case a single band of protein was detected when the gels were stained with amido black or Coomassie brilliant blue. The position of the protein bands corresponded to the bands of reduced tetrazolium dye when identical gels were incubated in glucose 6-phosphate dehydrogenase assay mixtures supplemented with tetrazolium chloride and phenazine methosulfate. The results indicated the preparations were homogeneous. When the mobilities of the holoenzyme in gels of different acrylamide concentration were compared with those of proteins of known molecular weight, as reported earlier (7), a molecular weight estimate of 120,000 was obtained.

**Subunit composition of the fatty acid-sensitive glucose 6-phosphate dehydrogenase.** Subunit preparations of the fatty acid-sensitive enzyme, which had been dissociated by treatment with SDS and 2-mercaptoethanol (see Materials and Methods), were resolved by acrylamide gel electrophoresis in the presence of SDS, and the molecular weight of the subunits was determined as detailed earlier (14). Only one size subunit was detected on the gels, which corresponded in position to a polypeptide chain of 60,000 molecular weight. The results indicated that the holoenzyme is a dimer composed of similar size subunits of about 60,000 daltons.

**Antigenic properties of the fatty acid-sensitive glucose 6-phosphate dehydrogenase.** Since the molecular weights of the subunits comprising the fatty acid-sensitive enzyme were similar to those of the ATP-sensitive glucose 6-phosphate dehydrogenase (14), we were interested in examining whether or not the two enzymes shared common antigenic determinants, as would be expected if they were different oligomeric forms of the same protein or if the genes encoding them had a common evolutionary origin. Antiserum prepared against preparations of the fatty acid-sensitive enzyme equivalent to step 4 of Table 1 was used in enzyme inhibition and immunodiffusion experiments to test the possibility of a relationship between the two isoenzymes. The data of Table 2 indicate that the antiserum markedly inhibited the homologous fatty acid-sensitive enzyme but failed to inhibit the activity of the ATP-sensitive glucose 6-phosphate dehydrogenase.

The results were confirmed by double-diffu-
and fatty acids (10). It might be expected that such a physiological role of the pathway would be reflected by inhibition of key pathway enzymes by fatty acids and/or reduced glutathione, or possibly by activation by oxidized glutathione or fatty acid precursors such as acetate and acetyl CoA. In the course of exploring these possibilities we noted that long-chain fatty acid acyl CoA esters strongly inhibited the enzyme. Similar results have been reported for glucose 6-phosphate dehydrogenase from other organisms (1, 15). Free fatty acids also inhibited the enzyme, but as can be seen from the data of Table 3, which compares the concentrations of the different fatty acids and several of their acyl CoA esters required for half-maximal inhibition, they were less effective than the CoA esters. The unsaturated fatty acids tested were more potent inhibitors than the corresponding saturated fatty acids. Neither acetyl, butyryl, propionyl, or free CoA (all tested at $10^{-4}$ M) nor the reduced or oxidized forms of glutathione (each tested at $10^{-2}$ M) influenced the activity of the enzyme.

The ATP-sensitive glucose 6-phosphate dehydrogenase was also inhibited by long-chain fatty acid acyl CoA esters. However, between 10- and 30-fold-higher concentrations were required for comparable inhibition. For example, the $K_i$ values for palmitoyl CoA were, respectively, $2 \times 10^{-4}$ and $6 \times 10^{-5}$ M for the NADP- and NAD-linked activities of the enzyme. At the levels tested (up to $2 \times 10^{-4}$ M) the free fatty acids listed in Table 3 were not inhibitory. The preferential inhibition of the ATP-insensitive glucose 6-phosphate dehydrogenase by long-chain fatty acids and their CoA esters suggests that this enzyme might play a key role in formation of NADPH for reductive steps.

### Table 3. Relative inhibition of the fatty acid-sensitive glucose 6-phosphate dehydrogenase by representative fatty acid acyl CoA esters and free fatty acids

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration required for half-maximal inhibition* (M)</th>
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<tbody>
<tr>
<td>Palmitoyl CoA</td>
<td>$7 \times 10^{-6}$</td>
</tr>
<tr>
<td>Stearoyl CoA</td>
<td>$4 \times 10^{-6}$</td>
</tr>
<tr>
<td>Oleoyl CoA</td>
<td>$5 \times 10^{-6}$</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>$3 \times 10^{-4}$</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>$2 \times 10^{-4}$</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>$3 \times 10^{-5}$</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>$9 \times 10^{-5}$</td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>$8 \times 10^{-5}$</td>
</tr>
</tbody>
</table>

* The assay mixtures contained 0.003 U of enzyme from a preparation equivalent to step 3 of Table 1. Inhibition curves from which these values were obtained were hyperbolic.
in fatty acid synthesis. Accordingly, we have redesignated the ATP-insensitive enzyme as the fatty acid-sensitive glucose 6-phosphate dehydrogenase.

Influence of palmitoyl CoA on the affinity of the fatty acid-sensitive glucose 6-phosphate dehydrogenase for NADP and glucose 6-phosphate. To examine the mechanism of inhibition of the fatty acid-sensitive enzyme by long-chain fatty acid acyl CoA esters, the kinetic properties of the enzyme were compared in the presence and absence of 10⁻⁵ M palmitoyl CoA. Double-reciprocal plots of enzyme activity as a function of NADP or glucose 6-phosphate concentration in the presence and absence of palmitoyl CoA are shown in Fig. 1. As can be seen, palmitoyl CoA decreased the affinity of the enzyme for both NADP and glucose 6-phosphate. In the presence of 10⁻⁵ M palmitoyl CoA, the Kₘ values for NADP and glucose 6-phosphate were increased five- and threefold, respectively. The influence of inhibitor on the affinity of the enzyme for NADP and glucose 6-phosphate fully accounted for the observed inhibition. Palmitoyl CoA at 10⁻⁵ M did not significantly affect Vₘₐₓ when the concentrations of glucose 6-phosphate and NADP were increased to 10⁻¹ and 2 × 10⁻³ M, respectively. The mechanism of inhibition of the enzyme by free fatty acids was presumably similar, since the inhibition by 5 × 10⁻⁵ M oleic acid was fully reversed by increasing the concentration of NADP and glucose 6-phosphate (results not shown).

Influence of glucose 6-phosphate on the apparent molecular weight of the fatty acid-sensitive enzyme. An unusual property of the fatty acid-sensitive enzyme which encouraged us to consider the possibility of its interconversion with the ATP-sensitive isoenzyme was its tendency to aggregate in the presence of glucose 6-phosphate (Lessie and Shuman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, P123, p. 165). We have examined this phenomenon in more detail and find that substrate-induced aggregation of the enzyme is strongly dependent upon enzyme concentration as well as glucose 6-phosphate concentration. Table 4 summarizes the results of experiments in which the apparent molecular weight of the enzyme was estimated by comparing its sedimentation in sucrose gradients relative to that of bovine hemoglobin. The data indicate that at relatively low concentrations of enzyme, glucose 6-phosphate did not significantly influence the apparent molecular weight of the enzyme. For example, at a concentration of 0.2 U/ml, which is equivalent to about 8 µg of pure enzyme per ml, increasing the concentration of glucose 6-phosphate to 2 × 10⁻² M did not markedly affect the sedimentation properties of the enzyme. However, as the concentration of the enzyme was progressively increased to 4 U or about 160 µg/ml, its apparent molecular weight in the presence of 2 × 10⁻² M glucose 6-phosphate increased to a value more than four times that in the absence of glucose 6-phosphate. Increasing the concentration of enzyme in the absence of glucose 6-phosphate did not significantly influence its sedimentation behavior. We have estimated the intracellular...
concentration of the fatty acid-sensitive glucose 6-phosphate dehydrogenase assuming values of $10^{-12}$ g for the amount of protein per cell and $10^{-12}$ ml for the intracellular volume. Using a value of 1/600 for the fraction of the cellular protein that is fatty acid-sensitive glucose 6-phosphate dehydrogenase, our calculations indicate a value of about 5 U or 200 $\mu$g/ml for the in vivo concentration of the enzyme, suggesting that the fatty acid-sensitive glucose 6-phosphate dehydrogenase may exist in an aggregated state in vivo. The influence of glucose 6-phosphate on the sedimentation properties of the fatty acid-sensitive enzyme was relatively specific, since glucose 1-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, and 6-phosphogluconate (each tested at $10^{-3}$ M) failed to significantly affect the sedimentation of the enzyme. Also $2 \times 10^{-3}$ M palmitoyl CoA, which completely inhibited the activity of the fatty acid-sensitive enzyme, had no influence on its apparent molecular weight.

Earlier work on the sedimentation behavior of the active form of the ATP-sensitive glucose 6-phosphate dehydrogenase from P. cepacia failed to show any influence of glucose 6-phosphate on its molecular weight (14). However, we have reexamined the sedimentation behavior of this enzyme and find that at sufficiently high concentrations of enzyme, glucose 6-phosphate also promotes a marked increase in its apparent molecular weight. For example, when ATP-sensitive enzyme obtained from step 3 of Table 1 was sedimented in one experiment at a concentration of 6 U/ml, its apparent molecular weight increased fourfold in the presence of $10^{-2}$ M glucose 6-phosphate. In contrast, at a concentration of 0.2 U/ml there was no significant increase in molecular weight of the enzyme. As with the fatty acid-sensitive glucose 6-phosphate dehydrogenase, a high concentration of enzyme was insufficient to increase the molecular weight unless glucose 6-phosphate was also present. The apparent failure to observe an influence of glucose 6-phosphate on the sedimentation of the enzyme in earlier experiments (in which the molecular weight of the active form of the enzyme was estimated in the presence of NAD and glucose 6-phosphate) can be attributed to the low concentration of enzyme used. Calculations of the in vivo concentration of the ATP-sensitive glucose 6-phosphate dehydrogenase, based on data obtained earlier (14), give a value of 18 U or 129 $\mu$g/ml. The results suggest that this species, like the fatty-acid sensitive isoenzyme, may exist in an aggregated state in vivo.

Activity of the fatty acid-sensitive glucose 6-phosphate dehydrogenase with NAD as co-factor. Earlier studies of the fatty acid-sensitive glucose 6-phosphate dehydrogenase indicated that the enzyme was not active with NAD as cofactor. However, in the course of purifying the enzyme we noted that preparations from which the ATP-sensitive isoenzyme had been removed still possessed significant activity with NAD, which amounted to about 5% of the activity observed with NADP. More extensive studies of the enzyme indicate that this activity is an intrinsic property of the fatty acid-sensitive enzyme. First, it was found that the NAD-linked activity of the enzyme could be increased to about 40% of that observed with NADP by increasing the concentration of NAD from its usual value of $5 \times 10^{-4}$ to $5 \times 10^{-3}$ M. The poor activity of the enzyme with NAD compared to NADP was found to reflect a nearly 100-fold-lower affinity of the enzyme for NAD compared to that for NADP (the respective $K_m$ values for NAD and NADP were $1.8 \times 10^{-5}$ and $2 \times 10^{-5}$ M). There was no difference in affinity of the enzyme for glucose 6-phosphate ($K_m$, $5 \times 10^{-3}$ M) whether NAD or NADP served as cofactor. Unlike the NADP-linked activity of the enzyme, its NAD-linked activity was subject to inhibition by ATP ($K_i$, $4 \times 10^{-3}$ M). Whereas the activity with NADP was optimal over a broad range of pH values between 7.5 and 9.0, the pH optimum for activity with NAD was relatively narrow, with a maximum at pH 8.5. The pH 8.5 Tris-chloride buffer used in routine assays gave optimal activity for both the NAD- and NADP-linked activities of the enzyme.

The NAD-linked activity of the fatty acid-sensitive enzyme differed in several ways from the major NAD-linked glucose 6-phosphate dehydrogenase activity found in extracts of P. cepacia, which is associated with the ATP-sensitive isoenzyme. First, antiserum prepared against the fatty acid-sensitive enzyme inhibited its NAD-linked activity, but not that of the ATP-sensitive enzyme. The concentration of antiserum required for 50% inhibition of the NAD-linked activity of the homologous enzyme (1:400-fold-diluted antiserum) was about 10-fold greater than that required for comparable inhibition of its NADP-linked activity. Second, the NAD-linked activities of the two enzymes had different pH optima. The activity of the fatty acid-sensitive enzyme decreased to less than 20% of its value at pH 8.5 as the pH was progressively increased to 9.5. In contrast, the NAD-linked activity of the ATP-sensitive enzyme exhibited a broad region of optimal activity between pH 8.5 and 9.5, with a slightly higher activity at pH 9.5 than at pH 8.5. Third, the kinetic properties of the two enzymes dif-
fered (see Table 5). The affinity of the fatty acid-sensitive glucose 6-phosphate dehydrogenase for NAD was lower than that of the ATP-sensitive enzyme, and the K\textsubscript{i} values for ATP, NADPH, and palmitoyl CoA also differed. The differences in kinetic properties of the two enzymes, which are summarized in Table 5, along with the differences in their pH optima and antigenic properties, indicate that the NAD-linked activity of preparations of the fatty acid-sensitive enzyme does not reflect the presence of trace amounts of the ATP-sensitive enzyme.

DISCUSSION

The main aims of the present study were (i) to attempt to define the physiological role of the ATP-sensitive glucose 6-phosphate dehydrogenase of \textit{P. cepacia} by examining the influence of potential regulatory ligands on its activity and (ii) to study the relationship of the ATP-insensitive enzyme to the ATP-sensitive isoenzyme. We were particularly interested in investigating the possibility that the two species of glucose 6-phosphate dehydrogenase might share common subunits or be different oligomeric forms of the same protein. To examine this possibility, it was necessary to purify the ATP-insensitive enzyme and compare its subunit composition and antigenic properties with those of the ATP-sensitive species.

Some insight into the probable physiological role of the ATP-insensitive glucose 6-phosphate dehydrogenase has been provided by the finding that long-chain fatty acids and their acyl coenzyme A esters inhibit the enzyme. The most marked inhibition of the enzyme was by the CoA esters, which inhibited the enzyme at concentrations as low as 5 x 10^{-6} M. In the light of this finding, the ATP-insensitive enzyme has been redesignated the fatty acid-sensitive glucose 6-phosphate dehydrogenase to distinguish it from the ATP-sensitive isoenzyme, which was relatively insensitive to such inhibition.

Since palmitoyl CoA both decreased the affinity of the enzyme for its substrate and cofactor and preferentially inhibited its activity compared with that of the ATP-sensitive isoenzyme, we consider the inhibition to be physiologically significant. However, in view of the finding by Taketa and Pogell (11) that palmitoyl CoA was a rather general inhibitor of several enzymes they examined from eucaryotic organisms, other supportive data will be necessary before it can be concluded that the inhibition reflects a primary role of the fatty acid-sensitive glucose 6-phosphate dehydrogenase in supplying NADPH for reductive steps in fatty acid synthesis. Experiments are currently in progress in our laboratory to attempt to demonstrate a role of long-chain fatty acids in repression of the enzyme or an influence of alterations of the enzyme on capacity of the bacteria to synthesize fatty acids or support the replication of lipid-containing bacterio- phage.

Our second objective, that of defining the relationship between the two glucose 6-phosphate dehydrogenase isoenzymes, has been accomplished by purification of the fatty acid-sensitive enzyme and comparison of its antigenic and kinetic properties with those of the ATP-sensitive enzyme. The possibility that the two species of \textit{P. cepacia} glucose 6-phosphate dehydrogenase were different oligomeric forms of the same enzyme was suggested by (i) the similar size of their subunits, (ii) the finding that under appropriate conditions the fatty acid-sensitive enzyme had significant NAD-linked activity, and (iii) the observation that the apparent molecular weight of the enzyme increased in the presence of glucose 6-phosphate. However, the failure of the ATP-sensitive glucose 6-phosphate dehydrogenase to interact with antiserum prepared against the purified fatty acid-sensitive enzyme, along with similar data indicating that antiserum prepared against the ATP-sensitive enzyme did not interact with the fatty acid-sensitive enzyme (14), strongly suggests that the subunits

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Conc (M) required for half-maximal activity</th>
<th>Conc (M) required for half-maximal inhibition</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>NAD</td>
<td>Glucose 6-phosphate (NAD)</td>
</tr>
<tr>
<td>Fatty acid-sensitive G6PD\textsuperscript{a}</td>
<td>$2 \times 10^{-3}$</td>
<td>$5 \times 10^{-3}$</td>
</tr>
<tr>
<td>ATP-sensitive G6PD\textsuperscript{a}</td>
<td>$2 \times 10^{-4}$</td>
<td>$5 \times 10^{-3}$</td>
</tr>
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\textsuperscript{a} The assay mixtures contained 0.003 U of enzyme from a preparation equivalent to step 3 of Table 1.

\textsuperscript{b} The assay mixture contained 0.002 U of enzyme obtained from step 2 of Table 1.
of the two species of glucose 6-phosphate dehydrogenase are distinct. Furthermore, the NAD-linked activity associated with preparations of the fatty acid-sensitive glucose 6-phosphate dehydrogenase from which the ATP-sensitive glucose 6-phosphate dehydrogenase had been removed has been found to be clearly different from that of the ATP-sensitive enzyme by both immunological and kinetic studies.

Further studies of the influence of glucose 6-phosphate on the apparent molecular weight of the fatty acid-sensitive enzyme have shown clearly that glucose 6-phosphate-mediated aggregation of the enzyme does not represent conversion of the enzyme to the ATP-sensitive isoenzyme. However, the studies do suggest that the two glucose 6-phosphate dehydrogenase species may exist in an aggregated form in vivo. The extent of the aggregation in the presence of glucose 6-phosphate depended upon the concentration of enzyme as well as substrate. Under the conditions optimal for aggregation of the fatty acid-sensitive enzyme, the ATP-sensitive isoenzyme, which had been previously thought not to aggregate in response to glucose 6-phosphate, was also found to aggregate. Estimates of the intracellular concentrations of the two glucose 6-phosphate dehydrogenase species (5 and 18 U/ml, respectively, for the fatty acid- and ATP-sensitive species) suggest that both species of glucose 6-phosphate dehydrogenase might exist in an aggregated state intracellularly, assuming a sufficiently high concentration of glucose 6-phosphate. Whether or not the aggregated forms of the two glucose 6-phosphate dehydrogenase species have different properties compared with those noted in vitro has not been examined, since it is not feasible to assay the enzymes under conditions that would maintain them in an aggregated state.

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