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Phosphoenolpyruvate Carboxykinase (Guanosine Triphosphate) from Rat Liver Cytosol. Separation of Homogeneous Forms of the Enzyme with High and Low Activity by Chromatography on Agarose–Hexane–Guanosine Triphosphate†

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ABSTRACT: Phosphoenolpyruvate carboxykinase (PEPCK) from rat liver cytosol has been purified to homogeneity by chromatography on agarose–hexane–GTP. The interactions responsible for PEPCK binding to agarose–hexane–GTP are shown to involve both the nucleotide ligand and the hydrophobic hexyl chain. This chromatography is able to separate PEPCK from contaminant proteins in heterogeneous preparations and/or a high activity form of the enzyme from low activity form(s) in apparently homogeneous preparations. The purified enzyme moves as a single component in polyacrylamide gel electrophoresis under nondenaturing or denaturing conditions. The molecular weight determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was found to be 71 500 ± 900. The enzyme appears to be composed of a single protein chain and catalyzes the reversible decarboxylation of oxalacetate to phosphoenolpyruvate and the irreversible decarboxylation of oxalacetate to pyruvate. The specific activity of homogeneous PEPCK in the presence of 2.25 mM Mn^{2+} is 18 ± 1 μmol of phosphoenolpyruvate carboxylated·min^{-1}·mg^{-1} at 25 °C. Data on amino acid composition have been obtained from acid hydrolysates of native and performic acid oxidized phosphoenolpyruvate carboxykinase. Independent measurements have been made for Trp, Tyr, Cys, and total nitrogen content of the enzyme. From the amino acid composition, a partial specific volume ($\bar{v}$) of 0.736 $cm^3 \cdot g^{-1}$ has been calculated. The homogeneous enzyme has a 280/260 nm ratio of 1.8–1.84. A molar extinction coefficient of 1.19×10^5 at 280 nm has been calculated based on the nitrogen composition of the protein and a molecular weight of 72 000.

The reversible decarboxylation of OAA[1] to PEP is the first committed step in the process of gluconeogenesis from pyruvate, amino acids, or intermediates of the Krebs tricarboxylic acid cycle. In animal systems, this reaction requires GTP (or ITP) and divalent metal ions and is catalyzed by the enzyme phosphoenolpyruvate carboxykinase (EC 4.1.1.32), which was discovered in avian liver by Utter & Kurahashi (1954). The enzyme occurs in many different animal, bacterial, and plant species (for a review, see Utter & Kolenbrander, 1972); the relative amounts in mitochondria and cytosol of animal tissues vary greatly from one species to another (Nordlie & Lardy, 1963). It is the cytosolic PEPCK that adaptively responds to hormonal and dietary influences (Lardy et al., 1964).

Phosphoenolpyruvate carboxykinase preparations from pig liver mitochondria (Chang & Lane, 1966) and baker's yeast (Cannata, 1970) have been reported to be homogeneous in sedimentation velocity experiments. Purified PEPCK isolated from sheep kidney cortex mitochondria (Barns & Keech, 1972) and chicken liver mitochondria (Noce & Utter, 1975) has been shown to be homogeneous by polyacrylamide gel electrophoresis under denaturing conditions. The molecular characteristics and amino acid composition of the yeast, and the pig and sheep liver mitochondrial enzymes have been reported.

Rat liver cytosolic PEPCK was first purified by Ballard & Hanson (1969) and the procedure was later modified by Philippidis et al. (1972). This preparation was reported to be homogeneous on Sephadex G-100 chromatography. However, on disc gel electrophoresis at pH 8.6 under nondenaturing conditions (Ballard & Hanson, 1969) some contamination was evident. To obtain direct chemical evidence for the enzyme

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[1] Abbreviations used; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TEA, triethanolamine; Nbs_2, 5,5'-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; PEP, phosphoenolpyruvate; OAA, oxalacetic acid; PEPCK, phosphoenolpyruvate carboxykinase; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate; GSH, gluthathione; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DEAE, diethylaminoethyl; Bis, *N,N'*-methylenebisacrylamide.

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groups involved in catalysis and to determine characteristics of their reactivity and chemical and physical properties, it was necessary to work with a homogeneous preparation. This paper reports the purification of rat liver cytosol PEPCK to homogeneity, and high specific activity, by chromatography on agarose-hexane-GTP. The factors contributing to the affinity of the enzyme for this matrix are also examined. Reference data for further analysis of the protein structure, as well as for studies of the involvement of critical amino acid residues in the structural and functional properties of the rat liver cytosol PEPCK, are reported.

Materials and Methods

Materials were from the following sources. Sodium salts of the nucleotides (ITP, IDP, ADP) and NADH were from P-L Biochemicals Inc.; GDP-Li_3 and glycerol solutions of pyruvate kinase, malate, and lactate dehydrogenase were from Boehringer Mannheim Biochemicals; PEP, Hepes, TEA, DTT, and Nbs_2 were from Sigma Chemical Co.; *N*-bromosuccinimide, EDTA, and 4,4′-dithiodipyridine were from Aldrich Chemical Co., Inc.; urea was from Mallinckrodt Chemical Works; ultrapure guanidine was from Schwarz/Mann; Tes (Ultrol), GSH, OAA, and GTP-Li_3 were from Calbiochem; agarose-hexane, agarose-hexane-GTP type 4, and agarose-hexane-ATP type 4 (9 μmol of nucleoside triphosphate/mL of gel) were from P-L Biochemicals, Inc.

Enzyme Preparation. PEPCK from rat liver cytosol was prepared as described previously by Philippidis et al. (1972) with the following modifications. Livers from rats fasted 24 h were perfused with 0.9% NaCl and homogenized with 2 volumes of 10 mM TEA-Cl buffer (pH 7.5) containing 0.25 M sucrose, 1 mM EDTA, and 1 mM DTT. All steps in the purification procedure were carried out at 0–4 °C. The precipitate of the initial ammonium sulfate fractionation (42.5–62.5% saturation at 25 °C) was resuspended and dialyzed in 50 mM Tris-Cl (pH 8) containing 1 mM EDTA and 1 mM DTT. This buffer was also used for the Sephadex G-100 chromatography. The combined fractions from Sephadex G-100 were adjusted to pH 7 with 1 M acetic acid and applied directly to DEAE-cellulose equilibrated with Tris-Cl (pH 7) containing 0.1 mM EDTA and 0.1 mM DTT. EDTA and DTT were added to the combined fractions eluted from DEAE-cellulose to give concentrations of 5 and 2 mM, respectively, and solid ammonium sulfate was added to 70% saturation. The pH was kept at 7.4 by addition of solid Tris base. After 1 h at 4 °C, the precipitate was collected by centrifugation. The pellet was extracted twice with 50 mM Tris-Cl (pH 7.5) containing 40% ammonium sulfate, 5 mM EDTA, and 2 mM DTT. Following overnight dialysis of this extract against 10 mM Tris-Cl (pH 7.5) containing 0.1 mM EDTA and 0.2 mM DTT, the pH was adjusted to 7 with 0.5 M potassium phosphate. The sample was applied to the hydroxylapatite column equilibrated with 10 mM potassium phosphate (pH 7.0) and eluted with a gradient of 10 to 250 mM potassium phosphate (pH 7.0) containing 0.1 mM EDTA and 0.2 mM DTT. The pooled fractions containing PEPCK activity were dialyzed for 3 h against saturated ammonium sulfate in 10 mM TEA-Cl buffer (pH 7.0) containing 5 mM EDTA and 2 mM DTT. The enzyme (6–8 mg/mL) was stored at 4 °C under N_2 atmosphere as a 90% ammonium sulfate suspension in this same buffer until further purification by chromatography on agarose-hexane-GTP (see Results).

Prior to use, a desired aliquot of the 90% ammonium sulfate suspension was diluted with an equal volume of buffer A [10 mM TEA-Cl, 10% glycerol, 1 mM GSH, and 0.1 mM EDTA (pH 7.2)] or buffer B [10 mM Tes-Na, 10% glycerol, 1 mM GSH, 0.1 mM EDTA (pH 7.2)] and dialyzed overnight at 4 °C against 500 volumes of the same buffer. In some experiments, 0.1 mM DTT replaced GSH.

Protein Determination. Protein in column effluents during the purification procedure was determined spectrophotometrically by the Warburg and Christian method as described by Layne (1957), except for fractions from the agarose-hexane-GTP column where protein is expressed as absorbance units at 280 nm. The protein concentration of solutions of homogeneous phosphoenolpyruvate carboxykinase was determined by its absorbance at 280 nm. The determination of the $A_{280nm}^{1\%,cm}$ is given under Results.

Assays of Phosphoenolpyruvate Carboxykinase. Unless otherwise stated, all reactions were started by the addition of the enzyme to the otherwise complete reaction mixture. The activity was determined spectrophotometrically at 25 °C by the decrease of absorbance at 340 nm.

The carboxylation of phosphoenolpyruvate to oxalacetate was measured at pH 7–7.2 in a 1-mL reaction mixture containing 56 mM Hepes, 1 mM IDP, 2 mM PEP, 47 mM $NaHCO_3$, 2.25 mM $MnCl_2$, 0.25 mM NADH, and 22 units of malate dehydrogenase. For the determination of the apparent Michaelis constants, 10 μM EDTA and 0.1 mM DTT were included in the reaction mixture; each substrate was varied in turn. K_m for PEP was determined in the presence of 45 mM $NaHCO_3$, 1 mM IDP, and 2.2 mM $MnCl_2$. Michaelis constants for IDP or GDP were determined in the presence of 45 mM $NaHCO_3$, 2 mM PEP, and 2 mM free $MnCl_2$ over equimolar MnIDP or MnGDP.

The decarboxylation of oxalacetate to PEP was determined at pH 7.5 in a reaction mixture containing 1 mL: 55 mM Hepes-KOH, 2 mM ITP, 4 mM $MgCl_2$, 300 μM OAA, 1 mM ADP, 0.25 mM NADH, and 30 μg each of pyruvate kinase and lactate dehydrogenase. The enzyme-catalyzed rates reported have been corrected for the spontaneous decarboxylation of OAA in the absence of PEPCK. For the determination of apparent Michaelis constants, 20 μM EDTA and 0.1 mM DTT were included in the reaction mixture. $MgCl_2$ was replaced by $Mg(CH_3COO)_2$. K_m for OAA was determined in the presence of 4 mM Mg^{2+}, 2 mM ITP, and 1 mM ADP. Michaelis constants for ITP or GTP were determined in the presence of 300 μM OAA and 1 mM free Mg^{2+} over equimolar MgITP or MgGTP.

The decarboxylation of OAA to pyruvate was determined at pH 7.5 in a reaction mixture containing in 1 mL final volume: 68 mM Hepes-NaOH, 1 mM IDP, 300 μM OAA, 0.24 mM NADH, and 6.2 units of lactate dehydrogenase. A control value for the spontaneous decarboxylation of OAA in the absence of PEPCK was used to correct the enzyme-catalyzed rate of pyruvate formation.

A unit of activity is defined as the amount of enzyme that catalyzes the formation of 1 micromole of product per minute under each assay condition. Specific activity is expressed as micromoles of product formed per minute per A_{280nm} for partially purified enzyme and as micromoles of product formed per minute per milligram of protein for homogeneous PEPCK.

Polyacrylamide Gel Electrophoresis. Analytical disc gel electrophoresis at pH 8.9 was conducted using 5% polyacrylamide, 0.17% Bis gels, according to Gabriel (1971), except that the stacking gel was not employed. The separating gels were preelectrophoresed using, in both compartments, the buffer employed in the polymerization of the gels (0.1 mM EDTA was included). The sample in 50 to 100 μL of 10 mM Tes buffer (pH 7.2), containing 10% glycerol, 0.1 mM EDTA, and a trace of Bromphenol blue, was layered directly on top of the

separating gel. Electrophoresis was performed at 4 °C for 5 h with Tris-glycine buffer (pH 8.3) containing 0.1 mM EDTA at 1.5 mA/tube.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to Weber & Osborne (1969) with the following modifications. All protein samples were denatured by incubation at 100 °C for 15 min in 10 mM sodium phosphate buffer (pH 7.0) containing 10% glycerol, 1% $NaDodSO_4$, and 60 mM DTT. After denaturation, Bromphenol blue was added. Electrophoresis was conducted at room temperature on 7.5% polyacrylamide gels with 100 mM sodium phosphate, 0.1% $NaDodSO_4$ buffer (pH 7.0) for 6 h at 7 mA/tube. The molecular weight of PEPCK was determined from a plot of relative mobility vs. log molecular weight of the following standard proteins (Weber & Osborne, 1969): phosphorylase *a* (94 000), bovine serum albumin (68 000), ovalbumin (43 000), lactate dehydrogenase (36 000), α-chymotrypsinogen (25 700), and β-lactoglobulin (18 400).

All gels were stained for protein with 0.25% Coomassie blue R, 50% methanol, and 9.2% acetic acid for 3 to 6 h and destained according to Greaser & Gergely (1971).

Amino Acid Analysis. A sample of homogeneous PEPCK (specific activity 19.6 μmol of PEP carboxylated·min^{-1}·mg^{-1}; ratio 280/260 nm = 1.84) purified by elution from agarose-hexane-GTP was dialyzed at 4 °C against several changes of N_2-gassed distilled H_2O. Aliquots of this dialyzed sample containing approximately 0.2 mg of protein were transferred to acid-washed, vacuum hydrolysis tubes (10 × 150 mm, Pierce no. 29562), and an equal volume of reagent-grade concentrated hydrochloric acid was added. To improve tyrosine recovery, 0.2% (v/v) phenol (88–90%) was included. Before sealing the tubes, traces of air dissolved in the HCl were removed by the procedure of Moore & Stein (1963). Hydrolysis was for 24 h at 108 °C. After hydrolysis, an equal volume of distilled water was added, and the samples were evaporated to dryness in a vacuum oven at 60 °C. The amino acid analysis of the samples resuspended in 0.2 N sodium citrate buffer (pH 2.2) was performed in a Durrum Model-500 automatic amino acid analyzer. The values for threonine, tyrosine, and serine were corrected for hydrolytic losses according to Moore & Stein (1963). The corrections applied were 5% for threonine and tyrosine and 10% for serine.

Determination of Cysteine. Cysteine (half-cystine) was determined after its conversion to cysteic acid by performic acid oxidation (Hirs, 1967). Purified PEPCK (0.2 mg) dialyzed against distilled water was taken to dryness in a rotary evaporator and resuspended in 0.1 mL of 88% formic acid. After 4 h of oxidation at 4 °C with 2 mL of freshly prepared performic acid, the reaction was terminated with 0.3 mL of cold 48% HBr. Bromine was eliminated and the solution evaporated to dryness according to Hirs (1967). The residue was suspended in 6 N HCl and traces of air were removed. Hydrolysis was for 24 h at 108 °C. Amino acid analysis of samples evaporated to dryness and resuspended in 0.2 N sodium citrate (pH 2.2) was performed in a Durrum Model-500 automatic amino acid analyzer previously calibrated with authentic cysteic acid and methionine sulfone. Half-cystine content was calculated assuming a 94% yield in the oxidation to cysteic acid (Moore, 1963). The conversion of Met to the sulfone was assumed to be quantitative (Hirs, 1967).

Spectrophotometric Determination of Cysteine in the Presence of Denaturing Agents. Titration of homogeneous PEPCK with excess Nbs_2 was carried out at 25 °C in 100 mM Tes (pH 7.8), containing 0.83 mM EDTA and 0.9% $NaDodSO_4$. The reaction was monitored by the change in absorbance at 412 nm against a blank lacking the enzyme. The number of SH groups titrated was calculated using an extinction coefficient of 13 600 M^{-1} cm^{-1} (Ellman, 1959) for the thiophenylate anion liberated. Determination of the cysteine content in 6 M guanidine hydrochloride was carried out at 25 °C by titration with excess 4,4′-dithiodipyridine in 30 mM potassium phosphate buffer (pH 6.5) containing 1 mM EDTA. The value of SH groups reacted was calculated from the absorption of the 4-thiopyridone liberated using an ϵ_{324} of 19 800 (Grasetti & Murray, 1967). The enzyme used in these titrations had been freed of thiols by desalting on Sephadex G-25.

Spectrophotometric Determinations of Tryptophan and Tyrosine. The number of Trp residues in purified PEPCK was determined by the change in absorbance at 280 nm upon oxidation of the indole chromophore of tryptophan with *N*-bromosuccinimide (Spande & Witkop, 1967) in the presence of 6.3 M urea adjusted to pH 3.6 with acetic acid.

Tyrosine and tryptophan content was determined according to Edelhoch (1967). Tryptophan content of PEPCK in 6 M guanidine hydrochloride was determined by the absorbance at 280 and 288 nm in 26 mM potassium phosphate (pH 6.5). The tyrosine content was calculated from the absorbance changes at 295 and 300 nm determined before and within 1 min after increasing the pH to 12.5 by the addition of a small volume of 50% KOH. Good agreement was obtained for the tyrosine values determined at 295 and 300 nm.

Antibodies against PEPCK purified on agarose–hexane–GTP were produced in rabbits by intradermal injections of 170 μg of homogeneous PEPCK in buffer A (pH 7.2) emulsified in an equal volume of complete Freund's adjuvant. A booster dose of antigen was given 6 weeks after the first. After 15 days, the animals were bled and the serum was collected.

Miscellaneous. All buffers used for chromatography were extensively degassed and then saturated with N_2 gas. All solutions used for activity determinations were prepared in glass-distilled, deionized water that had been degassed and saturated with N_2. Glycerol concentration is expressed in terms of volume to volume.

Results and Discussion

Purity and Stability of Partially Purified PEPCK. Rat liver cytosolic PEPCK was isolated essentially as described by Ballard & Hanson (1969) and Philippidis et al. (1972), with the exception that EDTA and DTT were included throughout the purification procedure. This resulted in an increased recovery of the units in the ammonium sulfate fractionation and dialysis steps. The overall yield improved from 15% (Ballard & Hanson, 1969) to 40%. $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 1, gel A) shows the presence of a major component (65% of the total stained protein) of molecular weight ~72 000 and at least five contaminating proteins. Initial attempts at further purification were limited by the instability of the enzyme. Losses of activity were particularly evident at the edges of protein peaks in chromatographic procedures.

The stability of partially purified PEPCK was explored. It appeared that enzyme inactivation was caused by the combination of at least two processes: first, loss of activity at low protein concentration which is not prevented by EDTA or thiols; secondly, loss of activity due to SH oxidation. The sensitivity of PEPCK from different sources to thiol reagents is well documented (Utter & Kolenbrander, 1972 and references therein). Heavy metals, normally present as contaminants in the solutions, are known to form mercaptides and to catalyze thiol oxidation (Jocelyn, 1972). This loss of activity could be prevented, at least partially, by EDTA and thiols.

Whenever possible, conditions that included a protein concentration higher than 0.25 absorbance at 280 nm, the presence

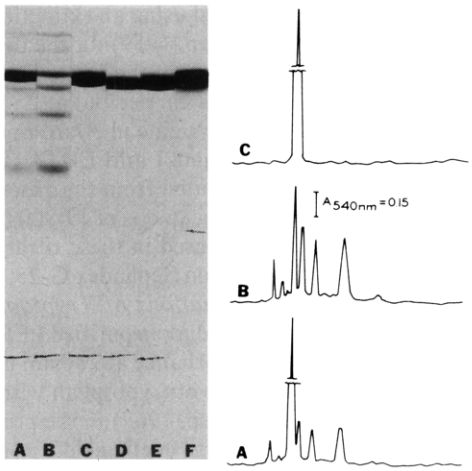

FIGURE 1: Disc gel electrophoresis of rat liver cytosol PEPCK. Gels A through E contain 21–25 μg of enzyme on 7.5% polyacrylamide gel in the presence of 0.1% $NaDodSO_4$. Gel A displays partially purified enzyme before chromatography on agarose–hexane–GTP, and gels B and C represent breakthrough (unadsorbed) and absorbed protein, respectively, corresponding to pooled fractions from Figure 2. Gel F contains 34 μg of the same sample as C but electrophoresis is on 5% polyacrylamide gel in the absence of denaturant. Gels D and E represent the unadsorbed and absorbed protein fractions from Figure 3A obtained after chromatography of low-activity homogeneous PEPCK on agarose–hexane–GTP. The gels were scanned on a Gilford Model 2410-S linear transport attachment, and the absorbance at 540 nm was monitored with a Beckmann DU monochromator, modified with a Gilford Model 2220 adapter and a Gilford 6040 chart recorder.

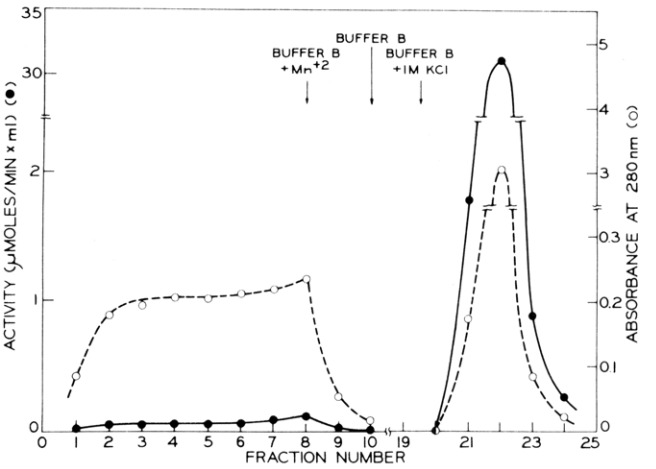

FIGURE 2: Chromatography of heterogeneous PEPCK on agarose–hexane–GTP. An aliquot of the 90% ammonium sulfate suspension of partially purified PEPCK was dialyzed overnight at 4 °C against buffer B. The enzyme was then diluted approximately tenfold with buffer B and filtered through glass wool, and $MnCl_2$ was added to a final concentration of 0.15 mM. This fraction (9.5 mL), containing 21 enzyme units and having an absorbance at 280 nm of 0.411, was applied at a flow rate of 7 mL/h to an agarose–hexane–GTP column (0.39 mL in a stylex 1-cm^3 tuberculin syringe) equilibrated with buffer B containing 0.15 mM $MnCl_2$. The column was washed with the same buffer until the absorbance at 280 nm was less than 0.015, followed by 30 bed volumes of buffer B. PEPCK was eluted with buffer B containing 1 M KCl. From 1 to 10 the fraction volume was 1.24 mL and from no. 21 on was 0.62 mL: (○–○) A_{280nm}; (●–●) enzymatic activity as described under Materials and Methods for the carboxylation of PEP.

of EDTA and a thiol reducing agent, and the absence (or low concentration) of divalent metal were used in further chromatographic procedures. These requirements were met with Buffer A or B.

Purification of PEPCK on Agarose–Hexane–GTP. Partially purified rat liver cytosol PEPCK (gel A, Figure 1) having a specific activity of 5.5 μmol of PEP carboxylated $min^{-1} \cdot A_{280nm}^{-1}$ and a 280/260 nm ratio of 1.73 was applied to the agarose–hexane–GTP column equilibrated with buffer B containing 0.15 mM $MnCl_2$. PEPCK was eluted from the GTP column with 1 M KCl in buffer B. A typical experiment is shown in Figure 2. A recovery of 87% of the enzyme units and 47% of the total protein A_{280nm} applied to the column was obtained in the fractions eluted by 1 M KCl. A twofold purification was achieved. The specific activity of this fraction was 10.2 μmol of PEP carboxylated $min^{-1} \cdot A_{280nm}^{-1}$. The absorbance ratio 280/260 nm was 1.82, Stability during elution was achieved by the relatively high protein concentration obtained as a result of eluting with a high concentration (1 M) of KCl. The first peak (fractions 1–10), which contained the protein that does not bind to the agarose–hexane–GTP column, accounted for 51% of the applied A_{280nm} and for a small amount of enzyme activity (2.3% of the applied units). The fractions of this unadsorbed peak have a 280/260 nm ratio of 1.46 and a relatively constant specific activity of around 0.25 μmol of PEP carboxylated $min^{-1} \cdot A_{280nm}^{-1}$.

Polyacrylamide Gel Electrophoresis. The purification of partially purified PEPCK achieved after chromatography on agarose–hexane–GTP is shown in Figure 1 by the use of polyacrylamide gel electrophoresis under denaturing and nondenaturing conditions. Prior to chromatography on the GTP column, polyacrylamide gel electrophoresis of the enzyme fraction incubated with sodium dodecyl sulfate exhibits a multibanded pattern (gel A). Upon chromatography on agarose–hexane–GTP, the contaminants appeared in the breakthrough of the column (gel B), and the enzyme protein eluted by 1 M KCl showed a single homogeneous component (gel C). It should be noted that in gel B a protein band having the same relative mobility as homogeneous PEPCK (gel C) is present. Scanning of gel B showed that 23% of the total protein stained with Coomassie blue is accounted for by this band.

PEPCK purified by chromatography on the GTP column also shows a single homogeneous band at pH 8.9 on polyacrylamide gel electrophoresis at 4 °C in the absence of denaturant (Figure 1, gel F). When gel electrophoresis under nondenaturing conditions was performed at room temperature, a multiple close-banding protein pattern was obtained (gel not shown). The reason for this phenomenon is unclear. The heterogeneity might be due to polymerization through formation of disulfide linkages or to nonenzymatic deamination of asparagine and glutamine residues which might be favored by the high pH and overheating of the gels in this electrophoresis.

Homogeneity of PEPCK eluted from the GTP column was further demonstrated by the following observations: a single precipitin band on Ouchterlony double-diffusion plates between antiserum against PEPCK and purified or partially purified enzyme, and a linear relationship between log fringe displacements vs. r^2 in high-speed sedimentation equilibrium experiments.

Molecular Weight Determination. The molecular weight of denatured PEPCK was determined by $NaDodSO_4$ disc gel electrophoresis of the purified enzyme eluted by 1 M KCl from agarose–hexane–GTP. A semilog plot of relative mobility vs. molecular weight of standard marker proteins yielded a molecular weight of 71 500 ± 900 for homogeneous PEPCK (average of eight determinations). This value is in close agreement with a molecular weight of 74 000 determined by Ballard & Hanson (1969) using gel filtration on Sephadex G-100. We conclude from these data that rat liver cytosol PEPCK is composed of a single polypeptide chain. Sheep

kidney (Barns & Keech, 1972) and chicken liver (Noce & Utter, 1975) mitochondrial PEPCK have molecular weights of 71 100 and 67 000, respectively, and also appear to be single polypeptide chains.

Amino Acid Composition, Extinction Coefficient, and UV Spectrum of Homogeneous PEPCK. The total amino acid composition of purified rat liver cytosol PEPCK is shown in Table I. Hydrophobic amino acids (Phe, Ala, Ile, Leu, Met, Pro, Val) account for 40.4% of the amino acid content of the enzyme. The content of aromatic amino acids is 9.8%.

A value of 13 mol of cysteic acid/mol of PEPCK was obtained after performic acid oxidation of the enzyme. This value is in close agreement with the number of cysteine residues determined by spectrophotometric titrations of the native enzyme in the presence of denaturants. Modification of purified PEPCK with Nbs_2 at pH 7.8 in the presence of 0.9% NaDodSO$_4$ or with 4,4′-dithiodipyridine at pH 6.5 in the presence of 6 M guanidine hydrochloride indicated the presence of 12.8 and 12.6 mol of cysteine/mol of enzyme, respectively. Therefore, the cysteine residues are all present in the reduced state.

Spectrophotometric determinations of the tryptophan content of purified PEPCK indicated the presence of 20.2 mol of Trp/mol of enzyme by the method of Spande & Witkop (1967) and 18.6 mol of Trp/mol of enzyme by the method of Edelhoch (1967). It is of interest that an average value of 18.1 mol of Trp/mol of enzyme was obtained by the method of Edelhoch (1967) for enzyme solutions in three different pH 7.2 buffer systems in the absence of guanidine hydrochloride. This may be an indication that Trp residues in PEPCK are exposed to the solvent in the native enzyme. The tyrosine content was found to be 14.9 mol/mol of enzyme by the method of Edelhoch (1967). This spectrophotometric determination of tyrosine agrees well with the value obtained after acid hydrolysis of the enzyme (Table I). The partial specific volume of the enzyme calculated from the amino acid composition according to Cohn & Edsall (1943) was 0.736 $cm^3 \cdot g^{-1}$.

The determination of the absorbancy index of purified PEPCK was based on its nitrogen content. PEPCK with a specific activity of 10.9 μmol of PEP carboxylated $min^{-1} \cdot A_{280nm}^{-1}$ was extensively dialyzed against 3 mM phosphate (pH 7.2), 6% glycerol, 2×10^{-4} M DTT, 2×10^{-5} M EDTA. The corrected nitrogen content of aliquots of this solution was determined by a micro-Kjeldahl procedure (Jirka et al., 1976) to be 0.093 mg of nitrogen/mL. This same solution of PEPCK had an absorbance at 280 nm of $0.929 \times cm^{-1}$. From the amino acid analysis (Table I) and the amide nitrogen one can calculate that the protein contains 16.6% nitrogen; therefore, the solution of PEPCK which was directly analyzed for nitrogen contained 0.56 mg of protein/mL. From these data, one obtains an absorbancy index ($1\% \times cm^{-1}$) of 16.6. The molar extinction coefficient (ϵ) of the purified PEPCK is 1.19×10^5. The specific activity is then 18 μmol $min^{-1} \cdot mg^{-1}$.

The high content of aromatic amino acids in PEPCK is consistent with its relatively high ultraviolet absorption. The value for ϵ_{280} of 1.19×10^5 appears compatible with the amino acid composition data shown in Table I. If it is assumed that each Tyr residue makes a contribution of 1280 to ϵ and each Trp a contribution of 5690 (Edelhoch, 1967), the calculated residue contributions to ϵ are 1.27×10^5 for 15 Tyr and 19 Trp. The value given above for PEPCK is 6% lower.

The ultraviolet absorption spectrum of purified PEPCK is shown in the inset of Figure 3B. The spectrum is typical of a protein with a maximum at 282.5 nm and a minimum at about 250 nm. The inflection at 276 nm is likely to be due to tyrosine, and the pronounced shoulder at 292 nm indicates the high tryptophan content of the enzyme. This assignment for tryptophan is also based on the abolition of the 282.5- and 292-nm bands when the enzyme is treated with the tryptophan-oxidizing agent *N*-bromosuccinimide (results not shown).

TABLE I: Amino Acid Composition of Rat Liver Cytosol PEPCK.[a]

amino acid	residues/mol of enz[b]	amino acid	residues/mol of enz[b]
Asp	56	Tyr[c]	15
Glu	76	Trp[c]	19
Lys	44	Phe	28
His	12	Ala	51
Arg	28	Ile	36
Gly	58	Leu	55
Ser[c]	34	Met[e]	18
Thr[c]	23	Pro	41
½-Cys[d]	13	Val	28

[a] The data correspond to the average of values obtained from duplicate acid hydrolysates of homogeneous PEPCK for 24 h at 108 °C. [b] On the basis of a molecular weight of 72 000. [c] Values include corrections for destruction during hydrolysis. The approximate corrections applied according to Moore & Stein (1963) were 5% for tyrosine and threonine and 10% for serine. [d] Value determined as cysteic acid after performic acid oxidation and hydrolysis of the protein (Hirs, 1967). [e] Value determined as methionine sulfone after performic acid oxidation and hydrolysis of the protein (Hirs, 1967). [f] Determined by spectrophotometric methods on the intact protein (Spande & Witkop, 1967; Edelhoch, 1967).

Catalytic Activities of Purified PEPCK. At 25 °C, an enzyme that catalyzed the carboxylation of 16.7 μmol of PEP·$min^{-1} \cdot mg^{-1}$ (28.3 at 36 °C), in the presence of 2.25 mM $MnCl_2$, decarboxylated 19.9 μmol of OAA·$min^{-1} \cdot mg^{-1}$ (32.2 at 36 °C) in the presence of 4 mM $MgCl_2$. When the reaction mixture contained 4 mM $MgCl_2$ and only 75 μM OAA, the rate of PEP formation was stimulated 5.7-fold by 40 μM Mn^{2+} to a specific activity of 78 and 3.9-fold by 40 μM Co^{2+}.

The effect of phosphoenolpyruvate carboxykinase ferroactivator on the activity of purified enzyme was quantitated as described by Bentle & Lardy (1976). When PEPCK (4.5 μg/mL) was incubated with 50 μM $FeCl_2$ and ferroactivator protein (4.4 μg/mL) before adding it to the reaction mixture, PEP synthesis was enhanced to 3.1 times the unstimulated rate.

The apparent K_m for PEP was 48.6 μM at pH 7.13 in the presence of 2.2 mM $MnCl_2$, 1 mM IDP, and 45 mM $NaHCO_3$. At the same $NaHCO_3$ concentration and with 2 mM PEP and 2 mM Mn^{2+} in excess over Mn–nucleotide diphosphate, the apparent Michaelis constants for IDP and GDP were 71.5 and 7.5 μM, respectively. For the decarboxylation of OAA to PEP, the apparent K_m for OAA was 26 μM. In the presence of 300 μM OAA and 1 mM Mg^{2+} over Mg–nucleotide triphosphate, the apparent K_m values for ITP and GTP were 279 and 21.1 μM, respectively.

In the presence of IDP, the purified rat liver cytosolic PEPCK catalyzes the decarboxylation of 3.05 μmol of OAA·$min^{-1} \cdot mg^{-1}$ to form pyruvate. EDTA (0.5 mM), $MgCl_2$ (1.5 mM), and DTT (5 mM) cause 86, 62, and 19% inhibition, respectively, of the pyruvate-forming activity. This activity and its metal-ion inhibition have been previously described for yeast (Cannata & Stoppani, 1963) and chicken liver mitochondrial (Noce & Utter, 1975) PEPCK. At variance with these three enzymes, the pig liver mitochondrial PEPCK decarboxylates OAA to pyruvate more rapidly in the presence of transition metal ions (Chang & Lane, 1966).

Stability of Homogeneous PEPCK. In the absence of metal

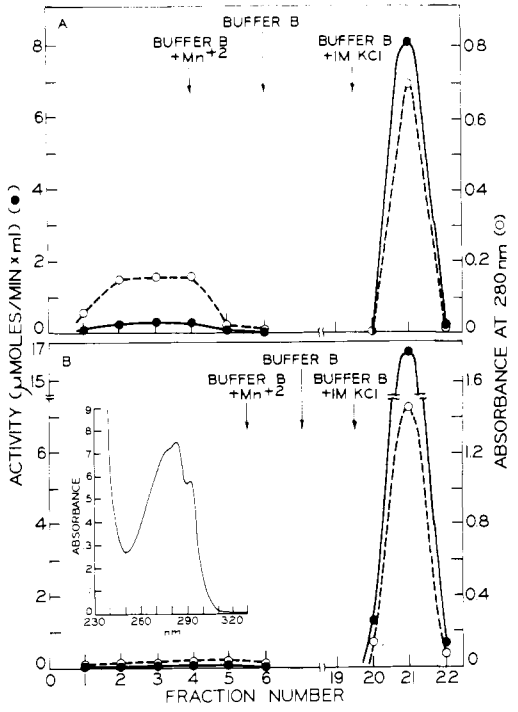

FIGURE 3: Chromatography of homogeneous PEPCK on agarose-hexane-GTP. The GTP columns were equilibrated with buffer B containing 0.15 mM $MnCl_2$. Enzymes were in the same buffer. (A) Homogeneous PEPCK of low specific activity (10.66 μmol of PEP carboxylated $min^{-1} \cdot mg^{-1}$). 4.3 mL of enzyme having an A_{280nm} of 0.37 and containing 10.5 units was applied to a 0.2-mL GTP column. Fraction volume 1.24 mL. As indicated by arrows, the column was first washed with buffer B containing 0.15 mM $MnCl_2$, followed by buffer B. After fraction number 19, PEPCK was eluted with buffer B containing 1 M KCl. (B) Homogeneous PEPCK of high specific activity (20.6 μmol of PEP carboxylated $min^{-1} \cdot mg^{-1}$). 6.5 mL of enzyme having an A_{280nm} of 0.27 and containing 21.7 units was applied to a 0.39-mL GTP column. Fraction volumes were 1.24 mL from 1 to 19 and 0.93 mL for fractions numbered 20 to 22. The washing and eluting buffers were as described in A above: (○-○) A_{280nm}; (●-●) PEPCK activity. Flow rate 7 mL/h. (B inset) Ultraviolet absorption spectrum of high-activity, homogeneous PEPCK in 10 mM TEA (pH 7.2), containing 10% glycerol, 0.1 mM EDTA, and 1 mM GSH taken with a Cary Model 15 spectrophotometer at a protein concentration of 0.33 mg/mL; 1-cm light path.

ions, PEPCK, as eluted from the agarose-hexane-GTP column (in either buffer A or B with KCl), was stable for at least 14 days if kept under N_2 at 4 °C at a protein concentration of 0.5 mg/mL or higher. Upon dilution in buffer A to an A_{280nm} of 0.094 (0.057 mg/mL) and a GSH concentration of 0.2 mM, the enzyme (sp act. 17) was stable for at least 3 h at 22 °C. If 2.5 mM $MnCl_2$ or 2.5 mM $MnCl_2$ plus 1 mM IDP was included in the dilution, 91 and 30% inactivation occurred, respectively, after 158 min. Very rapid inactivation was caused by 1.5 mM $FeCl_2$, which, after incubation with the enzyme for 1 and 26 min, inhibited by 69 and 93%, respectively. Inactivation of PEPCK by Fe^{2+} or Mn^{2+} correlates well with the relative abilities of these ions to catalyze thiol oxidation. Fe^{2+} is 21-fold more effective than Mn^{2+} in catalyzing cysteine oxidation and 1.5-fold more effective than Mn^{2+} in catalyzing GSH oxidation (Jocelyn, 1972). Any oxidation of GSH to GSSG by the metals would also facilitate PEPCK inactivation through a thiol-disulfide exchange mechanism. It has been shown that PEPCK incubated for 45 min with different mixtures of GSH/GSSG shows decreased activity in the mixtures containing higher proportions of GSSG (Johnston, 1970). Ballard & Hopgood (1976) reported that cystine stimulates microsomal inactivation of PEPCK in rat liver extracts; however, protective effects were found for Mn^{2+} and Co^{2+} at 2 mM.

Seubert et al. (1975) found that MgATP, Lys, or Gln stabilize PEPCK activity against inactivation in kidney cortex homogenates. In buffer A, neither Gln, MgATP, His, Met, Ala, nor Trp protected against inactivation of purified rat liver PEPCK caused by dilution to a protein concentration of 0.027 mg/mL. However, no loss of activity occurred over a 5-day period when the diluent contained proteins such as purified ferroactivator (Bentle & Lardy, 1977), bovine serum albumin, hemoglobin, or soybean trypsin inhibitor. Enzyme from which KCl and GSH had been removed by passage through a small Sephadex G-25 column, equilibrated with buffer B (GSH deleted), revealed no loss of activity over a 1-week period under N_2 atmosphere at 4 °C and at a protein concentration of 0.4 mg/mL or higher. Whenever possible, this was the method of choice to obtain enzyme free of KCl and thiols because dialysis produced some loss of activity.

Separation of Homogeneous PEPCK Species with High and Low Specific Activity. Partially purified rat liver cytosol PEPCK loses 14% of its activity in a 3-month period when stored at 4 °C as a suspension in ammonium sulfate in the presence of EDTA and DTT (Ballard & Hanson, 1969). Preparations that had been stored for different periods of time were applied to agarose-hexane-GTP columns in buffer A containing 0.1 mM $MnCl_2$ in excess over EDTA. The columns were washed with the same buffer and eluted with KCl. The resulting chromatographic pattern was dependent on the specific activity of the starting material. The first, unadsorbed component accounted for 82, 74, and 43% of the total amount of protein applied to the GTP columns when the initial specific activity was respectively 0.52, 3.6, and 5.8 μmol $min^{-1} \cdot A_{280nm}^{-1}$. The $NaDodSO_4$-gel electrophoresis pattern of the unadsorbed fractions was similar to that in Figure 1 (gel B). One of the protein bands had the same mobility as homogeneous PEPCK. The relative amount of unadsorbed protein is inversely proportional to the specific activity of the enzyme applied to the column. The specific activity of the enzyme bound to the columns and eluted by a KCl gradient was 10.3 μmol $min^{-1} \cdot A_{280nm}^{-1}$ (17 μmol $min^{-1} \cdot mg^{-1}$). Starting with partially purified enzyme preparations of different activities, the range of specific activity for homogeneous PEPCK eluted by KCl from 14 GTP columns was 16.3 to 20.6 with an average value of 18 μmol $min^{-1} \cdot mg^{-1}$. This is taken as an indication that high- and low-activity forms of the enzyme were separated in the chromatography. This was further substantiated by the fact that on Ouchterlony diffusion plates homologous precipitation reaction was obtained for partially purified enzyme (before GTP column), unadsorbed peak (low activity), and homogeneous PEPCK with antiserum from rabbits immunized against the purified enzyme. In spite of their different specific activity, the two forms of the enzyme are not immunologically different. The mobility of these forms appears to be identical in $NaDodSO_4$-gel electrophoresis. No differences in the apparent K_m values for OAA or IDP could be detected. No further characterization was attempted.

The following experiment was performed in order to ascertain whether the GTP column is able to separate a high-activity form of enzyme from a homogeneous PEPCK preparation of lower specific activity. Homogeneous PEPCK (gel C, Figure 1) of specific activity 16.8 μmol of PEP carboxylated $min^{-1} \cdot mg^{-1}$, obtained from chromatography on agarose-hexane-GTP (Figure 2), was inactivated to 64% of its original activity by dialysis for 17 h against buffer B in the presence of 0.15 mM $MnCl_2$ followed by 1.5-h incubation at 25 °C. When this homogeneous enzyme of specific activity 10.7 was re-

TABLE II: Interactions of Partially Purified PEPCK Preparations with Ligand and Spacer Arm.[a]

agrose deriv (col vol 0.2 mL)	sample applied to col: rel sp act.[b]	sample applied to col: total A_{280}	sample applied to col: total units	not adsorbed by col: % A_{280}	not adsorbed by col: % units	not adsorbed by col: rel sp act.[b]	adsorbed by col and eluted by KCl: % A_{280}	adsorbed by col and eluted by KCl: % units	adsorbed by col and eluted by KCl: rel sp act.[b]
agarose-hexane	5.34	0.752	4.04	7.8	0		75	88	6.2
agarose-hexane-GTP	6.44	0.664	4.28	32	3.2	0.64	67	104	10.6
agarose-hexane-ATP	6.01	0.734	4.41	60.6	24.3	2.21	34.3	49.5	8.6

[a] The columns were equilibrated with buffer A for the hexane and hexane-GTP and buffer B for the hexane-ATP in the presence of 0.15 mM $MnCl_2$. Sample (2.2, 2.1, and 2.4 mL, respectively) application and washing of the columns was as in Figure 1. Activity was eluted with a 0–0.3 M KCl gradient in the equilibration buffer in the absence of $MnCl_2$. [b] Relative specific activity = μmol of PEP carboxylated $min^{-1} \cdot A_{280nm}^{-1}$.

chromatographed on agarose-hexane-GTP, two peaks of activity of homogeneous PEPCK were recovered (Figure 3A; Figure 1, gels D and E). The unadsorbed protein of the first peak contained 10.5% of the activity units and 43% of the protein applied to the column. It had an average specific activity of 2.64 and a 280/260 nm ratio of 1.41. The adsorbed enzyme, eluted by 1 M KCl, contained 97% of the activity and 55% of the total protein applied. It had a specific activity of 19.4 μmol $min^{-1} \cdot mg^{-1}$ and a 280/260 nm ratio of 1.83. Both of these fractions, unadsorbed and adsorbed by the matrix, formed a single band in $NaDodSO_4$-gel electrophoresis (Figure 1, gels D and E) with identical electrophoretic mobility. After being dialyzed against buffer B (GSH deleted), these two homogeneous enzyme fractions displayed no major differences in their ultraviolet absorption spectra between 275 and 295 nm. The 280/290 nm absorbancy ratios are 1.33 and 1.35, respectively, for the unadsorbed and adsorbed enzyme forms. The former shows increased UV absorbance in the region above 295 nm. Its 280/310 nm ratio is 4.28 and that of the adsorbed enzyme is 11.2. The 250-nm trough is deeper in the adsorbed form than in the unadsorbed one. Their 280/250 nm ratios are 2.4 and 1.51, respectively.

Since the column apparently bound only a high-activity form of the enzyme, very little unadsorbed protein would be expected when homogeneous PEPCK of high specific activity is rechromatographed on the GTP column. The homogeneous PEPCK that eluted with 1 M KCl from the GTP column as in Figure 2 was freed of KCl by desalting in Sephadex G-25 equilibrated with buffer B. When this homogeneous enzyme of high specific activity (20.6 μmol $min^{-1} \cdot mg^{-1}$) is brought to 0.15 mM $MnCl_2$ and immediately rechromatographed on agarose-hexane-GTP, 2% of the applied units and only 11% of the applied protein appeared in the unadsorbed protein peak (Figure 3B); 88% of the protein applied to the GTP column was in the fractions eluted by 1 M KCl.

The results indicate that homogeneous PEPCK (as judged by $NaDodSO_4$-gel electrophoresis) represents a mixture of at least two enzyme forms with identical molecular weights but different specific activity. The proportion of the two forms appears to reflect the overall specific activity of the mixture. High and low specific activity forms of PEPCK can be separated on agarose-hexane-GTP in the presence of $MnCl_2$ (Figures 2 and 3A). The specific activity of the enzyme form that binds to the GTP column (eluted by KCl) is high and relatively constant. The one of the unadsorbed enzyme is low and more variable. Whether this unadsorbed enzyme is, in turn, a mixture of partially inactive and completely inactive forms or whether its activity is due to leaching of bound enzyme is not known at the present. The results from the experiment reported in Figure 3B suggest that it represents inactive enzyme plus 2–3% of the active enzyme that leaches from the column.

Perhaps the most interesting question is whether or not these forms of PEPCK are present in vivo and might have any physiological significance. At present this is not known. However, different "forms" of PEPCK have been reported in the literature. Two catalytically active peaks are eluted from DEAE-cellulose chromatography of baker's yeast PEPCK; crystals derived from these two fractions were similar in shape, size, and specific activity (Cannata, 1970). Rats which have been rendered acidotic by intragastric administration of NH_4Cl show increased assayable PEPCK activity in kidney cortex. This acidosis-induced rise of PEPCK is insensitive to actinomycin D and is accompanied by an increase in the apparent molecular weight of the enzyme, presumably to a dimeric species (Longshaw & Pogson, 1972). Foster et al. (1967) postulated that the increased assayable PEPCK activity in rat liver cytosol following administration of tryptophan occurs via an activation process of an enzyme form, which is inactive or is less active, to a PEPCK form that has a higher intrinsic activity. This activated enzyme form is unstable if stored in the absence of DTT, and it is no longer activated by Mn. No change in molecular weight was detected. Ballard & Hanson (1969) reported that chromatography of partially purified PEPCK from rat liver cytosol on a pH 5 to 8 electrofocusing column resulted in two PEPCK activity fractions: a peak with high activity focusing at pH 5 and a low activity peak focusing at higher pH. Studies by Ballard et al. (1974) regarding in vitro inactivation by a microsomal fraction of rat liver cytosol PEPCK indicated a sequence of events that involved a loss of catalytic activity, followed by decreased immunological reactivity and then loss of enzyme solubility. These three forms of the enzyme had an identical mobility on $NaDodSO_4$-gel electrophoresis as native PEPCK.

Type of Forces Involved in Agarose-Hexane-GTP Chromatography. The effects of the spacer arm and of the terminal substituent in a hexylagarose column were investigated in order to ascertain the specificity and the nature of the interactions existing between PEPCK and the matrix. Partially purified PEPCK (gel A, Figure 1) was chromatographed on agarose columns containing a ligand-free hexyl arm or containing an additional functional group (GTP or ATP) bound to the hexyl hydrocarbon chain. The density of hexyl chains was similar in the three columns, and the amount of nucleotide bound was the same in the substituted hexylagarose derivatives. The results (Table II) suggest that hydrophobic interactions must contribute greatly to the retention and discrimination power of the three columns. The modification of the hexyl side chain with the substrate greatly increases the specificity of the column for active PEPCK, inasmuch as 32% of the applied protein is not retained (mainly contaminant proteins and inactive enzyme) but almost all the enzyme units are (Table II). In contrast, nonspecific binding is obtained in the agarose-hexane column with no increase in specific activity. Enzyme activity

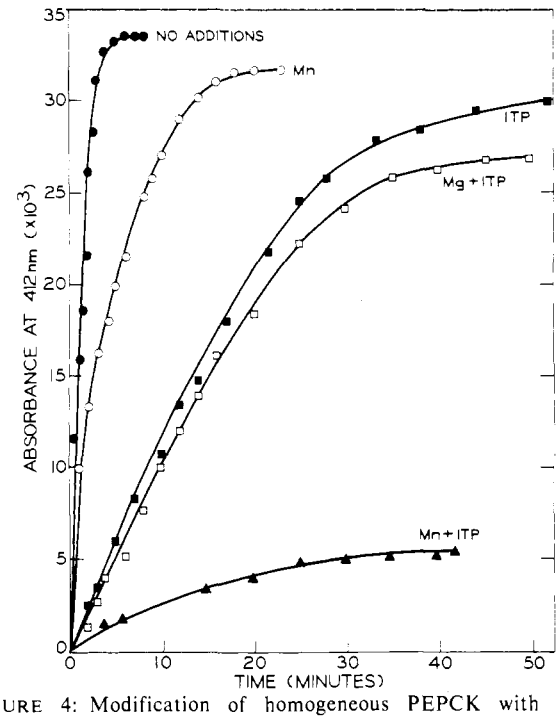

FIGURE 4: Modification of homogeneous PEPCK with 5,5′-dithiobis(2-nitrobenzoic acid). Enzyme was reacted at 25 °C with Nbs_2 in a reaction system containing 100 mM Tes, 0.1 mM EDTA (pH 7.6), and additions as follows: (●) control, no additions, 0.696 μM PEPCK and 1.24 μM Nbs_2; (○) 4 mM $MnCl_2$, 0.696 μM PEPCK, and 1.31 μM Nbs_2; (■) 4 mM ITP, 0.692 μM PEPCK, and 1.16 μM Nbs_2; (□) 4 mM ITP plus 5 mM $MgCl_2$, 0.58 μM PEPCK, and 0.99 μM Nbs_2; (▲) 4 mM ITP plus 4 mM $MnCl_2$, 0.59 μM PEPCK, and 1 μM Nbs_2. The reaction was monitored by following the absorbance change at 412 nm with a Gilford Model 2000 spectrophotometer. The absorbance of a blank containing all the components of the reaction system except the enzyme was continually subtracted from that of the sample. The enzyme used had been desalted in a Sephadex G-25 column equilibrated with 10 mM Tes (pH 7.2) containing 10% glycerol and 0.1 mM EDTA.

is also retained on ethyl- and butylagarose columns (results not shown). The agarose–hexane–ATP column has a lower affinity for PEPCK than the GTP-substituted matrix. The results support the idea of hydrophobic interactions as a significant mechanism of PEPCK binding to agarose–hexane–GTP with the nucleotide substitution on the arm providing specificity for binding. It is possible that the hexyl spacer chain could directly interact with a hydrophobic area on the enzyme. From the amino acid analysis, 40.4% of the residues are accounted for by hydrophobic amino acids.

Influence of Divalent Metals on Agarose–Hexane–GTP Chromatography. Divalent metals are required for PEPCK activity. Foster et al. (1967) reported that when rat liver cytosol containing PEPCK is assayed in the direction of PEP synthesis Mg^{2+} and Mn^{2+} show synergistic effects. With $MgCl_2$ and ITP present at equimolar concentrations, low concentrations of Mn^{2+} or other divalent transition metal ions (Snoke et al., 1971) activate the enzyme. Furthermore, in the presence of $MnGTP^{-2}$, free Mn^{2+} can act as the enzyme-activating metal (Jomain-Baum et al., 1976). The interaction of free metal with PEPCK at a site other than the active (metal–nucleotide substrate) site could possibly promote a specific enzyme conformation necessary for optimum catalytic activity. This conformational change also influences the tightness of PEPCK binding to agarose–hexane–GTP, as reflected in the concentration of KCl necessary for enzyme elution.

Chromatography of partially purified PEPCK in buffer A containing 0.2 mM $MnCl_2$ or 0.2 mM $Mg(CH_3COO)_2$ or in the absence of metal ions was carried out in GTP columns equilibrated with the respective buffers. After washing with equilibration buffer, the enzyme was eluted with a linear KCl gradient in the same buffer. The concentrations of KCl at the center of the activity peak were 800, 200, and 110 mM with Mn^{2+}, Mg^{2+}, and in the absence of metal, respectively. However, the concentration of KCl was 120 mM when the enzyme was applied in the presence of Mn^{2+}, but the column was then washed and eluted in the absence of Mn^{2+}. It follows then that the presence of Mn^{2+} (but not Mg^{2+}) in conjunction with the metal–nucleotide induces an enzyme conformation that binds tighter to agarose–hexane–GTP. This effect is further demonstrated by measurements of -SH group reactivity with Nbs_2 as an indicator of conformational changes. As shown in Figure 4, titration of homogeneous PEPCK with Nbs_2 in the absence of effectors or in the presence of $MnCl_2$ leads to a very rapid modification of cysteine residues. Under these conditions, disulfides are formed with loss of enzyme activity (Carlson et al., 1978). The presence of ITP or Mg^{2+} plus ITP (conditions in which low KCl elutes the enzyme from GTP columns) decreases the rate of Cys modification; ITP was somewhat less effective than Mg^{2+} plus ITP. This slower reaction is perhaps with another set of -SH groups because the modified enzymes were partially active. The presence of Mn^{2+} plus ITP (a condition in which high KCl is necessary to elute the enzyme from the GTP column) caused a dramatic decrease in the rate of reaction of -SH groups with Nbs_2. No loss of PEPCK activity occurred under this condition after 50 min of modification. It thus appears that Mn^{2+} plus MnITP induces a rather large conformational change. In this conformation, the enzyme is bound tighter to agarose–hexane–GTP, has its cysteine residues less exposed (Figure 4), and is optimal for catalytic activity (Jomain-Baum et al., 1976).

In conclusion, PEPCK from rat liver cytosol has been purified to homogeneity by chromatography on agarose–hexane–GTP in the presence of Mn^{2+}. Under these conditions, the binding of PEPCK to the GTP column is quite specific inasmuch as low activity and/or inactive forms of the enzyme and the contaminating proteins present in the partially purified preparations (Philippidis et al., 1972) are not retained by the matrix. The retained PEPCK that was effectively eluted by 1 M KCl in the absence of Mn^{2+} was homogeneous and of high specific activity. The availability of this highly purified enzyme will facilitate further physicochemical studies designed to correlate structural and functional parameters of PEPCK with its mechanism of action.

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A Vicinal Dithiol Containing an Essential Cysteine in Phosphoenolpyruvate Carboxykinase (Guanosine Triphosphate) from Cytosol of Rat Liver†

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ABSTRACT: The highly purified form of phosphoenolpyruvate carboxykinase (PEPCK) contained 13 thiols (all in the reduced state) per 72 000 daltons. Modification of the enzyme with equimolar 5,5′-dithiobis(2-nitrobenzoate) (Nbs_2) caused rapid formation of a cystine disulfide bridge and an even more rapid loss of enzymatic activity. Formation of the cystine bridge proceeded about 25 times faster than formation of the analogous intramolecular disulfide of dithiothreitol induced by Nbs_2. *o*-Iodosobenzoate, Cd^{2+}, and the 2,3-dimercapto-1-propanol complex of arsenite were potent, time-dependent, irreversible inhibitors of PEPCK. The inactivation by arsenite-2,3-dimercapto-1-propanol and *o*-iodosobenzoate was first order with respect to both time and inhibitor concentration. The sum of these data indicates the existence in PEPCK of a critical cysteine that is in a vicinal dithiol grouping with a second cysteine. PEP protected against cystine bridge formation induced by equimolar Nbs_2 but not against the extent of inactivation. In the presence of PEP, the modification by Nbs_2 of one cysteine/mol of enzyme ($k = 1.2 \times 10^6$ M^{-1} min^{-1} at pH 7.2) caused nearly complete inactivation. Replacing the bulky 5-thio-2-nitrobenzoate moiety with cyanide did not result in any reactivation. This critical, cyanylated cysteine was determined to be 44% of the distance from the amino terminus.

Phosphoenolpyruvate carboxykinase (GTP) (PEPCK, EC 4.1.1.32)[1] from a variety of species and tissues is sensitive to sulfhydryl reagents. *p*-Hydroxymercuribenzoate inhibits PEPCK isolated from the mitochondria of chicken liver (Utter

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§ Recipient of the Evelyn Steenbock Fellowship.

[1] Abbreviations used: PEPCK, phosphoenolpyruvate carboxykinase (GTP); Nbs_2, 5,5′-dithiobis(2-nitrobenzoate); Nbs, 5-thio-2-nitrobenzoate; BAL, 2,3-dimercapto-1-propanol; EDTA, ethylenediaminetetraacetate; OAA, oxalacetate; PEP, phosphoenolpyruvate; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); TEA, triethanolamine; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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