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Partial Purification and Characterization of Pyruvate,Orthophosphate Dikinase from *Rhodospirillum rubrum*†

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We confirmed an earlier report (B. B. Buchanan, J. Bacteriol. 119:1066–1068, 1974) that the nonsulfur purple photosynthetic bacterium *Rhodospirillum rubrum* contains pyruvate,orthophosphate dikinase (EC 2.7.9.1) activity that is absolutely dependent upon all three substrates by performing enzyme assays in both the forward (phosphoenolpyruvate formation) and reverse (ATP formation) directions. Of the various carbon sources tested, photoheterotrophic growth on DL-lactate plus bicarbonate proved to be best for the production of dikinase activity units. A four-step protocol, which included batch DEAE-cellulose processing, ammonium sulfate fractionation, and chromatography on hydroxylapatite and Blue A Dyematrex gels, was devised for partially purifying the enzyme from such cells. The protein was purified about 80-fold to an apparent electrophoretic purity of about 60% and a final specific activity of 3.6 U/mg of protein, with about a 35% overall recovery of activity units. Estimations of native and monomeric relative molecular weights by sucrose density gradient centrifugation, high-pressure liquid chromatography-based size exclusion chromatography, denaturing electrophoresis, and immunoblotting suggested that the holoenzyme was most likely a homodimer of 92.7-kilodalton subunits. The results are compared with related previous data on the nonphotosynthetic bacterial dikinase and the C_4 mesophyll chloroplast enzyme.

Pyruvate,orthophosphate dikinase (PPDK; EC 2.7.9.1) catalyzes the reversible formation of phosphoenolpyruvate (PEP), AMP, and PP_i from pyruvate, ATP, and P_i (11) as follows:

$$\text{Pyruvate} + \text{ATP} + P_i \xrightleftharpoons{Mg^{2+},\ NH_4^+} \text{PEP} + \text{AMP} + PP_i$$

Although the enzyme in certain nonphotosynthetic bacteria, including *Bacteroides symbiosus* (synonym of *Clostridium symbiosum*), *Propionibacterium shermanii*, *Acetobacter aceti*, and *A. xylinum*, has been studied extensively (4, 10, 11, 19, 20, 22, 23), relatively little is known about the presence and characteristics of PPDK in photosynthetic bacteria. Buchanan (6) detected PPDK activity in crude cell lysates of *Chlorobium thiosulfatophilum*, *Chromatium* sp. strain D, and *Rhodospirillum rubrum* after ammonium sulfate fractionation, but no properties of the enzyme were reported.

The main objectives of our work were to (i) confirm the earlier sole report (6) that certain photosynthetic bacteria do, indeed, contain PPDK, (ii) develop a protocol for at least partially purifying active PPDK from a photosynthetic bacterium for further analysis, and (iii) characterize several gross physicochemical properties of the heretofore-unstudied photosynthetic bacterial dikinase for comparison with those of the extensively studied 387-kilodalton (kDa) tetrameric C_4 mesophyll chloroplast enzyme (12, 25) and 170-kDa dimeric protein from *B. symbiosus* (10, 11). Our studies with PPDK isolated from photoheterotrophically grown *R. rubrum* are described here.

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MATERIALS AND METHODS

Materials. Sephadex G-50 and Sephacryl S-200 and S-300 were purchased from Pharmacia, Inc., Blue A Dyematrex gel was purchased from Amicon Corp., and sodium dodecyl sulfate (SDS; sequanal grade) was purchased from Pierce Chemical Co. Protein standards for SDS-polyacrylamide gel electrophoresis (PAGE), acrylamide, enzyme immunoassay-grade gelatin, nitrocellulose membrane, protein A-horseradish peroxidase conjugate, and hydroxylapatite (HAP; Bio-Gel HT) were obtained from Bio-Rad Laboratories, and high-pressure liquid chromatography (HPLC)-grade native-molecular-weight protein standards were obtained from U.S. Biochemical Corp. AcrylAide and GelBond were purchased from FMC Corp., and ultrapure ammonium sulfate (enzyme grade) and sucrose (density gradient grade) were purchased from Schwarz/Mann. All other biochemical reagents, DEAE-cellulose, and coupling enzymes were obtained from Sigma Chemical Co., except for PEP carboxylase, which was obtained from Boehringer Mannheim Biochemicals.

Organism and growth conditions. The stock culture of *R. rubrum* (strain S-1) was kindly provided by the Photosynthetic Bacteria Group at Indiana University. Cells were grown photoheterotrophically on a synthetic medium based on the receipe described by Ormerod et al. (21), with the following modifications (per liter of medium): DL-malic acid, 4.0 g, or DL-lactic acid, 6.0 ml of 85% syrup, plus $NaHCO_3$, 2.0 g, as needed; thiamine hydrochloride, 1.0 mg; $MnSO_4 \cdot H_2O$, 1.6 mg; and K_2HPO_4, 1.18 g. For large-scale-growth cultures, 12 liters of lactate-$NaHCO_3$ medium was inoculated with 880 ml of 3-day-old cells grown photoheterotrophically on malate at ~70 klx. Cells were grown for 5 days at pH 6.8 ± 0.2 and 28 to 30°C in a 14-liter Magnaferm fermentor (New Brunswick Scientific Co., Inc.) illuminated at ~90 klx. The washed and pelleted cells were stored at −80°C until used (1 day to 3 months).

Lysis of cells. Cells were lysed by the osmotic shock protocol described by Ludden and Burris (16). Frozen cell

TABLE 1. Purification of PPDK from *R. rubrum*[a]

Purification step	Total protein (mg)	Total PPDK activity (U)	PPDK sp act (U/mg of protein)	Purification (fold)	Yield (%)
1. HSSF	701	31.0	0.044	1.00	100
2. Batch DEAE-cellulose eluant	148	19.6	0.132	3.00	63
3. 0 to 55% $(NH_4)_2SO_4$ pellet	90	18.8	0.209	4.75	61
4. Sephadex G-50 eluant	85	18.1	0.213	4.84	58
5. HAP eluant[b]	10	10.6	1.06	24.1	34
6. Blue A eluant	2.9	10.4	3.59	81.6	34

[a] PPDK was isolated from 50 g of wet packed cells grown photoheterotrophically on DL-lactate plus bicarbonate. PPDK activity is based on the PEP carboxylase-malate dehydrogenase coupling scheme (i.e., PEP formation) at pH 8.0 and 30°C.

[b] The values given are based on a desalted preparation of the HAP pool because PPDK activity is inhibited by the high phosphate levels present after elution from the HAP column.

paste (50 g [wet weight]) was thawed for ~90 min at 4°C in 200 ml of dehydration buffer (buffer A), which contained 0.1 M 4-morpholinepropanesulfonic acid (MOPS)–KOH (pH 7.5 at 4°C), 0.4 M glycerol, 75 mM KCl, 2.5 mM disodium EDTA, 2 mM $MgCl_2$, and 5 mM dithiothreitol (DTT). The thawed and dehydrated cells were then centrifuged for 15 min at 11,950 × *g*, and 10 ml of digestion buffer was added to the pellet. The digestion buffer consisted of 10 ml of buffer A plus 10 mg (21,500 U) of DNase II, 10 mg (750 U) of RNase A, 20 mg (1.18×10^6 U) of lysozyme, 1 mM phenylmethylsulfonyl fluoride (freshly prepared in 95% ethanol as a 40 mM stock solution), and 50 μM leupeptin. After the slurry had remained at 23°C for 15 min, 200 ml of lysis buffer (4°C), which consisted of buffer A minus the glycerol but plus 1 mM phenylmethylsulfonyl fluoride and 50 μM leupeptin, was added. This extract was then centrifuged at 38,720 × *g* for 30 min to remove cellular debris. A second centrifugation followed at 143,800 × *g* for 90 min in a Beckman 60 Ti fixed-angle rotor. The resulting supernatant fraction, referred to as the high-speed supernatant fluid (HSSF), was saved for further processing (see below).

Enzyme assays. PPDK activity in the forward direction (PEP formation) was measured spectrophotometrically at 340 nm and 30°C via the oxidation of NADH in the presence of exogenous PEP carboxylase and NADH-malate dehydrogenase (12). A typical assay mixture contained, in a 1-ml final volume, 0.1 M Tris hydrochloride (pH 8.0 at 30°C), 0.11 mM disodium EDTA, 20 mM NH_4Cl (NH_4^+ is an essential monovalent cation for PPDK activity [11]), 10 mM $MgCl_2$, 5 mM DTT, 4 mM pyruvate, 1.5 mM ATP, 3 mM KH_2PO_4, 5 mM $NaHCO_3$, 0.2 mM NADH, 0.2 U of PEP carboxylase, 2.9 U of malate dehydrogenase, and 0.006 U of PPDK.

PPDK activity in the reverse direction (ATP formation) was measured spectrophotometrically at 340 nm and 30°C via the reduction of NADP in the presence of exogenous hexokinase and glucose-6-phosphate dehydrogenase (4). A typical assay mixture contained, in a 1-ml final volume, 0.1 M MOPS-KOH (pH 7.0 at 30°C), 5 mM $MgCl_2$, 25 mM NH_4Cl, 5 mM DTT, 1 mM $(NH_4)_6Mo_7O_{24}$ (to inhibit inorganic pyrophosphatase activity), 1 mM PEP, 1 mM AMP, 1 mM tetrasodium PP_i, 5 mM glucose, 0.3 mM NADP, 5.6 U of hexokinase, 2 U of glucose-6-phosphate dehydrogenase, and 0.006 U of PPDK.

One unit of PPDK activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of PEP or ATP per min at 30°C.

Enzyme purification. PPDK was purified by the following procedure (Table 1); all steps were performed at 4°C.

(i) Batch DEAE-cellulose processing. The HSSF (175 ml) was mixed with 180 ml of DEAE-cellulose equilibrated with wash buffer (buffer A minus glycerol). The resultant slurry was thoroughly mixed and stored for ~5 min at 4°C before being taken to dryness by vacuum filtration. The DEAE-cellulose cake was then mixed with 200 ml of fresh wash buffer, followed by another 5 min of incubation and vacuum filtration. This wash step was repeated once again. PPDK was batch eluted from the DEAE-cellulose by two 5-min rinses with 200 ml of 0.1 M MOPS-KOH (pH 7.5 at 4°C)–0.2 M KCl–2.5 mM disodium EDTA–2 mM $MgCl_2$–5 mM DTT.

(ii) Ammonium sulfate precipitation. The 0.2 M KCl eluant from the batch DEAE-cellulose processing was taken to 55% saturation (4°C) with pulverized $(NH_4)_2SO_4$. The resultant precipitate was collected at 11,950 × *g* and stored overnight at −80°C.

(iii) HAP column chromatography. The 0 to 55% $(NH_4)_2SO_4$ fraction was dissolved in 17 ml of buffer containing 10 mM potassium phosphate (pH 7.0 at 4°C), 5 mM KCl, 0.5 mM disodium EDTA, and 5 mM DTT and desalted on a Sephadex G-50 column (2.5 by 46 cm) at a flow rate of 1 ml/min. The eluant (~30 ml) was placed onto an HAP column (2.5 by 46 cm) and eluted at a flow rate of 1 ml/min with a linear 10 to 400 mM gradient of potassium phosphate (pH 7.0 at 4°C) containing the above additions. Peak PPDK activity eluted at ~75 mM potassium phosphate. Fractions (2 ml) were pooled and stored overnight at −80°C as a 60% $(NH_4)_2SO_4$ pellet (4°C) or processed immediately through Blue A Dyematrex.

(iv) Affinity column chromatography. The 60% $(NH_4)_2SO_4$ pellet was dissolved in 9 ml of buffer containing 20 mM Tris hydrochloride (pH 7.5 at 4°C), 5 mM $MgCl_2$, and 5 mM DTT and desalted as described above. The eluant (~24 ml) was passed through a Blue A Dyematrex affinity column (2.5 by 3 cm) at a flow rate of 0.6 ml/min. Wash fractions (1 ml) were collected, and those containing PPDK activity were pooled (~30 ml, total) and concentrated by precipitation to 80% $(NH_4)_2SO_4$ saturation (4°C).

Estimation of native molecular weight. Centrifugation on linear 10 to 30% sucrose density gradients was used to estimate the native relative molecular weight of *R. rubrum* PPDK (18). Gradients (22 ml) of ultrapure sucrose were prepared in 25 mM MOPS-KOH (pH 7.0 at 4°C) containing 2 mM β-mercaptoethanol. The gradients were loaded with 1-ml mixtures containing desalted enzyme standards (in concentrations of 1 mg each) and *R. rubrum* PPDK which had been purified through Blue A (Table 1, step 6). Spinach leaf ribulosebisphosphate carboxylase/oxygenase (RuBisCO [533 kDa]), *R. rubrum* RuBisCO (101 kDa), and bovine liver catalase (250 kDa) served as marker enzymes. Centrifugation was performed at 4°C and 177,500 × *g* for 3.5 to 4.5 h in a 60 Ti fixed-angle rotor. Fractions (1 ml) were collected

from the bottom of the tubes and assayed for the respective enzyme activities. Radioisotopic RuBisCO assays were performed at 25°C as described by Jordan and Chollet (14). Spectrophotometric catalase assays were performed at 240 nm and 25°C as described by Beers and Sizer (3).

The native relative molecular weight was also estimated by HPLC-based size exclusion chromatography (1) on a Spherogel-TSK column (7.5 by 300 mm; Altex) with an approximate fractionation range of 30 to 300 kDa. HPLC-grade native-molecular-weight standards included glutamate dehydrogenase (290 kDa), lactate dehydrogenase (140 kDa), enolase (67 kDa), and adenylate kinase (32 kDa). The buffer system consisted of 25 mM MOPS-KOH (pH 6.8 at 25°C) and 0.2 M KCl. The retention times of the standards were based on A_{280} profiles of an injection mixture containing all four proteins. The retention time of *R. rubrum* PPDK was based on activity assays performed on collected fractions. The column flow rate and temperature were maintained at 0.5 ml/min and 25°C, respectively, on the Waters HPLC apparatus.

Denaturing electrophoresis. The relative electrophoretic mobility of monomeric PPDK (26) and the electrophoretic purity of the PPDK preparations were evaluated by denaturing electrophoresis. Continuous linear gradient SDS vertical slab gels (1.5 mm thick) of 5 to 20% polyacrylamide were prepared by a modified Laemmli procedure (15). AcrylAide cross-linker was substituted for *N,N'*-methylenebisacrylamide in gels covalently bound to GelBond PAG plastic support films.

Immunoblotting. The denatured protein samples were separated by SDS-PAGE on a 5 to 15% polyacrylamide gel and transblotted onto a nitrocellulose membrane (15 by 15 cm) by high-field-intensity transfer at 100 V for 80 min (in cold buffer composed of 25 mM Tris, 192 mM glycine [pH 8.3], and 20% methanol) with a Bio-Rad Trans-Blot Cell equipped with a cooling coil operated at 0°C. The membrane was blocked with 3% gelatin, incubated with maize leaf anti-PPDK immunoglobulin G (7) for 1 h at 30°C, and probed with protein A-horseradish peroxidase conjugate. Buffers were made and washes were performed exactly as described in the "Bio-Rad Immunoblot Protein A-Horseradish Peroxidase Instruction Manual."

Protein assay. Protein concentrations were determined by the Bio-Rad dye-binding assay (5). Crystalline bovine serum albumin in distilled water was used as the standard.

RESULTS

Enzyme occurrence. Given the earlier report (6) of PPDK activity in a 35 to 45% $(NH_4)_2SO_4$ fraction of autotrophically grown *Chromatium vinosum* cells and our interest in this microorganism (14), our initial efforts were directed at isolating PPDK from this sulfur purple photosynthetic bacterium. We were unable, however, to detect P_i-dependent PEP (or oxaloacetate) formation or PP_i-dependent ATP formation in the HSSF or a 35 to 45% $(NH_4)_2SO_4$ fraction of cells grown either autotrophically or photoheterotrophically on pyruvate plus HCO_3^-. Given this inability to detect in *C. vinosum* PPDK activity that was dependent upon all three substrates, we next attempted to isolate the enzyme from the nonsulfur purple photosynthetic bacterium *R. rubrum*. We confirmed Buchanan's earlier report (6) that this organism contains PPDK activity that is absolutely dependent upon all three substrates (pyruvate-, ATP-, and P_i-dependent PEP formation) by performing enzyme assays in both the forward (PEP formation) and reverse (ATP formation) directions. When all substrates were present, 3.86 U of PEP was formed per mg of protein at pH 8.0 and 30°C, and 3.13 U of ATP was formed per mg of protein at pH 7.0 and 30°C. These values are averages of two separate purifications through Blue A (Table 1, step 6). There were no coupling enzyme limitations during the assay. When any of the three substrates (pyruvate, ATP, or P_i for PEP and PEP, AMP, or PP_i for ATP) was absent, no activity was detected.

Photoheterotrophic growth of *R. rubrum* on DL-lactate plus HCO_3^- in 1-liter Roux bottles produced cells with a high PPDK specific activity (per gram [wet weight]) relative to a number of other carbon sources tested. These results were based on PPDK activity (PEP formation at pH 8.0 and 30°C) determined in the HSSF of cells grown on acetate, malate, succinate, pyruvate, alanine, or lactate, with values ranging from ~1.4 (malate) to ~2.3 (succinate and lactate) U/g (wet weight). Bicarbonate was included in all cultures except those grown on malate. Although photoheterotrophic growth on lactate or succinate produced cells with similar PPDK specific activities, lower yields of cells were produced on the latter. As a result of these comparative studies, we subsequently used the lactate-bicarbonate medium.

Comments on purification protocol. PPDK from *R. rubrum* was purified ~80-fold to a final specific activity of 3.6 U/mg of protein, with about a 35% overall recovery after Blue A processing (Table 1, step 6).

Low PPDK total activity in the initial HSSF (Table 1, step 1) necessitated the conservation of units at each stage in the purification protocol. Although the bulk of the PPDK activity units (94% of the total recovered units) was present in a 40 to 55% $(NH_4)_2SO_4$-saturated (4°C) fraction of the HSSF, a rapid one-step cut from 0 to 55% (Table 1, step 3) was preferable to two successive 0 to 40% and 40 to 55% fractionations. Two steps yielded only a ~50% overall recovery, versus a 96% yield with a single-step fractionation. At step 5 (HAP processing), the conservation of activity units was sacrificed for the fivefold increase in purity. DEAE-cellulose and HAP column materials were used only once before being discarded, owing to irreversible pigment binding.

After either step 3 [$(NH_4)_2SO_4$ fractionation] or step 5 (HAP processing) and storage as an ammonium sulfate pellet at −80°C, enzyme activity was stable for up to 1 month without appreciable loss. However, after step 6 (Blue A processing) and storage as an ammonium sulfate pellet at −80°C, enzyme activity diminished greatly. Within 1 week, PPDK activity after storage decreased by ~25%; after 2 weeks of such storage, PPDK activity decreased by ~50%.

The purity of the final PPDK preparation after step 6 (Table 1) was evaluated by SDS-PAGE and densitometry of the Coomassie blue R-250-stained gels. Protein in a ~92-kDa band represented ~60% of the total protein applied to the gel (Fig. 1, lanes 1 and 2). Several of the lower-molecular-weight bands increased or appeared anew at the later stages of purification, suggesting that they may be proteolytic products.

Native relative molecular weight. Sucrose density gradient centrifugation was used to estimate the native M_r of *R. rubrum* PPDK. A value of 169,300 ± 3,900 was calculated based on the spinach leaf and *R. rubrum* RuBisCO markers (Fig. 2). The standard-state sedimentation coefficient ($s_{20,w}$) for *R. rubrum* PPDK was estimated to be 8.6S. Experimental verification of the linearity of the sucrose gradient indicated calculated native M_rs of 105,800 and 509,100 for *R. rubrum* RuBisCO and spinach leaf RuBisCO, respectively. These values agree well with the previously published values of

101,000 (13) and 533,256 (17, 27), respectively, calculated from the primary sequences of the component subunits.

HPLC-based size exclusion chromatography was also used to estimate the native M_r of *R. rubrum* PPDK. The value determined by this independent technique was 246,800 (Fig. 3).

Subunit relative molecular weight and quaternary structure. SDS-PAGE revealed numerous soluble polypeptides in the initial HSSF. The increasing prominence during purification of a ~92-kDa band which was clearly the dominant polypeptide after Blue A processing (Fig. 1, lanes 1 and 2) suggested that it corresponded to the monomeric form of *R. rubrum* PPDK. This view was corroborated by immunoblot analysis, which indicated that the ~92-kDa polypeptide in the Blue A eluant (Table 1, step 6) cross-reacted with C_4 leaf anti-PPDK antiserum (Fig. 4, lanes 2 and 3). A more precise monomeric M_r of 92,700 was estimated for *R. rubrum* PPDK on the basis of a plot of the relative electrophoretic mobilities of six standard proteins and PPDK versus molecular-weight (Fig. 1, 4, and 5 and reference 26). The nearly exclusive phosphorylation of this 92.7-kDa polypeptide was also evident in autoradiographs after reaction with the dikinase-specific C_4 leaf PPDK regulatory protein and [β-^{32}P]ADP plus ATP (9), further supporting the view that the 92.7-kDa band is, indeed, the monomeric form of *R. rubrum* PPDK.

Taken collectively, the various relative molecular-weight determinations suggest that *R. rubrum* PPDK is most likely a dimer or possibly a trimer, based on a monomeric M_r of 92,700 (Fig. 2 through 5). This view was further supported by gel filtration of the native protein on calibrated columns of Sephacryl S-200 and S-300.

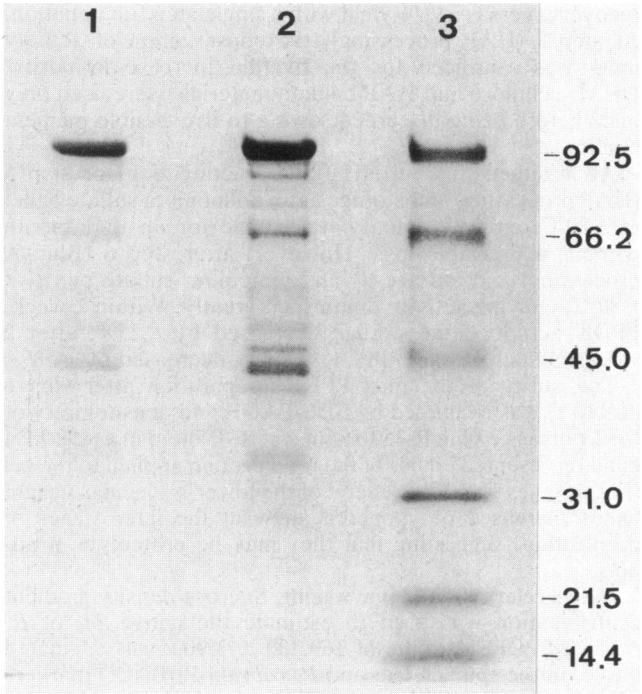

FIG. 1. SDS-PAGE of PPDK from *R. rubrum* and protein standards in 5 to 20% polyacrylamide. Lanes 1 and 2 contain 25 and 50 μg of protein, respectively, after affinity chromatography with Blue A (Table 1, step 6). Lane 3 contains molecular weight markers (values in kilodaltons). Proteins were visualized by staining with Coomassie blue R-250.

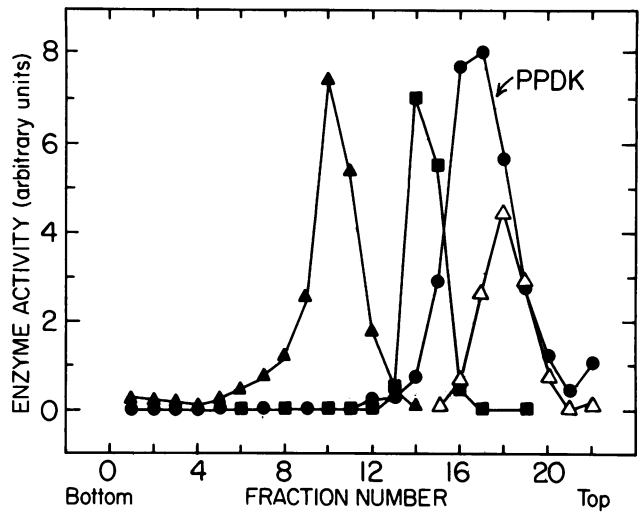

FIG. 2. Sucrose density gradient centrifugation of PPDK from *R. rubrum*. Activities (arbitrary units) of marker enzymes and *R. rubrum* PPDK (●) processed through Blue A (Table 1, step 6) are plotted versus the fraction number. Marker enzymes included spinach leaf RuBisCO (M_r, 533,300; ▲), bovine liver catalase (M_r, 250,000; ■), and *R. rubrum* RuBisCO (M_r, 101,000; △). Fractions (1 ml) were collected from the bottom of the tubes for measurement of enzyme activities.

DISCUSSION

The purification protocol for *R. rubrum* PPDK described here resembles that for the C_4 leaf enzyme (25) and those for the *B. symbiosus* (10, 11) and *A. aceti* (23) enzymes in that ammonium sulfate fractionation, DEAE-cellulose processing, and HAP processing were all performed. Our protocol

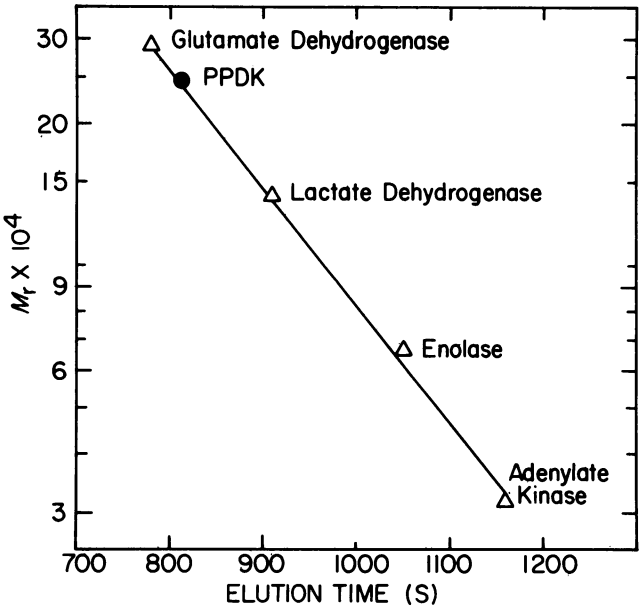

FIG. 3. Estimation of the native M_r of PPDK from *R. rubrum* (●) by HPLC-based size exclusion chromatography. Native-molecular-weight standards (△) included glutamate dehydrogenase (M_r, 290,000), lactate dehydrogenase (M_r, 140,000), enolase (M_r, 67,000), and adenylate kinase (M_r, 32,000).

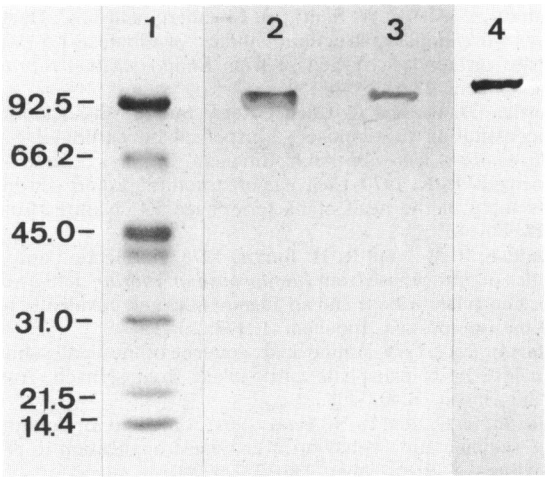

FIG. 4. Identification of monomeric PPDK from *R. rubrum* by immunoblotting. The denatured proteins were separated by SDS-PAGE (5 to 15% polyacrylamide gradient gel), and the proteins in lanes 2 through 4 were transferred to nitrocellulose, probed with maize leaf anti-PPDK antiserum, and visualized with protein A-horseradish peroxidase conjugate. Lanes 1, molecular weight markers (values in kilodaltons) visualized by staining with Coomassie blue R-250; 2 and 3, two different preparations of *R. rubrum* PPDK processed through Blue A (Table 1, step 6); 4, total soluble C_4 leaf extract from *Z. mays*.

places the DEAE-cellulose step before $(NH_4)_2SO_4$ fractionation because this sequence markedly decreases the time and number of steps involved; the HSSF is immediately mixed with DEAE-cellulose without prior desalting, and the 0.2 M KCl batch eluant is then immediately fractionated in a single step and stored for up to 1 month at −80°C as a 0 to 55% $(NH_4)_2SO_4$ precipitate. Unlike the above protocols, which end with molecular sieve chromatography, ours terminates with a rapid negative purification step with Blue A, which yields a 98% recovery and a three- to fourfold increase in specific activity. The utility of this Dyematrex gel was recently reported by Ashton et al. (2) and Budde et al. (7) in the purification of PPDK from the C_4 plant maize (*Zea mays*).

The final specific activity of the dikinase enzyme from *B. symbiosus* has been reported to be ~30 U/mg of protein (11), and that of the isolated C_4 mesophyll chloroplast enzyme has been reported to be ~17 U/mg of protein (2). PPDK purified from *A. aceti* and crude C_4 leaf extracts, however, has reported final specific activities of 4 to 6 U/mg of protein (7, 23, 25). These latter values are in the same range as that reported here for PPDK partially purified from *R. rubrum*. With respect to the bacterial dikinases, that from *B. symbiosus* has been purified 490-fold (11), whereas those from *A. aceti* (23) and *R. rubrum* have been purified only ~65-fold or ~80-fold, respectively.

Perhaps a more interesting comparison between the results described here and data for the related dikinases pertains to the subunit composition and presumed quaternary structure of the PPDK holoenzyme. Although the protein from most sources examined appears to be either a dimer (*B. symbiosus* [10, 11]) or a tetramer (C_4 leaf tissue [25]) of ~94-kDa subunits, the enzyme from *A. aceti* is reportedly a hexamer of 58-kDa polypeptides (23). Clearly, *R. rubrum* PPDK falls into the former grouping, having a monomeric M_r of 92,700. Although the native M_rs estimated for the *R. rubrum* protein by two independent techniques differ, for unknown reasons, by about 75 kDa, neither value is consistent with a tetrameric or hexameric structure. The average native M_r of 208,000 is most consistent with the *R. rubrum* holoenzyme being a homodimer of 92.7-kDa subunits, much like the *B. symbiosus* enzyme (10, 11). This gross structural homology between these two bacterial dikinases is of considerable interest in light of the recent heterologous phosphorylation-inactivation studies with the C_4 leaf PPDK regulatory protein. Burnell (8) reported that PPDK from the nonphotosynthetic bacterium *B. symbiosus* could not serve as an alternate protein substrate for the C_4 leaf regulatory protein, whereas our related studies with the *R. rubrum* enzyme indicate that it is both phosphorylated and inactivated in a qualitatively identical manner as the dikinase from C_4 leaf tissue (9). These comparative findings clearly indicate that more subtle properties than gross quaternary structure must determine whether a given PPDK protein can serve as an effective substrate for the dikinase-specific C_4 leaf regulatory protein.

A final area of comparison related to the quaternary structure of the various PPDK holoenzymes pertains to the effect of the aggregation state of the protein on activity. It is known that the C_4 leaf enzyme is reversibly inactivated in vitro at temperatures below about 12°C (24, 25) and that this inactivation is correlated with the reversible dissociation of the active 387-kDa tetramer into inactive dimers (24). In contrast, our preliminary cold lability studies with the dimeric *R. rubrum* enzyme suggest that this protein is not low-temperature sensitive in that upon transfer from 4°C to 25 to 30°C for 1 h, no increase in activity was observed. Under similar conditions, the activity of the cold-inactivated C_4 leaf enzyme increases by more than 10-fold (25), owing to reformation of the active tetramer (24). Exactly why the

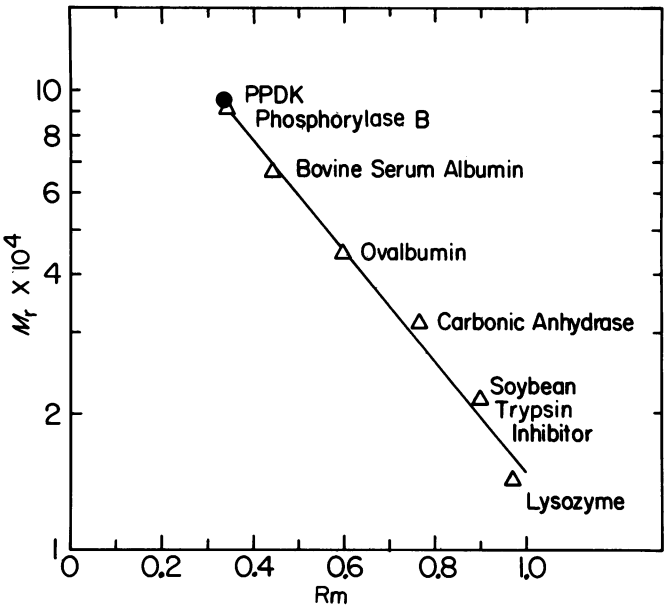

FIG. 5. Estimation of subunit M_r of PPDK from *R. rubrum* (●) by SDS-PAGE. The M_rs are plotted versus the corresponding relative electrophoretic mobilities (Rm) (26). The molecular weight standards (△) included phosphorylase B (M_r, 92,500), bovine serum albumin (M_r, 66,200), ovalbumin (M_r, 45,000), carbonic anhydrase (M_r, 31,000), soybean trypsin inhibitor (M_r, 21,500), and lysozyme (M_r, 14,400). The estimated M_r of the PPDK monomer is 92,700 (average of four different lanes [see also Fig. 1 and 4]).

dimeric dikinases from *R. rubrum* and *B. symbiosus* are catalytically competent, whereas the C_4 leaf dimer is not, is an intriguing structure-function question which awaits future investigation.

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LITERATURE CITED

1. **Andrews, P.** 1964. Estimation of the molecular weights of proteins by Sephadex gel-filtration. Biochem. J. **91**:222–233.
2. **Ashton, A. R., J. N. Burnell, and M. D. Hatch.** 1984. Regulation of C_4 photosynthesis: inactivation of pyruvate, Pi dikinase by ADP-dependent phosphorylation and activation by phosphorolysis. Arch. Biochem. Biophys. **230**:492–503.
3. **Beers, R. F., Jr., and I. W. Sizer.** 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. **195**:133–140.
4. **Benziman, M.** 1975. Pyruvate, orthophosphate dikinase from *Acetobacter xylinum*. Methods Enzymol. **42**:192–199.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
6. **Buchanan, B. B.** 1974. Orthophosphate requirement for the formation of phosphoenolpyruvate from pyruvate by enzyme preparations from photosynthetic bacteria. J. Bacteriol. **119**: 1066–1068.
7. **Budde, R. J. A., G. P. Holbrook, and R. Chollet.** 1985. Studies on the dark/light regulation of maize leaf pyruvate, orthophosphate dikinase by reversible phosphorylation. Arch. Biochem. Biophys. **242**:283–290.
8. **Burnell, J. N.** 1984. Regulation of C_4 photosynthesis: proximity of a histidine involved in catalysis and a threonine involved in regulation of pyruvate, Pi dikinase. Biochem. Int. **9**:683–689.
9. **Ernst, S. M., R. J. A. Budde, and R. Chollet.** 1985. *In vitro* phosphorylation/inactivation of pyruvate, Pi dikinase (PPDK) from *Rhodospirillum rubrum*. Plant Physiol. **77**(Suppl.):46.
10. **Goss, N. H., C. T. Evans, and H. G. Wood.** 1980. Pyruvate phosphate dikinase: sequence of the histidyl peptide, the pyrophosphoryl and phosphoryl carrier. Biochemistry **19**: 5805–5809.
11. **Goss, N. H., and H. G. Wood.** 1982. Covalent chemistry of pyruvate, orthophosphate dikinase. Methods Enzymol. **87**: 51–66.
12. **Hatch, M. D., and C. R. Slack.** 1975. Pyruvate, Pi dikinase from leaves. Methods Enzymol. **42**:212–219.
13. **Janson, C. A., W. W. Smith, D. Eisenberg, and F. C. Hartman.** 1984. Preliminary structural studies of ribulose-1,5-bisphosphate carboxylase/oxygenase from *Rhodospirillum rubrum*. J. Biol. Chem. **259**:11594–11596.
14. **Jordan, D. B., and R. Chollet.** 1985. Subunit dissociation and reconstitution of ribulose-1,5-bisphosphate carboxylase from *Chromatium vinosum*. Arch. Biochem. Biophys. **236**:487–496.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
16. **Ludden, P. W., and R. H. Burris.** 1978. Purification and properties of nitrogenase from *Rhodospirillum rubrum*, and evidence for phosphate, ribose and an adenine-like unit covalently bound to the iron protein. Biochem. J. **175**:251–259.
17. **Martin, P. G.** 1979. Amino acid sequence of the small subunit of ribulose-1,5-bisphosphate carboxylase from spinach. Aust. J. Plant Physiol. **6**:401–408.
18. **Martin, R. G., and B. N. Ames.** 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. **236**:1372–1379.
19. **Milner, Y., G. Michaels, and H. G. Wood.** 1975. Pyruvate, orthophosphate dikinase of *Bacteroides symbiosus* and *Propionibacterium shermanii*. Methods Enzymol. **42**:199–212.
20. **Milner, Y., and H. G. Wood.** 1976. Steady state and exchange kinetics of pyruvate, phosphate dikinase from *Propionibacterium shermanii*. J. Biol. Chem. **251**:7920–7928.
21. **Ormerod, J. G., K. S. Ormerod, and H. Gest.** 1961. Light-dependent utilization of organic compounds and photoproduction of molecular hydrogen by photosynthetic bacteria; relationships with nitrogen metabolism. Arch. Biochem. Biophys. **94**:449–463.
22. **Phillips, N. F. B., N. H. Goss, and H. G. Wood.** 1983. Modification of pyruvate, phosphate dikinase with pyridoxal 5′-phosphate: evidence for a catalytically critical lysine residue. Biochemistry **22**:2518–2523.
23. **Schwitzguébel, J.-P., and L. Ettlinger.** 1979. Pyruvate, orthophosphate dikinase from *Acetobacter aceti*. Arch. Microbiol. **122**:103–108.
24. **Shirahashi, K., S. Hayakawa, and T. Sugiyama.** 1978. Cold lability of pyruvate, orthophosphate dikinase in the maize leaf. Plant Physiol. **62**:826–830.
25. **Sugiyama, T.** 1973. Purification, molecular, and catalytic properties of pyruvate phosphate dikinase from the maize leaf. Biochemistry **12**:2862–2868.
26. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406–4412.
27. **Zurawski, G., B. Perrot, W. Bottomley, and P. R. Whitfeld.** 1981. The structure of the gene for the large subunit of ribulose 1,5-bisphosphate carboxylase from spinach chloroplast DNA. Nucleic Acids Res. **9**:3251–3270.