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Organization of Phosphoribulokinase and Ribulose Bisphosphate Carboxylase/Oxygenase Genes in *Rhodopseudomonas (Rhodobacter) sphaeroides*

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A heterologous phosphoribulokinase (PRK) gene probe was used to analyze two recombinant plasmids isolated from a *Rhodopseudomonas (Rhodobacter) sphaeroides* gene library. These plasmids were previously shown to carry the genes for form I and form II ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBPC/O). Southern blot hybridization analysis indicated that there were two PRK genes linked to the RuBPC/O coding sequences. Restriction mapping showed the arrangement of the duplicate sets of PRK and RuBPC/O to be distinct. Subcloning of the hybridizing PRK sequences downstream of the *lac* promoter of pUC8 allowed expression of the two PRK enzymes in *Escherichia coli*. Analysis of the purified proteins by sodium dodecyl sulfate-slab gel electrophoresis revealed polypeptides with molecular weights of 32,000 and 34,000 corresponding to the form I and form II PRKs, respectively. Preliminary experiments on sensitivity to NADH regulation suggested that the two PRK enzymes differ in catalytic properties.

In most photosynthetic and chemosynthetic organisms, CO_2 assimilation is mediated by the reductive pentose phosphate pathway. Key regulatory enzymes of this pathway include ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBPC/O), which catalyzes the primary CO_2 fixation reaction, and phosphoribulokinase (PRK), which catalyzes the regeneration of ribulose 1,5-bisphosphate, the CO_2 acceptor molecule. In photosynthetic bacteria, most studies have focused on the regulation and synthesis of RuBPC/O, whereas PRK has been largely ignored.

In the genus *Rhodopseudomonas (Rhodobacter)*, the control of CO_2 fixation is complicated by the occurrence of two structurally distinct RuBPC/O enzymes (5–7, 21) that are encoded by separate genes (8, 18, 19). Form I RuBPC/O is composed of eight large subunits and eight small subunits (L_8S_8) (5, 6), and similar to other L_8S_8 enzymes, these polypeptides are the products of the *rbcL* and *rbcS* genes, respectively. In contrast, form II RuBPC/O is composed of large subunits only, and the form II polypeptide is the product of one gene, designated *rbpL* to distinguish it from *rbcL*, the gene which encodes the large subunit of the form I L_8S_8 enzyme. The genes encoding both form I and form II RuBPC/O have been cloned, and fully active and correctly assembled enzymes were expressed in *Escherichia coli* (8, 18, 19). The two RuBPC/O enzymes are subject to independent control in vivo and respond differently to physiological stimuli such as the concentration of CO_2 and the nature of the organic electron donor in the medium (5, 11, 21, 26). In addition, markedly different kinetic and regulatory properties have also been observed in vitro (5, 10). Although the role of the two RuBPC/O enzymes is not clear, a broad range of studies has revealed notable differences in the regulation and expression of the isofunctional proteins. These differences probably reflect the physiological function of the proteins (11, 21, 26). In light of the complexity involved in the regulation of RuBPC/O, the question of how PRK is expressed and controlled in *Rhodobacter* spp. naturally arises.

While examining PRK from *Rhodopseudomonas (Rhodobacter) sphaeroides*, we noted that highly purified PRK preparations from this organism contained two distinct polypeptide chains. The inability to obtain a single polypeptide species led us to suspect that, as with RuBPC/O, there may be two distinct kinases in this organism. In this paper, we show that there are two PRK genes in *R. sphaeroides*, each of which encodes one of the polypeptides purified from extracts of this organism. We also show that the two sets of PRK and RuBPC/O genes are arranged in a unique fashion and that the PRK genes may be expressed in *E. coli* such that highly purified recombinant PRK may be obtained.

MATERIALS AND METHODS

Bacterial strains and plasmids. *R. sphaeroides* HR is a heat-resistant, streptomycin-resistant derivative of NCIB 1853 (28). *R. sphaeroides* WS22 is a lysine auxotroph, kindly provided by William Sistrom (23). *E. coli* HB101 (3) was used for the construction and maintenance of the *R. sphaeroides* gene library (28), and *E. coli* JM107 (29) was used for the identification and expression of *R. sphaeroides* genes cloned into pUC vectors as previously described (8). The plasmids used in this study are listed in Table 1.

Media and growth conditions. *R. sphaeroides* cultures were grown photoautotrophically as described elsewhere (7). *E. coli* was grown aerobically at 37°C in LB medium (4). Supplements were added as needed at the following concentrations: ampicillin, 50 μg/ml; streptomycin, 25 μg/ml; tetracycline, 25 μg/ml; 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), 40 μg/ml; and isopropyl-β-D-thiogalactoside (IPTG), 1 mM in liquid cultures and 0.1 mM in agar plates.

DNA preparation. For routine analysis of subclones, plasmid DNA was isolated from *E. coli* by the sodium dodecyl sulfate (SDS)-NaOH rapid lysis method (9). Large-scale plasmid preparations obtained by the same method were further purified by cesium chloride-ethidium bromide den-

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TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristics	Source or reference
pVK102	Tc^r Km^r *cos*	14
pUC8	Ap^r *lacZ*ΔM15	27
pAEP204	Ap^r pUC9 containing *A. eutrophus* PRK gene within 1.8-kb *Pst*I fragment	B. Bowien
pJG106	Tc^r pVK102 containing form II RuBPC/O gene within 24-kb *Hin*dIII insert of *R. sphaeroides* DNA	8
pJG336	Tc^r pVK102 containing form I RuBPC/O genes within 22-kb *Hin*dIII insert of *R. sphaeroides* DNA	8
pJG7	Ap^r pUC8 containing 3.4-kb *Eco*RI fragment of pJG336	This study
pJG6	Ap^r pUC8 containing 3.4-kb *Eco*RI fragment of pJG7 in opposite orientation	This study
pJG3	Ap^r pUC8 containing 3.8-kb *Eco*RI fragment of pJG106	This study
pJG5	Ap^r pUC8 containing 4.0-kb *Eco*RI fragment of pJG106	This study
pJG8	Ap^r pUC8 containing 3.7-kb *Pst*I fragment of pJG106	This study
pJG9	Ap^r pUC8 containing 3.7-kb *Pst*I fragment of pJG8 in opposite orientation	This study
pRQ52	Ap^r pUC8 containing form II RuBPC/O gene within 3.0-kb *Eco*RI fragment	19

sity gradient centrifugation. The 1.4-kilobase (kb) *Eco*RI-*Sma*I fragment of pJG7, used as a probe in Southern hybridizations, was isolated by electroelution from an agarose gel (8).

Construction of plasmids. Restriction endonucleases and T4 DNA ligase were used according to the instructions of the supplier. For subcloning, DNA fragments derived from complete restriction digests of pJG336 or pJG106 were ligated with pUC8 that had been digested with the appropriate enzyme. Restriction digests of plasmid DNA were used to screen colonies, obtained by transformation of ligation mixes, for insert size and orientation.

Southern hybridization. Nick translation of probes, DNA transfer, and conditions of hybridization were done as described previously (8). Basically, hybridizations were carried out under conditions of moderate stringency at 37°C in 35% formamide, after which the blots were washed extensively in 5× SSC–0.1% SDS (1× SSC is 0.15 M sodium chloride plus 0.015 M trisodium citrate [pH 7.0]) at 55°C.

Purification of PRK. Washed cells (5.5 g) of photoautotrophically grown *R. sphaeroides* HR or WS22 were suspended 1:1 (wt/vol) in TEM (25 mM Tris hydrochloride (pH 7.5)–1 mM EDTA–5 mM 2-mercaptoethanol) and passed twice through a French pressure cell at 12,000 lb/in^2. The broken-cell extract was centrifuged at 12,000 × *g* for 10 min, 1 M $MgCl_2$ was added to the supernatant to a final concentration of 50 mM, and the extract was heated at 50°C for 10 min. Denatured protein was removed by centrifugation as described before (5). The supernatant fraction was diluted 1:5 with TEM, HCO_3^- was added to 10 mM, and the extract was applied to a green A agarose column (Amicon Corp., Lexington, Mass.) equilibrated with TEMMB (TEM plus 10 mM $MgCl_2$ and 10 mM $NaHCO_3$). The column was washed successively with 5 column volumes of TEMMB, 10 mM 6-phosphogluconate in TEMMB, 10 mM ATP in TEMMB, TEM, 10 mM ATP in TEM, and 0.5 M NaCl in TEM. Peak fractions containing PRK activity were pooled and applied to a column of reactive red 120-agarose (Sigma Chemical Co., St. Louis, Mo.) after dialysis or dilution to reduce the ATP concentration. The red-dye column was successively washed with TEM plus 10 mM $MgCl_2$, 10 mM ATP in TEMM, 0.2 M NaCl in TEM, and 10 mM ATP in TEM. PRK-containing fractions were collected and examined by SDS-polyacrylamide gel electrophoresis. The purification of PRK from IPTG-induced cultures of *E. coli* harboring pJG7 or pJG8 was similar to that described for *R. sphaeroides* PRK except that cells were routinely broken by sonication and the HCO_3^- in the loading buffer and the phosphogluconate wash for green agarose chromatography were omitted. Subsequent chromatography through red agarose as described above resulted in highly purified enzyme.

Other analytical techniques. The PRK assay was performed as described elsewhere (25). Protein concentrations were determined by a modification of the Lowry procedure (16). SDS-polyacrylamide slab gels were run by the method of Lugtenberg et al. (15).

RESULTS

Purification of PRK from *R. sphaeroides*. The purification scheme we employed for the isolation of PRK from *R. sphaeroides* is similar to that previously developed for the purification of PRK from the closely related organism *Rhodopseudomonas* (*Rhodobacter*) *capsulata* (25), except that green A agarose was substituted for Affi-gel blue and additional washes were included to allow the simultaneous isolation of form I and form II RuBPC/O from the same extract. Cell extracts prepared from photoautotrophically grown cells were routinely applied to the green A column in buffer containing Mg^{2+} and HCO_3^-. After specific elution of form II RuBPC/O with 6-phosphogluconate, the column was washed with TEM buffer containing ATP and Mg^{2+} and then with TEM alone. No PRK activity was eluted in any of these washes. However, a single peak of PRK activity was eluted with TEM buffer containing ATP in the absence of Mg^{2+}. A final wash of 0.5 M NaCl removed form I RuBPC/O from the column. At this stage in the purification, analysis of fractions containing PRK activity by SDS-polyacrylamide gel electrophoresis revealed two major polypeptides with molecular weights of 32,000 and 34,000 along with several minor polypeptides. Peak fractions containing PRK activity were pooled and chromatographed on DEAE-cellulose and red agarose columns. These steps eliminated the minor polypeptide constituents of the preparation, but the two major polypeptides were found to cochromatograph through these columns. Gradients of either ATP or NaCl applied to green A columns, or agarose A 1.5-m columns for chromatography, did not effect separation of the two polypeptides. In all cases, the elution profile indicated a single protein species, and the ratio of the bands in the doublet remained constant across the peak. Because the specific activity of PRK at this point was 44, similar to that reported for the purified enzyme from *R. capsulata* (25), we considered the possibility that both polypeptides were PRK related, representing either posttranslationally modified and unmodified forms of the same protein or separate gene products as had been demonstrated for the RuBPC/O enzymes of *R. sphaeroides*. To discriminate between these explanations, we examined the

possibility that there might be two PRK genes in *R. sphaeroides*.

Evidence for two PRK genes in *R. sphaeroides*. Plasmid pAEP204 contains a PRK gene isolated from *Alcaligenes eutrophus* (B. Bowien, personal communication). This plasmid was nick translated and used as a probe for homologous PRK sequences in *R. sphaeroides*. As a first step in identifying the PRK gene in *R. sphaeroides*, we considered the possibility that a PRK-coding sequence might be situated near one of the RuBPC/O genes since we had previously isolated plasmids with considerable sequence surrounding the RuBPC/O genes. These two plasmids, pJG336 and pJG106, were isolated from an *R. sphaeroides* gene library and contain form I and form II RuBPC/O genes, respectively, within 22- to 24-kb *Hin*dIII inserts in the broad-host-range vector pVK102 (8). Initial hybridization tests between *Eco*RI restriction digests of plasmids pJG336 and pJG106 and the ^{32}P-labeled PRK probe pAEP204 showed that homologous sequences were present on both plasmids. A 3.4-kb *Eco*RI fragment of pJG336 and a band of approximately 4 kb from pJG106 gave positive hybridization signals when they were probed with pAEP204. For further analysis of the homologous regions in the absence of extraneous DNA, individual *Eco*RI fragments were cloned into the expression vector pUC8. Subcloning also allowed separation of the two 4-kb *Eco*RI fragments of pJG106 that corresponded to the region of the hybridizing band. Plasmids pJG336 and pJG106 were completely digested with *Eco*RI and ligated with *Eco*RI-linearized pUC8. The ligation mixture was used to transform *E. coli* JM107; plasmids isolated from white colonies on IPTG–X-Gal indicator plates were screened for insert size by analysis of restriction digests. The selected recombinant plasmids obtained from this screening were designated pJG7, which contained the 3.4-kb *Eco*RI fragment of pJG336, and pJG3 and pJG5, which, respectively, carried 3.8- and 4.0-kb *Eco*RI fragments of pJG106 inserted into pUC8.

Subcloning of *prkA*. The *A. eutrophus* PRK gene probe hybridized strongly to the 3.4-kb *Eco*RI insert of pJG7, which verified results obtained with the parent plasmid pJG336 (Fig. 1A, lane 2). Hybridization to the 2.7-kb fragment is due to pUC sequences in both plasmids. To examine the homology between pAEP204 and pJG7 in more detail, the plasmid was digested with *Eco*RI and *Sma*I, which cuts at a site 1.4 kb into the insert, and the double restriction digest was probed with the heterologous PRK gene. The strongest hybridization was associated with the 1.4-kb *Sma*I-*Eco*RI frament, and a very faint hybridization band corresponding to the larger *Sma*I-*Eco*RI fragment was observed (Fig. 1A, lane 4). Interestingly, the 3.4-kb *Eco*RI fragment has been shown to contain DNA sequences corresponding to form I RuBPC/O (8). Southern hybridizations have previously positioned the form I RuBPC/O coding region to the right of the *Sma*I restriction site within the insert in pJG7 (Fig. 2), and from expression studies with *E. coli*, it can be inferred that the direction of transcription is from left to right (8). Consequently, the presumptive PRK sequences are located a short distance upstream from the form I RuBPC/O genes. For expression studies with *E. coli*, the 3.4-kb *Eco*RI fragment of pJG7 was obtained in the opposite orientation with respect to the *lac* promoter in pUC8, and this plasmid was designated pJG6. The fragment was further mapped, and additional restriction sites are shown in the map of pJG7 (Fig. 3). The presumptive PRK coding sequence contained within pJG7 is referred to as *prkA*.

Subcloning of *prkB*. When the subclones derived from pJG106 were probed with pAEP204, both pJG3 and pJG5 gave positive hybridization signals. *Bam*HI digests of the two plasmids were probed with the PRK gene to localize more precisely the hybridizing sequences. There are two *Bam*HI sites in each of the *Eco*RI inserts, one located at a distal end and one situated approximately in the middle of each fragment (Fig. 2). The multiple-cloning region of pUC8 provides the third *Bam*HI site, which generates the fragments seen in the restriction pattern in Fig. 1. The autoradiograph (Fig. 1) shows the results of this hybridization. A moderate level of hybridization to a 1.4-kb *Bam*HI fragment of pJG3 and a faint but reproducible signal associated with the 1.8-kb *Bam*HI fragment of pJG5 were consistently observed (Fig. 1A, lanes 6 and 8). Although the hybridizations were not done under conditions of high stringency, the weak hybridization was specific, as no hybridization was observed to the 2.0-kb *Bam*HI fragments of the same plasmids (Fig. 1, lanes 6 and 8) or to the 3.0-kb *Eco*RI fragment of pRQ52 that contained the form II RuBPC/O gene (data not shown). When the *Bam*HI digests of pJG3 and pJG5 were probed with the 1.4-kb *Sma*I-*Eco*RI fragment of pJG7 containing the presumptive form I PRK gene (*prkA*), the hybridization pattern was the same but the signal was stronger (Fig. 1B, lanes 4 and 5). Hybridization of the *prkA* probe to the *A. eutrophus* PRK gene contained within the 1.8-kb *Pst*I fragment (Fig. 1B, lane 6) is shown for comparison. Because the hybridization results with the pJG106 *Eco*RI subclones indicated that *Eco*RI and *Bam*HI sites were within the PRK gene, pJG106 was digested with other restriction enzymes and probed with the 1.4-kb *Sma*I-*Eco*RI *prkA* fragment to find a DNA sequence that would produce a single hybridizing band presumably encompassing the entire PRK gene. Digestion with *Pst*I was chosen for subcloning pJG106, as a single band of 3.7 kb was observed in Southern hybridizations when the sequence was probed with the *prkA* fragment. The *Pst*I fragment was cloned into pUC8 in both orienta-

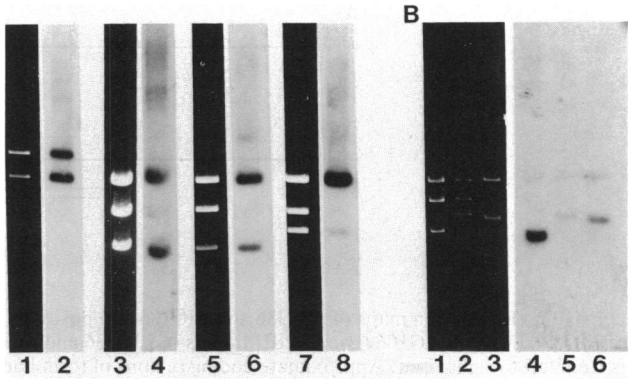

FIG. 1. Restriction and Southern blot analyses of recombinant plasmids containing PRK sequences. DNA fragments electrophoresed through a 0.8% agarose gel were transferred to nitrocellulose filters and hybridized with nick-translated probes. (A) Ethidium bromide-stained agarose gels (lanes 1, 3, 5, and 7) and corresponding autoradiograms after hybridization to ^{32}P-labeled pAEP204 (lanes 2, 4, 6, and 8). The restricted DNAs were pJG7 cut with *Eco*RI (lanes 1 and 2), pJG7 cut with *Eco*RI and *Sma*I (lanes 3 and 4), pJG3 cut with *Bam*HI (lanes 5 and 6) an pJG5 cut with *Bam*HI (lanes 7 and 8). (B) Ethidium bromide-stained gels (lanes 1 to 3) and corresponding autoradiograms after hybridization with the ^{32}P-labeled 1.4-kb *Sma*-*Eco*RI fragment of pJG7 (lanes 4 to 6). The restricted DNAs were pJG3 digested with *Bam*HI and *Eco*RI (lanes 1 and 4), pJG5 digested with *Bam*HI and *Eco*RI (lanes 2 and 5), and pAEP204 digested with *Pst*I (lanes 3 and 6).

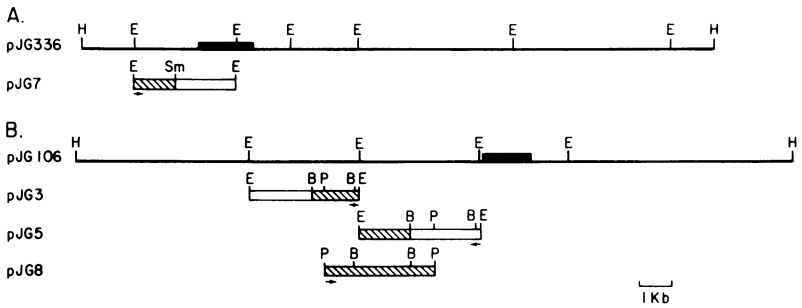

FIG. 2. Restriction maps of pJG336 and pJG106 and positions of PRK subclones. *Eco*RI restriction sites are shown within *Hin*dIII inserts of pJG336 (A) and pJG106 (B). *Hin*dIII inserts of pJG336 and pJG106 are drawn so that the direction of transcription for the RuBPC/O genes is from left to right. ▬, Approximate coding regions of form I and form II RuBPC/O genes; ▭, locations and sizes of individual restriction fragments used in this study; →, direction of transcription from the *lac* promoter within the recombinant plasmids; ▨, regions of homology to pAEP204. The vector pUC8 used in subcloning is not drawn. Additional restriction sites are shown within the fragments relevant for orientation and subcloning purposes. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I, Sm, *Sma*I.

tions, and the resulting subclones were designated pJG8 and pJG9. A restriction map of pJG8 is shown in Fig. 3. The PRK sequence associated with these subclones is referred to as *prkB*.

Localization of PRK genes relative to form I and form II RuBPC/O. Since we knew that the sequences showing homology to pAEP204 within pJG336 were located a short distance transcriptionally upstream from the form I RuBPC/O genes, it was of interest to determine the position of the 3.7-kb *Pst*I fragment relative to the form II RuBPC/O gene within pJG106. Restriction maps of pJG336 and pJG106 were constructed from single and double digests, and the *Eco*RI restriction maps are illustrated in Fig. 2, with the positions of the relevant subclones and hybridizing fragments indicated below the maps. It is interesting to note the position of the PRK homologous sequences within pJG106. The *prkB* sequence is situated 3.5 to 4 kb upstream from the form II *rbpL* gene.

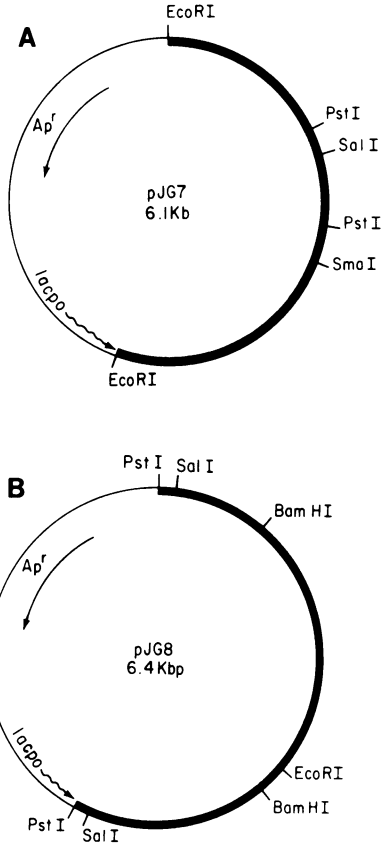

FIG. 3. Restriction endonuclease maps of plasmids expressing PRK in *E. coli*. (A) pJG7 consists of the 3.4-kb *Eco*RI fragment of pJG336 cloned into the *Eco*RI site of pUC8. This fragment carries the *prkA* gene encoding form I PRK. (B) pJG8 carries the 3.7-kb *Pst*I fragment of pJG106 cloned into the *Pst*I site of pUC8. This fragment contains the *prkB* gene encoding form II PRK. The maps show pertinent restriction sites and relevant features of the vector. ▬, *R. sphaeroides* insert DNA; ⇝, direction of transcription from the *lac* promoter; Kbp, kilobase pairs.

Expression of PRK genes in *E. coli*. To verify that the DNA fragments that hybridized to the *A. eutrophus* PRK gene probe carried intact structural genes encoding PRK, it was necessary to express these genes in *E. coli* and identify the corresponding translation products by enzyme assay. To test for expression in *E. coli*, cells transformed with each of the four constructs, pJG6, pJG7, pJG8, and pJG9, were incubated with IPTG to induce transcription from the *lac* promoter in pUC8. Crude extracts for enzyme assays were prepared, and substantial PRK activity was present in extracts of cells harboring pJG7 and pJG8, with 0.074 and 0.162 μmol of ribulose 1,5-bisphosphate produced per min per mg of protein, respectively. Virtually no activity was detected in extracts of cells containing pJG6 or pJG9, in which the DNA inserts are simply in the reverse orientation with respect to the vector promoter. The orientation dependence of PRK expression indicates that transcription is being directed exclusively from the *lac* promoter of pUC8. For simplicity, the PRK activity associated with pJG7 is referred to as form I PRK (*prkA* gene product), and the enzyme encoded by *prkB* in pJG8 is referred to as form II PRK, in concert with their proximity to the corresponding RuBPC/O genes.

Identification of *prkA* and *prkB* gene products. The purification protocol developed for isolation of PRK from *R. sphaeroides* was followed for isolation of the translation products of pJG7 and pJG8 produced in *E. coli*. As already noted for the PRK activity in *R. sphaeroides* extracts, form I and form II PRKs expressed in *E. coli* were eluted from green A columns by ATP in the absence of divalent cation. Analysis of peak fractions eluted from the green-dye column by SDS-polyacrylamide gel electrophoresis showed the presence of multiple bands. Subsequent chromatography through a column of red agarose resulted in relatively homogeneous protein. SDS-polyacrylamide gel electropho-

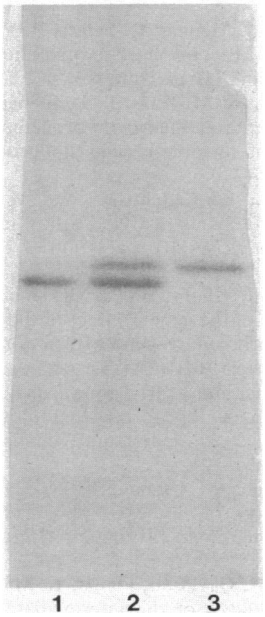

FIG. 4. SDS slab gel of purified PRK preparations. Denatured protein samples were electrophoresed in 12% SDS slab gels and visualized with Coomassie blue. Lane 1, Form I PRK isolated from IPTG-induced cultures of JM107(pJG7); lane 2, a mixture of form I and form II PRKs isolated from *R. sphaeroides* extracts; lane 3, form II PRK isolated from IPTG-induced cultures of JM107(pJG8).

resis of active fractions eluted from the second column revealed a single polypeptide of M_r = 32,000 derived from extracts of pJG7 and a polypeptide of M_r = 34,000 from extracts of pJG8 (Fig. 4). In addition, the two polypeptides exactly comigrated with the lower and upper bands of the doublet associated with PRK activity in *R. sphaeroides* (Fig. 4). The specific activities determined for purified form I and form II PRKs were 50 and 36, respectively. From these results, the two polypeptides originally isolated from extracts of *R. sphaeroides* can be said to represent PRK isozymes that are the products of two genes, the lower-molecular-weight polypeptide corresponding to form I PRK (*prkA*) and the higher-molecular-weight polypeptide corresponding to form II PRK (*prkB*). A similar pattern was obtained after Western (immunoblot) analysis of the *R. sphaeroides* doublet and *prkA* and *prkB* gene products with antiserum against form I PRK (results not shown).

Activation of PRK by NADH. PRK isolated from a variety of bacterial sources has in many cases been shown to be activated by NADH (1, 12, 17, 20, 22, 25, 26). We compared the extent of NADH activation of purified form I and form II PRK isozymes. The results show that form II PRK retains 75% of its activity in the absence of 0.6 mM NADH compared with form I PRK, which exhibits an almost absolute dependence on NADH for activity (10% activity in the absence of NADH).

DISCUSSION

Our initial attempts to purify PRK from *R. sphaeroides* led to ambiguous results. After a variety of chromatographic separations based on size, charge, and substrate affinity failed to separate two polypeptides of M_rs 32,000 and 34,000, we sought evidence at the molecular level to determine which of the polypeptides was the product of a single PRK gene or whether both were. Heterologous hybridization analysis of pJG336 and pJG106 with a PRK probe from *A. eutrophus* strongly suggested the presence of two PRK genes in *R. sphaeroides* that were linked to the form I and form II RuBPC/O genes. Subsequent restriction analysis of pJG336 and pJG106 demonstrated that although PRK and RuBPC/O sequences were clustered, the relative positions and linkages of the carboxylase and kinase genes were somewhat different. In pJG336, *prkA* is located a short distance upstream from the *rbcL* and *rbcS* sequences. Although sequence analysis is necessary to firmly establish the distance between the two coding regions, hybridization data allow a maximum estimate of 1 kb of intervening sequence. Within pJG106, *prkB* is located approximately 4 kb upstream from the *rbpL* gene. The divergent arrangement of the form I and form II gene clusters may be important in regulation of the duplicate sets of CO_2 assimilatory genes. It will be very interesting to examine the intervening regions for open reading frames and potential regulatory signals and to compare flanking sequences to determine the extent of homology throughout these regions.

The reason for the production of isofunctional proteins involved in CO_2 fixation in *R. sphaeroides* is not clear but may reflect the diverse metabolic modes of growth characteristic of the purple photosynthetic bacteria. Duplication of these two key enzymes allows differential activity or stability in a variety of environments. Subcloning of the appropriate restriction fragments of pJG336 and pJG106 into the expression vector pUC8 provided confirmation of the presence of two functional PRK genes based on expression of fully active PRK in *E. coli*. Form I PRK was expressed in an orientation-dependent fashion from a 3.4-kb *Eco*RI fragment derived from pJG336, which suggested that transcription proceeds in the same direction as that of the form I RuBPC/O genes. The polypeptide produced comigrates with the 32,000-M_r polypeptide of the doublet observed in SDS gels associated with PRK activity in *R. sphaeroides*. Form II PRK expression was also dependent on the *lac* promoter of pUC8, and *prkB* appears to be transcribed in the same direction as form II RuBPC/O, even though the genetic regions are separated by almost 4 kb. This vector promoter dependence has been demonstrated for a number of *R. sphaeroides* genes as well as for genes from other organisms containing DNA of high G+C content (8, 18, 19, 24). This dependence probably reflects an inability of *E. coli* RNA polymerase to recognize the promoter sequences of these genes.

The expression of PRK isozymes in *E. coli* provides a means of evaluating various properties of individual enzymes since we have been unable to separate the proteins from extracts of *R. sphaeroides*. Certainly, preliminary characterization of the NADH requirement indicates that form I and form II PRK isozymes may exhibit quite different regulatory features at the enzymatic level. Finally, it is interesting to point out that for *A. eutrophus*, a duplication of RuBPC/O and PRK genes has also been described; one set is chromosomal, and one set is associated with an indigenous megaplasmid (13). In both cases, the gene encoding PRK is located 3.5 kb downstream from the RuBPC/O coding sequence (2).

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LITERATURE CITED

1. **Abdelal, A.T. H., and H. G. Schlegel.** 1974. Purification and regulatory properties of phosphoribulokinase from *Hydrogenomonas eutropha* H16. Biochem. J. **139:**481–489.
2. **Bowien, B., M. Huseman, R. Klintworth, and U. Windhovel.** 1987. Metabolic and molecular regulation of the CO_2-assimilating enzyme system in aerobic chemoautotrophs, p. 21–27. *In* H. W. Van Verseveld and J. A. Ovine (ed.), Microbial growth on C_1 compounds. Martinus Nijhoff Publishers, Dordrecht.
3. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459–472.
4. **Davis, R. W., D. Botstein, and J. R. Roth (ed.).** 1980. Advanced bacterial genetics: a manual for genetic engineering. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
5. **Gibson, J. L., and F. R. Tabita.** 1977. Different molecular forms of D-ribulose-1,5-bisphosphate carboxylase from *Rhodopseudomonas sphaeroides*. J. Biol. Chem. **252:**943–949.
6. **Gibson, J. L., and F. R. Tabita.** 1977. Isolation and preliminary characterization of two forms of ribulose 1,5-bisphosphate carboxylase from *Rhodopseudomonas capsulata*. J. Bacteriol. **132:**818–823.
7. **Gibson, J. L., and F. R. Tabita.** 1985. Structural differences in the catalytic subunits of form I and form II ribulose 1,5-bisphosphate carboxylase/oxygenase from *Rhodopseudomonas sphaeroides*. J. Bacteriol. **164:**1188–1193.
8. **Gibson, J. L., and F. R. Tabita.** 1986. Isolation of the *Rhodopseudomonas sphaeroides* form I ribulose 1,5-bisphosphate carboxylase/oxygenase large and small subunit genes and expression of active hexadecameric enzyme in *Escherichia coli*. Gene **44:**271–278.
9. **Ish-Horowitz, D., and J. F. Burke.** 1981. Rapid and efficient cosmid vector cloning. Nucleic Acids Res. **9:**2989–2998.
10. **Jordan, D. B., and W. L. Ogren.** 1981. Species variation in the specificity of ribulose bisphosphate carboxylase/oxygenase. Nature (London) **291:**513–515.
11. **Jouanneau, Y., and F. R. Tabita.** 1986. Independent regulation of synthesis of form I and form II ribulose bisphosphate carboxylase-oxygenase in *Rhodopseudomonas sphaeroides*. J. Bacteriol. **165:**620–624.
12. **Kiesow, L. A., B. F. Lindsley, and J. W. Bless.** 1977. Phosphoribulokinase from *Nitrobacter winogradskyi*: activation by reduced nicotinamide adenine dinucleotide and inhibition by pyridoxal phosphate. J. Bacteriol. **130:**20–25.
13. **Klintworth, R., M. Husemann, J. Salnikow, and B. Bowien.** 1985. Chromosomal and plasmid locations for phosphoribulokinase genes in *Alcaligenes eutrophus*. J. Bacteriol. **164:** 954–956.
14. **Knauf, V. C., and E. W. Nester.** 1982. Wide host range cloning vectors: a cosmid clone bank of an *Agrobacterium* Ti plasmid. Plasmid **8:**45–54.
15. **Lugtenberg, B., J. Meijers, R. Peters, P. Van der Hoek, and L. Van Alpen.** 1975. Electrophoretic resolution of the major outer membrane protein of *E. coli* into bands. FEBS Lett. **58:**254–258.
16. **Markwell, M. A., S. M. Haas, L. L. Bieber, and N. E. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87:**206–210.
17. **McElroy, R. D., E. J. Johnson, and M. K. Johnson.** 1969. Control of ATP-dependent CO_2 fixation in extracts of *Hydrogenomonas facilis*: NADH regulation of phosphoribulokinase. Arch. Biochem. Biophys. **131:**272–275.
18. **Muller, E. D., J. Chory, and S. Kaplan.** 1985. Cloning and characterization of the gene product of the form II ribulose-1,5-bisphosphate carboxylase gene of *Rhodopseudomonas sphaeroides*. J. Bacteriol. **161:**469–472.
19. **Quivey, R. J., Jr., and F. R. Tabita.** 1984. Cloning and expression in *E. coli* of the form II ribulose 1,5-bisphosphate carboxylase/oxygenase gene from *Rhodopseudomonas sphaeroides*. Gene **31:**91–101.
20. **Rindt, K.-P., and E. Ohmann.** 1969. NADH and AMP as allosteric effectors of ribulose-5-phosphate kinase in *Rhodopseudomonas sphaeroides*. Biochem. Biophys. Res. Commun. **36:**357–364.
21. **Shively, J. M., E. Davidson, and B. L. Marrs.** 1984. Derepression of the synthesis of the intermediate and large forms of ribulose 1,5-bisphosphate carboxylase/oxygenase in *Rhodopseudomoans capsulata*. Arch. Microbiol. **138:**233–236.
22. **Siebert, K., P. Schobert, and B. Bowien.** 1981. Purification, some catalytic and molecular properties of phosphoribulokinase from *Alcaligenes eutrophus*. Biochim. Biophys. Acta **658:**35–44.
23. **Sistrom, W. R., A. Macaluso, and R. Pledger.** 1984. Mutants of *Rhodopseudomas sphaeroides* useful in genetic analysis. Arch. Microbiol. **138:**161–165.
24. **Somerville, C. R., and S. C. Somerville.** 1984. Cloning and expression of the *Rhodospirillum rubrum* ribulose bisphosphate carboxylase gene in *E. coli*. Mol. Gen. Genet. **193:**214–219.
25. **Tabita, F. R.** 1980. Pyridine nucleotide control and subunit structure of phosphoribulokinase from photosynthetic bacteria. J. Bacteriol. **143:**1275–1280.
26. **Tabita, F. R.** 1981. Molecular regulation of carbon dioxide assimilation in autotrophic microorganisms, p. 70–81. *In* H. Dalton (ed.), Microbial growth on C_1 compounds. Heydon & Sons, London.
27. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19:**259–268.
28. **Weaver, K. E., and F. R. Tabita.** 1985. Complementation of a *Rhodopseudomonas sphaeroides* ribulose bisphosphate carboxylase-oxygenase regulatory mutant from a genomic library. J. Bacteriol. **164:**147–154.
29. **Yanisch-Perron, C., J. Vieira, and J. Messing** 1985. Improved M13 cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.