A periplasmic, α -type carbonic anhydrase from *Rhodopseudomonas palustris* is essential for bicarbonate uptake

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Intact cells of the purple non-sulfur bacterium *Rhodopseudomonas palustris* growing anaerobically, but not aerobically, contain carbonic anhydrase (CA) activity. The native enzyme was purified >2000-fold to apparent homogeneity and found to be a dimer with an estimated molecular mass of 54 kDa and a subunit molecular mass of 27 kDa. The CA gene (*acaP*) was cloned and its sequence revealed that it was homologous to α -type CAs. The upstream region of *acaP* was fused to the *lacZ* gene and β -galactosidase activity was measured under different growth conditions. Acetazolamide inhibited purified CA with an IC_{50} in the range of 10^{-8} M, and in the culture media concentrations as low as 30 μ M inhibited phototrophic growth under anaerobic, light conditions when bicarbonate was used. An *acaP::Kan^r* mutant strain was constructed by insertion of a kanamycin-resistance cassette and showed a growth pattern similar to wild-type cells grown in the presence of CA inhibitor. CO₂ gas supplied as an inorganic carbon source reversed the effect of mutation or acetazolamide. CA activity measurements, fusion and Western blot experiments confirmed that CA is expressed under different anaerobic conditions independently of bicarbonate or CO₂ and that there is no expression under aerobic conditions.

Keywords: carbonic anhydrase, inorganic carbon uptake, *Rhodopseudomonas palustris*, periplasmic enzyme

INTRODUCTION

Carbonic anhydrase (CA; EC 4.2.1.1) is a zinc-containing metalloenzyme catalysing the reversible hydration of CO₂. CA is important in biological systems because the uncatalysed interconversion between CO₂ and HCO₃⁻ is slow around neutral pH. Its high efficiency catalysis is fundamental to many biological processes, such as photosynthesis, respiration, pH homeostasis and ion transport (Badger & Price, 1992; Tashian, 1989; Vandenberg *et al.*, 1996). Presently, three distinct classes of CAs are recognized based on amino acid sequence comparisons (Hewett-Emmett & Tashian,

1996). Among these gene families, significant sequence homologies cannot be recognized. CA has been described in photosynthetic organisms, including higher plants, micro- and macroalgae, and cyanobacteria (Burnell *et al.*, 1990; Fukuzawa *et al.*, 1992; Hatch & Burnell, 1990; Kimpel *et al.*, 1983; Price *et al.*, 1992). Some photosynthetic organisms have multiple types of CA enzyme found in different cellular locations that serve different functions in the photosynthetic process. In cyanobacteria and algae, different types of CA have been found. The role of extracellular CA is to improve the efficiency of inorganic carbon (C_i) transport and it is located in the periplasm or in the cell membrane (Price *et al.*, 1992). The function of the intracellular CA in these organisms is to convert HCO₃⁻ to CO₂ for fixation by ribulose-bisphosphate carboxylase-oxygenase (RubisCo) (McKay *et al.*, 1993; Rawat & Moroney, 1995). In plants, intracellular CA enhances the rate of CO₂ to HCO₃⁻ conversion, for fixation by phosphoenolpyruvate carboxylase (Hatch & Burnell, 1990). CA has been proposed to be involved in C_i utilization in

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Abbreviations: AZ, acetazolamide; CA, carbonic anhydrase; C_i, inorganic carbon; PNSB, purple non-sulfur bacterium; RubisCo, ribulose-bisphosphate carboxylase-oxygenase.

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most anaerobic bacteria and archaea (Alber & Ferry, 1994; Kaplan *et al.*, 1991). CA function in processes other than C_i utilization has also been verified in several bacteria. In *Escherichia coli* the *cynT* gene, encoding CA, is part of the cyanate degradation operon. Its physiological role is to prevent depletion of cellular HCO_3^- during cyanate decomposition due to loss of CO_2 (Guilloton *et al.*, 1992). In acetogenic bacteria the function of CA might be to participate in increasing the intracellular CO_2 concentration and to regulate intracellular pH during acetate production (Braus-Stromeier *et al.*, 1997).

Purple non-sulfur bacteria (PNSB) can grow either heterotrophically under aerobic conditions or phototrophically under anaerobic light conditions using bicarbonate or CO_2 as C_i source and hydrogen or organic compounds as electron donors (Imhoff, 1995). They exhibit remarkable versatility in aromatic compound transformations and their use as biocatalysts for bioremediation has been suggested (Harwood & Gibson, 1988). Recently, the use of PNSB for waste treatment and subsequent fixation of CO_2 released during industrial processes was proposed (Brenner *et al.*, 1998). Little information exists with regard to the mechanism of CO_2 utilization in these bacteria, and their C_i uptake mechanism is unexplored. Because CA is known to play an important role in C_i transport and in photosynthetic processes in other organisms, in the present study we intended to verify the presence and the possible function of this enzyme in PNSB. Intracellular, but not periplasmic, CA has been detected in *Rhodospirillum rubrum* (Gill *et al.*, 1984); however, the biological function of this enzyme was not investigated.

Here we report the identification of a periplasmic, α -type CA from *Rhodopseudomonas palustris*. This is the only type found in animals, but it can also be found in the periplasm and the thylakoid membranes of the eukaryotic unicellular green alga *Chlamydomonas reinhardtii* (Coleman *et al.*, 1984; Karlsson *et al.*, 1998) and in the eubacterium *Neisseria gonorrhoeae* (Chirica *et al.*, 1997). In the PNSB *Rhodobacter sphaeroides*, *Rhodobacter capsulatus* and *Rsp. rubrum* β - and γ -type, but not α -type, CAs have been detected by immunoblotting with polyclonal antisera and based on sequence searches of the incomplete genome sequences (Smith *et al.*, 1999). We describe the cloning of CA gene, designated *acaP*, encoding CA protein (AcaP) in *Rps. palustris*.

METHODS

Cultivation of bacteria. *Rps. palustris* No. 7, *Rba. sphaeroides* and *Rsp. rubrum* were cultivated at 30 °C in a minimal medium (Fujii *et al.*, 1983) containing 5 mM 1-propanol and 20 mM $NaHCO_3$ (pH 7.5) under anaerobic conditions using a light incubator when the activity of the strains were compared, when the effect of different concentrations of acetazolamide (AZ; Sigma) was determined and for purification of CA from *Rps. palustris* No. 7. When comparing CA activities using different organic compounds, the same minimal media was used but instead of 1-propanol, 2 mM hexanoic acid or

butylamine was added. When comparing CA activities in different C_i concentrations, 1, 5, 10 and 20 mM sodium bicarbonate and 2 mM 1-propanol was used in minimal media (pH 7.5). When the effect of CO_2 was studied instead of $NaHCO_3$, CO_2 was bubbled into the media (3% CO_2 in argon). Photoautotrophic growth was studied by growing cells on agar plates anaerobically using CO_2 and H_2 gas. Comparison of growth of mutant and wild-type cells was also performed on Petri plates set in a small chamber and filled with CO_2 with or without H_2 at the concentration mentioned above. Aerobic cultivation was performed in van Niel medium (van Niel, 1944) containing 5 mM 1-propanol on a rotary shaker (150 r.p.m.) with or without 20 mM sodium bicarbonate. Fermentative growth of cells was carried out anaerobically in the dark using van Niels minimal medium with 4 mM pyruvate on agar plates.

Sample preparation and enzyme assays. Cells were collected by centrifugation, washed and suspended in 50 mM sodium phosphate buffer (pH 7.2) containing 1 mM zinc sulfate. For measurement of CA of intact cells, 200 ml of this suspension ($15 OD_{660} U ml^{-1}$) was used directly. Cells were treated to obtain the periplasmic and cytoplasmic fractions as previously published (Brandner *et al.*, 1989; Tai & Kaplan, 1985). For total-cell CA activity and for purification of CA from *Rps. palustris* No. 7, cell suspension ($0.4 g$ wet weight ml^{-1}) was passed through a French pressure cell (20000 p.s.i.; 138 MPa) in the presence of DNase I. The cell debris was centrifuged at 16000 r.p.m. for 20 min at 4 °C and the supernatant was used.

CA activity was measured from the pH decrease after the addition of 1 ml CO_2 -saturated water to 20 mM Veronal buffer (pH 8.5) (Wako Chemicals) containing the test material in a total volume of 2.8 ml. Units of enzyme activity (U) were calculated using the equation $U = (t_0 - t)/t$, where t and t_0 represent the time for a pH change from 8.5 to 7.0 with and without sample (Wilbur & Anderson, 1948). Protein concentrations were determined colorimetrically by the method of Bradford (1976) using Bio-Rad dye reagents and standards. β -Galactosidase activity was determined by measuring the initial rate of hydrolysis of ONPG (Malamy & Horecker, 1964). Cytochrome c_2 levels were determined by measuring the difference in the A_{550} between the fully reduced and oxidized forms (Brandner *et al.*, 1989).

Enzyme purification and N-terminal amino acid sequence determination. Cells from 2 l total culture volume were collected and disrupted with a French press. Centrifugation of the cell extract was then performed for 20 min at 16000 g. About 90% of the total CA activity was obtained in the supernatant fraction. Ammonium sulfate was added to the supernatant ($15 g 100 ml^{-1}$) and the mixture stirred for 1 h at 4 °C. After centrifugation for 20 min at 16000 g, the same amount of ammonium sulfate was added and the extract treated as above. The precipitate was collected by centrifugation, dissolved in sodium phosphate buffer (50 mM, pH 7.2 containing 1 mM zinc sulfate) and stored on ice for 2 h. The mixture was loaded onto a *p*-aminomethylbenzene-sulfonamide-agarose (Sigma) (3 ml bed volume) column equilibrated with the same buffer. The column was washed with buffer (20 ml), buffer containing 0.7 M NaCl (30 ml) and 0.3 M sodium azide in the same buffer (30 ml). CA activity was eluted with 0.6 M sodium azide. The eluate was concentrated using a 50 kDa cut-off Amicon column. The concentrated eluate was diluted with phosphate buffer, reconcentrated with the same column and rediluted. The diluted enzyme solution was loaded onto Q-Sepharose (Pharmacia) and the flow-through was collected. This step was repeated using a cation-exchange column, CM-Sepharose (Pharmacia). Under various

pH conditions CA activity would not bind to anion or cation exchange resins. Although CA could not bind to these columns, collections of the flow-through served to purify CA an additional 1.2-fold. Ammonium sulfate was added to 1.7 M and the sample was loaded onto a Phenyl-Sepharose (Pharmacia) column equilibrated with phosphate buffer (50 mM, pH 7.2) containing 1.7 M ammonium sulfate. After a 50 ml washing step (in the same buffer as used in equilibration), the activity was eluted with 1.6 M ammonium sulfate solution. The fractions containing CA activities were dialysed against phosphate buffer and concentrated as described in the affinity chromatography step.

After purification, 8 μ g CA enzyme was transferred onto a nitrocellulose membrane (Millipore) and the N-terminal sequence of the protein was determined commercially by the automated Edman degradation procedure (Takara).

Immunoblot analysis of *Rps. palustris* CA. The soluble cell fractions were analysed by immunoblotting for CA content after separating the proteins on polyacrylamide gels under denaturing (using SDS) or native conditions. Proteins were electrophoretically transferred to nitrocellulose (Millipore). Immunoblotting was performed with mouse antiserum against purified CA and goat anti-mouse antibody-alkaline phosphates conjugate as a detection method, as described previously (Blake *et al.*, 1984).

Cloning and nucleotide sequence determination of *acaP*. Based on the amino acid sequence of the N terminus of CA from *Rps. palustris* (AEGAYHW) the following degenerate primer was synthesized: *acap1*, 5'-GC(CG)GA(AG)GG(CT)-GC(CG)TGGGG-3'. The second primer for PCR was designed according to the highly conserved amino acid motif GSLTTPP among α -type CAs: *acap2*, 5'-CGGCGG(CG)-GT(CG)GT(CG)AG(CG)GA(CG)CC-3'. PCR was carried out in a total volume of 100 μ l with 10 ng *Rps. palustris* genomic DNA, 0.2 mM dNTPs, 2% DMSO and 1 $\times$ *Taq* polymerase buffer with MgCl₂ and 4 U *Taq* DNA polymerase (Takara) for 30 cycles at temperatures of 95 °C for denaturation (1 min), 57 °C for annealing (1 min) and 72 °C for extension (1 min). After agarose gel electrophoresis of the PCR mixture, the desired fragment (516 nt long) was purified with a PCR purification kit (Pharmacia) and cloned into the pGEMT vector (Promega) and sequenced by the dye-terminator method using ABI reagents and protocol (Perkin Elmer). Using the same primers and PCR conditions, a full-length genomic clone was identified using PCR from a *NaeI* genomic library of *Rps. palustris* DNA in the pBluescript SK(II) vector (Stratagene) by screening serial dilutions of pooled plasmids as template. The nucleotide sequence of the whole *NaeI* fragment was determined as described below. The nucleotide sequence and the 255 aa ORF encoding *Rps. palustris* CA, designated *acaP*, is available in the GenBank nucleotide sequence database with the accession number AB022175.

Construction of *acaP* mutant strain. A kanamycin-resistance gene cassette was excised from vector pUC4K (Pharmacia) by *PstI* restriction enzyme cleavage and gel purification, and inserted into the *PstI* site within the coding region of *acaP* (into the 225th nt position). The recombinant fragment was amplified with two specific primers complementary to regions flanking the genomic *NaeI* sites, which also contained *BamHI* sites at their 5' ends. The resulting fragment was digested with *BamHI* and cloned into the pGP-704 vector that had been digested with *BglIII*. Procedures used to obtain the *Rps. palustris* double crossover *acaP::kanR* insertion mutant strain were as described by Inui *et al.* (1999).

Construction of *acaP-lacZ* fusion vector. The coding region of the *lacZ* gene was excised from the plasmid pMC1871 (Pharmacia) with *PstI/BamHI* and cloned into the pBluescript SK (II) multicloning site resulting in the plasmid pBlac. A 500 bp upstream region of *acaP* (from the first Ala codon of the mature CA enzyme) was amplified with primers having *EcoRI* and *PstI* sites, cut with the same enzymes and cloned into pBlac upstream of the *lacZ* gene. The *EcoRI-BamHI* fragment was cut out and cloned into the corresponding sites of pMG103 to produce plasmid pMlaca. pMlaca was electroporated into *Rps. palustris* cells or transformed into *E. coli* cells and the transformants were selected with kanamycin and used in β -galactosidase fusion studies.

DNA sequencing. DNA sequencing was performed using Thermo Sequenase dye-terminators and samples were run on an ABI 373 fluorescent sequencer. All plasmid inserts produced by PCR in this study were confirmed by sequencing.

Materials. All chemicals were purchased from Wako Chemicals unless otherwise mentioned. Goat anti-mouse antibody-alkaline phosphatase conjugate was purchased from Boehringer Mannheim Biochemicals. Restriction endonucleases were obtained from Takara Biochemicals and were used according to the manufacturer's specifications.

RESULTS

CA activity in *Rps. palustris* cells

Protein preparations of *Rps. palustris* cells cultivated under anaerobic conditions exhibited total CA activities from total cell protein [2.95–3.20 U (mg protein)⁻¹], similar to those of other CA-containing bacteria (Alber & Ferry, 1994; Braus-Stromeyer *et al.*, 1997), whereas cells grown aerobically showed little or no activity (<0.1 U) (Table 1). Intact cells showed CA activity

Table 1. CA activities of *Rps. palustris*

Activities of cell extracts cultivated under different growth conditions and substrates. Activity is expressed as U (mg protein)⁻¹.

Condition	Activity
Anaerobic conditions	
Photoheterotrophic light (minimal medium)	
Ethanol + bicarbonate	3.05
1-Propionate + bicarbonate	3.20
1-Butylamine + bicarbonate	3.02
Ethanol + CO ₂	3.08
Photoautotrophic light (minimal medium)	
CO ₂ + H ₂	2.98
Fermentative dark (rich medium)	
Pyruvate	2.95
Aerobic conditions	
Heterotrophic dark (rich medium)	
Ethanol + bicarbonate	0.05
Ethanol – bicarbonate	0.07
1-Butylamine	0.03
Ethanol (minimal medium)	0.07
Pyruvate (rich medium)	0.08

Table 2. Purification of CA from *Rps. palustris*

Purification step	Volume (ml)	Protein (mg)	Total activity (U)	Specific activity (U mg ⁻¹)	Recovery (%)	Purification (fold)
Total cell extract	100	1575	4798	3.05	100	1.0
Soluble fraction	98	1160	3980	3.43	83	1.1
Ammonium sulfate fraction	80	710	3360	4.73	70	1.6
Affinity column	15	1.5	2845	1896.7	59	621.9
Ion-exchange column	20	1.03	2342	2276.7	49	746.5
Phenyl-Sepharose column	15	0.29	1818	6269.0	38	2055.4

[0.6 U (OD₆₀₀ U bacteria)⁻¹] due to periplasmic or membrane-bound CA enzyme. Activity was measured from cells that were cultivated under different anaerobic conditions: fermentative dark conditions using pyruvate as carbon source, photoheterotrophic light conditions using different organic compounds and CO₂ or bicarbonate as the C₁ source, photoautotrophic light condition using H₂ and CO₂. Cells that were cultivated under aerobic heterotrophic conditions with or without bicarbonate in the media showed no activity. This result did not depend on the applied organic carbon source (Table 1).

Enzyme purification

Under a variety of conditions, CA did not bind to cation- or anion-exchange columns. However, these steps were useful to remove certain contaminants. Major purification was achieved with affinity chromatography. Strong binding of the enzyme to the *p*-aminomethyl-benzenesulfonamide-agarose column was observed and this is consistent with the high affinity of CA for AZ. The enzyme was purified more than 2000-fold, with an overall recovery of 38 % (Table 2). The specific activity of the purified enzyme (6269 U mg⁻¹) is comparable to that of commercially available human erythrocyte isozyme II (7870 U mg⁻¹).

Properties of the purified enzyme

The enzyme appeared as a single band of about 27 kDa on a 4–20 % gradient SDS polyacrylamide gel after gel electrophoresis and Coomassie brilliant blue staining (Fig. 1a). On a native gel (acrylamide gradient 4–20 %) CA also gave a single band (Fig. 1b). To examine the presence of CA enzyme in cells cultivated under different conditions and for localization studies, mouse polyclonal antisera were raised against the purified CA from *Rps. palustris*. As shown in Fig. 2, the antibodies detected a polypeptide of the same size as CA in a Western blot analysis and could detect very small amounts of purified CA protein. When antiserum was

Fig. 1. Denaturing (a) and native (b) PAGE of CA from *Rps. palustris*. The molecular mass of the markers is indicated. One microgramme protein from the Phenyl-Sepharose chromatography step was applied.

used against whole-cell extracts, no other protein showed reactivity, thus it seems that the antiserum is specific to CA from *Rps. palustris*.

The migration of the purified enzyme was analysed on a series of denaturing and native gels containing different polyacrylamide concentrations and the apparent size of the enzyme was determined by the method of Ferguson (1964). Under denaturing conditions the estimated molecular mass of CA is about 27 kDa while under native conditions the molecular mass is approximately 54 kDa, suggesting that the native enzyme is a dimer. The NH₂ terminus of the purified CA protein was determined by the automated Edman degradation pro-

Fig. 2. Immunoblot analysis of protein extracts from *Rps. palustris* cultivated under different conditions. Lane 1 contains purified CA as a control. Under photoautotrophic (lane 2) and photoheterotrophic (lanes 3 and 4) conditions, cells were cultivated in minimal media at 35 °C in the light using bicarbonate or CO₂ as the C_i source as indicated. Under fermentative conditions (lane 5) cells were grown in the dark in an anaerobic chamber using pyruvate in rich media at 35 °C. When cells were cultivated under heterotrophic conditions in the presence of oxygen (lanes 6, 7 and 8), the same media, and organic and inorganic carbon compounds were used as in the case of the corresponding photoheterotrophic conditions (lanes 3, 4 and 5). Aerobic growth in the dark was at 35 °C with shaking. Blotting was performed with mouse antiserum against purified CA and goat anti-mouse antibody-alkaline phosphatase conjugate as a detection method.

cedure using a band isolated from a native gel. NH₂-terminal analysis of the first 12 aa revealed a single homogeneous sequence (AEGAYHWGYEGE), so the CA dimer is probably composed of identical subunits.

We determined the IC₅₀ values (concentration required for 50% inhibition of activity) of several inhibitors of CA reported from studies in other organisms (Maren & Sanyal, 1983; Sugrue, 1996) (N₃⁻, 6.0 × 10⁻³ M; Cl⁻, > 10⁻² M; AZ, 3.1 × 10⁻⁸ M). AZ was a more effective inhibitor of this CA than of other bacterial CAs (Braus-Stromeyer *et al.*, 1997; Yu *et al.*, 1992). The values obtained here are more characteristic of human CAs and other CAs from the α family (Armstrong *et al.*, 1966; Lindskog *et al.*, 1971; Price *et al.*, 1992). Monovalent ions were significantly less effective than for some bacterial CAs (Braus-Stromeyer *et al.*, 1997; Yu *et al.*, 1992). The enzyme also has an esterase activity detected with *p*-nitrophenylacetate as the substrate (data not shown).

Cellular localization of CA

Rps. palustris is a Gram-negative bacterium and contains two cellular compartments, the cytoplasm and the periplasm. CA activity was found only in the periplasmic space (the cytoplasmic fraction exhibited 0.49 U, while the periplasmic fraction showed 11.5 U). The purity of the periplasmic extract was evaluated by

using marker enzymes with known localizations. We chose cytochrome *c*₂ as the periplasmic, and β -galactosidase as the cytoplasmic marker. Fractionation and measurements of marker enzyme activities were performed as published elsewhere (Brandner *et al.*, 1989; Tai & Kaplan, 1985). In the cells grown under photosynthetic conditions, cytochrome *c*₂ and CA were found only in the periplasmic fraction (997 pmol Cyt *c* in the periplasm, and 173 pmol Cyt *c* in the cytoplasmic fraction), while β -galactosidase activity appeared only in the cytoplasmic fraction (260 Miller U in the cytoplasm and 38 Miller U in the periplasmic fraction). Since wild-type *Rps. palustris* No. 7 strain does not show β -galactosidase activity, we constructed a plasmid which contains the *E. coli lacZ* gene using an *Rps. palustris* shuttle vector (Inui *et al.*, 2000). To examine the localization of CA by another method we used immunoblot analysis. We used specific antisera against the protein fractions from the periplasm and the cytosol. We were able to confirm the periplasmic location of CA as observed with enzymic assays (data not shown).

Cloning and sequence data comparison of CA gene, *acaP*

Based on the sequence of the first 12 NH₂-terminal amino acid residues of the purified CA and from studies of inhibitor specificity, we assumed that *Rps. palustris* CA belongs to the α class. Two degenerate primers were designed for PCR based on the first NH₂-terminal 8 aa and on one internal conserved sequence region present in all α -CAs (Hewett-Emmett & Tashian, 1996) (see Methods). PCR was used to amplify a DNA fragment of 516 bp which was cloned and sequenced. Nucleotide sequence similarity comparisons showed that this fragment had significant homology to other α -CAs in the database. To identify an *E. coli* clone having the full-length *Rps. palustris* CA gene, PCR amplification was used to screen a *NaeI* genomic library from *Rps. palustris* in pBluescript SK II. A 1443 bp *NaeI* fragment was found to contain the full-length CA gene. From the DNA sequence and the NH₂-terminal amino acid determination we assume that there is a 25 aa signal sequence which is responsible for the periplasmic export of the enzyme. A positively charged part in the amino terminus of the possible signal motif and a following hydrophobic core are recognized characteristics of bacterial signal peptides (von Heijne, 1984). The 18 residue hydrophobic region ends in the +1, -1 AXA motif characteristic of the recognition site of signal peptidase (Perlman & Halvorson, 1983). The protein is thus predicted to be made as a 27,302 Da precursor which is cleaved between Ala-25 and Ala-26 to produce a mature 230 residue protein of 24772 Da. The putative initiation codon (ATG) is preceded by a possible ribosome-binding site sequence (GGAGA), 5 nt upstream. Downstream from the ORF is a possible rho-independent transcription terminator. The nucleotide sequence and the ORF, designated *acaP*, encodes the 255 aa *Rps. palustris* CA. The deduced amino acid

Fig. 3. (a) Effects of different concentrations of AZ on photoheterotrophic growth of wild-type *Rps. palustris* cells. Without AZ, ●; with 60 μM AZ, ■; 30 μM AZ, ▲. The growth pattern of the *acaP* mutant (○) without the inhibitor under the same conditions using bicarbonate as the C_i source and ethanol as the organic carbon compound is also shown. Cells were incubated at 35 °C in the light in minimal medium. (b) Photoheterotrophic growth of the wild-type (▲) and mutant (△) cells in the presence of CO₂ and of the wild-type (●) and mutant cells (○) in the presence of bicarbonate. (c) The effects of pH on photoheterotrophic growth; wild-type (●) and mutant cells (○) at pH 7; wild-type (▲) and mutant cells (△) at pH 6 using the same conditions as in (a).

sequence of *acaP* was aligned with the sequences of CA from *Anabaena*, *Synechococcus*, *Erwinia carotovora*, *Klebsiella pneumoniae*, *N. gonorrhoeae* and human CA isozymes I and II. *Rps. palustris* CA showed the highest homology to bacterial CAs (35.7–45.7% identities). Comparing *Rps. palustris* CA with the two human CA isozymes I and II, the identities were 33% and 34.6%, respectively. The highest identity was found with the β -*Proteobacterium* *Neisseria* (42.9%) and the γ -*Proteobacteria* *Erwinia* (45.7%) and *Klebsiella* (42.9%).

Regulation of *acaP* expression

CA activity was measured and was the same when cells were grown under different anaerobic conditions: photoheterotrophic light, photoautotrophic light or fermentative dark conditions. In the presence of oxygen, CA activity could not be detected using the media listed in Table 1. The presence of a CA band as demonstrated by immunoblot analysis under each condition (Fig. 2) was in agreement with the activity data. The enzyme was detected under different anaerobic conditions. No difference was detected in the amount of CA enzyme in cells grown in low and high concentrations of bicarbonate under different anaerobic conditions (data not shown).

To investigate the transcriptional regulation of *acaP*, an *acaP::lacZ* fusion was constructed. The non-coding, upstream region with the proposed translation initiation signals of *acaP* was fused translationally in-frame to the first codon of *lacZ*. In *E. coli* the β -galactosidase activity remained unchanged when cells were shifted from aerobic to anaerobic conditions, while a dramatic

change was observed in the case of *Rps. palustris*, where activity was more than 20-fold higher when cells were incubated in the absence of oxygen (0.9 compared to 19.7 Miller U). Expression of β -galactosidase occurred within several hours and cells plated in the presence of X-Gal became blue when examined on LB plates containing the indicator. In *E. coli* the expression of *acaP* gene seemed to be constitutive compared to expression from the native *lac* promoter, and the strength of the *acaP* promoter was approximately 13-fold weaker than the *lac* promoter both in *E. coli* and in *Rps. palustris*. The immunoblotting and β -galactosidase fusion studies suggest that CA expression is regulated at the transcriptional level.

Inhibition of phototrophic growth by CA inhibitors

Since AZ was an effective *in vitro* inhibitor of CA, we studied the effect of AZ on the growth of *Rps. palustris* under anaerobic light conditions. Preliminary studies showed that CA activity could be completely inhibited in intact cells using 0.2 mM AZ. Lower concentrations of AZ inhibited photosynthetic growth in a concentration-dependent manner when bicarbonate was used as the C_i source (Fig. 3a). Even higher concentrations of AZ than these had no effect on aerobic growth (data not shown). These results are in good agreement with the observation that extracts from cells harvested under aerobic conditions exhibited no CA activity. Cells showed CA activity when grown under photoautotrophic and fermentative conditions. When CO₂ was used instead of bicarbonate under photoheterotrophic conditions, AZ had no inhibitory effect on growth (data not shown).

Insertion mutation of *acaP* and characterization of the mutant strain

To obtain more information regarding the physiological role of CA, we made a mutant strain of *Rps. palustris* by insertion of a kanamycin-resistance cassette into the coding region of *acaP*. The single-crossover mutants showed resistance to another antibiotic, gentamicin, while strains that displayed only kanamycin resistance were double-crossover mutants and these were selected for further analysis. The homologous recombination event was confirmed by PCR and Southern blotting (data not shown). The mutant strain showed a similar growth pattern to wild-type cells cultivated in the presence of 60 μ M AZ in minimal medium containing bicarbonate as the C_i source (Fig. 3a). Phototrophic growth was inhibited for several days when bicarbonate was used. The very slow growth can be explained by the slow conversion of bicarbonate to CO_2 without catalysis at close-to-neutral pH in liquid media. Using CO_2 with different organic compounds or with H_2 , no difference in growth was observed between the wild-type and the mutant cells using solid media in small anaerobic chambers (data not shown).

Effect of CO_2 and pH on *acaP* mutant and wild-type cells

When cell growth was arrested by AZ under phototrophic conditions using bicarbonate, the inhibitory effect could be diminished by bubbling CO_2 gas into the media (data not shown). An *acaP* mutant strain could also grow when the media was supplemented with CO_2 (Fig. 3b). The same effect could be seen when the pH of the media was lowered with buffers. Although wild-type cells grew more slowly in lower pH (pH 5–6) media, mutant bacteria showed increased growth, probably due to the increased CO_2 conversion from bicarbonate (Fig. 3c).

DISCUSSION

One report on CA from the PNSB has so far appeared where a cytoplasmic CA from *Rsp. rubrum* was purified and some features of the enzyme were studied (Gill *et al.*, 1984). The physiological role of this enzyme was not studied, nor was a periplasmic location established. In this report, CA from *Rps. palustris* is studied in detail. We have found that intact cells exhibit CA activity when cultivated under photosynthetic growth conditions. Enzyme activity could be localized mainly to the periplasmic space and this was confirmed by using cytochrome c_2 and β -galactosidase enzymes as control markers localized to the periplasm and cytoplasm, respectively. CA activity was unchanged when cells were grown under different anaerobic conditions: photoheterotrophic light, photoautotrophic light or fermentative dark conditions. Under photoheterotrophic conditions, the levels of CA are not regulated in response to the growth substrate, and enzymic activities are similar in alcohol, organic acid or alkylamine-grown cells. No correlation was established between CA activity and the

concentration of C_i in the media. Little [<0.1 U (mg protein) $^{-1}$] or no activity was detected when cells were cultured under aerobic conditions.

CA protein structural relationships

Cloning of the *acaP* gene and determination of the amino acid sequence demonstrates that *Rps. palustris* CA is homologous to α -type CAs. Stronger homology and other distinctive features characteristic of bacterial CAs of the same family were recognized, which suggest a common molecular structure and also a role in the localization of the enzyme. The NH_2 terminus of all bacterial CAs contains a possible signal sequence and the AXA signal peptidase recognition motif can be seen in all of the cases except in the *Klebsiella* enzyme. Four distinct deletions in the sequence can be seen when compared to human CA isozymes I and II (data not shown). Three of them are located in the regions that connect β strands D, E and F, which are central characteristics of the dominating structural element, the 10-stranded β sheet of all α -CAs (Eriksson *et al.*, 1988). A fourth gap is located close to the C terminus and might result in a shorter corner loop which is longer in the human isoenzymes molecular periphery region (Eriksson *et al.*, 1988). Thirty-six amino acids are found to be highly conserved among all α -CAs. Three histidines (His-94, His-96, His-119), which are the three zinc ligands in the enzyme, as well as other conserved amino acids (Gln-92, Glu-117, Ala/Val-121, Val-143) are unchanged. Interestingly, there are two positions where all bacterial CAs have cysteine residues (Cys-53 and Cys-200; *Rps. palustris* numbering) while human isozymes have alanine and leucine in those positions. In the case of the newly discovered α -type CA from the thylakoid membrane of *C. reinhardtii*, cysteines are also found in these positions (Karlsson *et al.*, 1998); this may suggest a close evolutionary relationship to bacterial CAs.

Localization and expression of CA

CA and RubisCo are associated in the carboxysomes of cyanobacteria and possibly in the pyrenoids of algae (Price *et al.*, 1992). The carboxysomes are postulated to contain a specialized CA at the active site to provide CO_2 . It is also possible that intracellular CA aids in the conversion of CO_2 to HCO_3^- for fixation by phosphoenolpyruvate carboxylase (Hatch & Burnell, 1990). Both biochemical pathways are found in photosynthetic bacteria (Buchanan *et al.*, 1967; Inui *et al.*, 1997; Tabita, 1995) although carboxysomes are not found in PNSB and our results suggest that CA enzyme is not associated with RubisCo, because the main CA activity was found in the periplasmic fraction.

Periplasmic localization and the presence of the enzyme only under anaerobic conditions suggests that CA participates in C_i utilization in this bacterium. We also tested two other PNSB, *Rba. sphaeroides* and *Rsp. rubrum*, and found the same phenomenon (data not shown). This suggests that an α -type CA exists in these PNSB as well. We cannot exclude the presence of other

intracellular accumulation systems for C_i , because when different C_i concentrations were used under phototrophic conditions, the expression and activity of the CA studied here did not change. Other C_i transport proteins probably act under phototrophic conditions, either in cooperation with CA or independently. These aspects of the uptake mechanism await clarification.

Anaerobic induction and transcriptional regulation of the *acaP* gene is suggested by the analysis of the *acaP::lacZ* fusion. A 20-fold change in β -galactosidase activity was observed in the case of *Rps. palustris*, when cells were incubated anaerobically. Induction occurred within several hours and was also detected on plates where the cells became blue in the presence of X-Gal. In *E. coli*, the β -galactosidase activity remained unchanged when cells were shifted from aerobic to anaerobic conditions, suggesting that the anaerobic regulation of CA expression is specific for *Rps. palustris*. In *E. coli*, β -galactosidase expression from the *acaP-lacZ* fusion gene was much weaker than in the case of the full length *lacZ* gene with its own promoter. These results suggest that the promoter of *acaP* is quite weak and may be regulated at the transcriptional level by oxygen via an oxygen-responsive transcriptional regulatory system as found in other bacteria (Monson *et al.*, 1995; Uden & Schirawski, 1997). However, FixL-like and Fnr-like recognition sequences found in other oxygen regulatory systems could not be discerned. It is also possible that the expression of CA is controlled by a global regulatory system, like the one recognized to control aerobic and anaerobic CO_2 metabolism in *Rsp. rubrum* and *Rba. sphaeroides*. In these bacteria the RegA/RegB system was found to regulate the aerobic and anaerobic expression of genes encoding enzymes of primary and alternative CO_2 fixation and integrates photosynthesis and nitrogen fixation (Qian & Tabita, 1996; Joshi & Tabita, 1996). However, a RegA-like consensus recognition motif (Du *et al.*, 1998) was not found in the sequence of *acaP*, nor upstream of the coding region, which suggests that a different regulatory system is involved.

Physiological role of CA

In PNSB, CA has an important role under anaerobic growth conditions when bicarbonate is the C_i source. Growth inhibition could be detected by inhibiting the extracellular CA of *Rps. palustris* by the specific CA inhibitor AZ, and a growth defect was observed in the *acaP* mutant. Growth of wild-type cells in the presence of AZ or the *acaP* mutant was slowed by several days but this inhibitory effect could be largely reversed using CO_2 , or partially reversed when the pH of the media was lowered and the non-enzymic conversion of bicarbonate to CO_2 is much faster. From these results we assume that the cell membrane is a barrier to the bicarbonate ion, but that CO_2 can penetrate by diffusion into the cytoplasm. The function of CA in *Rps. palustris*, and probably in other PNSB, is to accelerate the bicarbonate- CO_2 conversion in the periplasmic space. This process might

be analogous to CA function in cyanobacteria and algae; however, intact cells of cyanobacteria exhibit very low CA activity and inactivation of the cyanobacterial *ecaA* gene, which encodes extracellular CA, did not give a dramatic change in photosynthetic growth (Soltes-Rak *et al.*, 1997). Although there are slight differences in periplasmic CA activities, CA seems to function universally in C_i utilization in photosynthetic microorganisms.

We believe that this work contributes to a basic understanding of the key reactions in C_i utilization and of the nature of anaerobic/aerobic gene regulation in *Rps. palustris*. We also hope that this knowledge will prove useful in efforts to improve CO_2 fixation and photosynthetic ability in this species for a variety of biotechnological applications.

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