Amino Acid Sequence Homology between N- and C-terminal Halves of a Carbonic Anhydrase in Porphyridium purpureum, as Deduced from the Cloned cDNA*

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Carbonic anhydrase (CA) from Porphyridium purpureum, a unicellular red alga, was purified >209-fold to a specific activity of 1,147 units/mg protein. cDNA clones for this CA were isolated. The longest clone, comprising 1,960 base pairs, contained an open reading frame which encoded a 571-amino acid polypeptide with a calculated molecular mass of 62,094 Da.

The N- and C-terminal halves of the putative mature Porphyridium CA have amino acid sequence homology to each other (>70%) and to other prokaryotic-type CAs. Both regions contain, at equivalent positions, one set of three possible zinc-liganding amino acid residues conserved among prokaryotic-type CAs. CA purified from Porphyridium contained two atoms of zinc per molecule. We propose that the Porphyridium CA has evolved by duplication of an ancestral CA gene followed by the fusion of the duplicated CA gene. The CA truncated into the putative mature form was overexpressed in Escherichia coli, and the expressed protein was active. Clones expressing separately the N- and C-terminal halves of the CA were constructed. CA activity was present in extracts of E. coli cells expressing the N-terminal half, while no detectable activity was found in cells expressing the C-terminal half.

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‡ The abbreviations used are: CA, carbonic anhydrase; low-CO2 cells, algal cells grown in air (containing 0.04% CO2); high-CO2 cells, algal cells grown in air containing 1–5% CO2; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; kb, kilobase(s); bp, base pair(s).

Experimental Procedures

General Procedures—Restriction enzyme digestion, transformation, and other standard molecular biological techniques were carried out as described by Sambrook et al. (17). Restriction and DNA-modifying enzymes were obtained from Takara Shuzo Co., Ltd. (Kyoto, Japan) and Toyobo Co., Ltd. (Osaka, Japan).

Cells and Culture Conditions—Cells of P. purpureum R-1 (formerly
named *P. cruentum* R-1, Culture Collection of the Institute of Molecular Cell Biology, University of Tokyo, were grown as described by Yasuwa *et al.* (15) except that the culture medium was buffered with 10 mM TES-NaOH (pH 7.5). To obtain low-CO2 cells, the culture medium with 50 mM Tris-HCl (pH 8.5) containing 80 mM NaClO4. The fraction (Hybond-N method. Plaque hybridization was carried out using nylon membrane from 8.3 to 7.3 (was run at ambient temperature. The precipitate obtained at 30–65% saturation was resuspended and dialyzed against 5 mM Tris-HCl (pH 9.5). The dialysate was charged onto an anion-exchange column of DEAE-Sepharose 6B which was prepared according to Yang *et al.* (18) and pre-equilibrated with 5 mM Tris-HCl (pH 9.5). After extensive washing with 5 mM Tris-HCl (pH 8.5), the fraction with CA activity was eluted with 50 mM Tris-HCl (pH 8.5) containing 80 mM NaClO4. The flow rate of 1.0 ml/min, and fractions containing CA activity were collected and kept at 4°C. All purification procedures were carried out at 4°C, with the exception of anion-exchange chromatography, which was run at ambient temperature.

The CA activity was measured as the time needed for a pH change from 8.3 to 7.3 (t) after addition of 2 ml of CO2 saturated water to 12 ml sodium 5,5-diethylbarbiturate-HCl buffer (pH 8.3) containing an enzyme solution and 5 mM NaCl (final concentration in a total volume of 5 ml). The reaction was carried out at 2°C. An enzyme activity unit was calculated using the equation, unit = t/t, where t is the time required for the pH change using buffer without enzyme. SDS-PAGE was carried out according to the method of Laemmli (19) using 12.5% gel. Protein concentrations were determined by the method of Bradford (20) using Bio-Rad dye reagents and bovine serum albumin as a standard.

**Plasmid Construction for Expression of CA in E. coli**—In order to construct a plasmid expressing a truncated CA (encoded in pPCA1, the cloned cDNA) as a fusion peptide, cDNA encoding the N-terminal portion of the CA was amplified by PCR using two primers as follows: M1, 5’-GGGGAAAGCTT-GAAGCTCAGCCGGCAGCTGAG-3’; M2, 5’-GTCAGAAGCTT-GCTACCC-3’. M1 partially corresponds to the sense strand bases from 229 to 248, determining the N-terminal end of the putative mature CA. M2 corresponds to the antisense strand bases flanking a HindIII site inside the cDNA. M1 was tagged by the HindIII cleavage site, therefore the amplified product was cleaved by HindIII into a segment of 0.8-kb. The 1.0-kb HindIII-HindIII fragment of plasmid pUC119 containing the entire cDNA insert was replaced by the 0.8-kb HindIII fragment and the absence of base substitution in the amplified region was confirmed by a DNA sequence analysis. The resultant plasmid containing the 0.8-kb HindIII fragment in correct orientation, encodes 7 amino acid residues of LacZ fused to the CA lacking 76 amino acid residues at the N-terminal. The plasmid called pPCA was used to transform *Escherichia coli* JM109 cells.

**Plasmid Constructions for the Expression of the N- and C-terminal Halves of the CA**—As described under “Results,” the *Porphyridium* CA consists of homologous N- and C-terminal halves, each of which exhibits a sequence similarity to other prokaryotic-type CAs. To construct plasmids for the expression of the N- and C-terminal halves, EcoRI and HindIII sites were introduced into the two halves by using PCRs. The primers used are as follows: N1, 5’-CTGGTCTCTGAGTACGGG-3’; N2, 5’-GTCCGACAACGTGATTTGTCCGTCAGGATAGCTTT-3’; N3, 5’-GTCAGAAGCTT-GCTAAGGATGTTCAAGGA-3’; C1, 5’-CTCCAGATGTTTCTAACCAGGAGCTCAATTT-3’; C2, 5’-CTTACACTTGGATTGTACACGGG-3’. N1 and N2 correspond to the sense and antisense strands, bases from 557 to 574 and partial bases from 927 to 951, respectively, while C1 and C2 correspond to the sense and antisense strands, partial bases from 943 to 966 and bases from 1333 to 1356, respectively. N2 and C1 contain nucleotide mismatch to create EcoRI and HindIII site. First, two PCRs were performed either with N1 and N2 or with C1 and C2. Next, using a mixture of the products of these two PCRs, the second PCR was carried out with N1 and C2, and the product was cloned into a pCR vector (pCR-EH). The introduction of EcoRI and HindIII restriction sites and the absence of mutation in the amplified region were confirmed by a DNA sequence analysis. The plasmid pCR-EH digested with PrA1 and the 0.8-kb fragment was gel-purified and ligated into the PrA1-cleaved pPCA plasmid. The resultant plasmid containing the pCR-EH-derived 0.8-kb PrA1 fragment in correct orientation was designated as pPCA-EH.

For expression of the N-terminal half (molecular mass approximately 28.1 kDa) of the CA, the 0.9-kb EcoRI-EcoRI fragment of pPCA-EH encoding the C-terminal half was replaced by the omega fragment (23). The omega fragment terminates transcription and translation coming into the region at the both ends (23). The resultant plasmid called pPCA was used to transform *E. coli* strain JM109. For the expression of the C-terminal half (molecular mass approximately 28.9 kDa) of the CA, the pPCA-EH plasmid was digested with HindIII and the 4.1-kb fragment was self-ligated, and the resultant plasmid called pPCA was used to transform *E. coli* strain JM109.

**Expression and Measurement of the Activity of CA and Polypeptides of N- and C-terminal Halves**—*E. coli* clones containing a control plasmid lacking the insert, pPCA, pNCA, or pCWA were grown overnight, diluted into fresh Luria-Bertani broth containing 100 μg of ampicillin/ml, and grown at 37°C until the cultures reached an A600 of 0.2. Subsequently, the cultures were incubated in the presence or absence of
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TABLE I

| Steps                | Total activity | Protein | Specific activity | Recovery | Purification |
|----------------------|----------------|---------|------------------|----------|--------------|
| Cell extract         | 1,369          | 248.90  | 5.5              | 100      | 1            |
| (NH₄)₂SO₄ 30–65%     | 703            | 80.80   | 8.7              | 51       | 1.6          |
| saturated fraction   |                |         |                  |          |              |
| Affinity             | 402            | 3.39    | 118              | 29       | 21           |
| DEAE-5PW             | 368            | 0.32    | 1147             | 27       | 209          |

Purification of CA—CA from P. purpureum cells was purified by 209-fold (27% recovery) with a specific activity of 1,147 units/mg protein (Table I). It was thought that the sulfanilamide-conjugated affinity chromatography used in the purification of Chlamydomonas CA would not be suitable for that of Porphyridium CA, since this compound showed little inhibitory effect on the CA activity of the latter alga (15). However, under weak ionic strength and alkaline conditions, namely in 5 mM Tris-HCl (pH 9.5), Porphyridium CA was bound to the affinity column and efficiently purified. Using almost the same conditions for binding and elution, the chloroplastic CA had been effectively purified from pea (24). This observation suggests that both Porphyridium CA and pea chloroplastic CA exhibit similar interaction with sulfanilamide.

After the step of anion-exchange HPLC, the active protein fraction migrated as a doublet band with an apparent molecular mass of around 59 kDa on SDS-PAGE (Fig. 1). These bands showed similar amino acid compositions (data not shown). In addition, these bands reacted with the antisera raised against pea chloroplastic CA and showed similar amino acid compositions (data not shown). It is therefore possible that the smaller band represents either proteolytic product generated during the purification or CA isomer present in P. purpureum cells. The latter possibility was supported by the isolation of cDNA clones encoding two CAs different in 4 amino acid residues (see below).

cDNA Cloning of CA—In order to obtain a partial cDNA fragment for screening of the clones with full-length cDNA insert, two PCRs were carried out using a combination of degenerated primers, either PrS1-PrA2 or PrS2-PrA1. Amplification occurred only when the set of PrS1-PrA2 was used. The PCR product (0.8-kb) was cloned and sequenced. It contained two primer sequences at both ends and five amino acid sequences deduced from the nucleotide sequence of the PCR product.

The cDNA insert of the second group having 2 HindIII sites as gPCA/2 was designated as gPCA1 and another clone with the longest inserts were analyzed by restriction mapping (see below). It was, therefore, concluded that the PCR product was amplified from the cDNA of Porphyridium CA, and is suitable for use as a probe to screen a cDNA library for the clone encoding the CA.

A 28P-labeled PCR-amplified partial cDNA fragment, and more than 100 independent plaques were positive. Twenty clones having the strongest signals were purified. The EcoRI fragment inserts of positive clones were from 1.4 to 2.0 kb in length. Using these phage DNAs as a template and two primers mentioned above, 0.8-kb DNAs were PCR-amplified. Several clones with the longest inserts were analyzed by restriction mapping and classified into two groups based on the number of HindIII sites. A clone having the longest insert with one HindIII site was designated as gPCA1 and another clone with the longest insert of the second group having 2 HindIII sites as gPCA2.

Nucleotide and Deduced Amino Acid Sequence of CA cDNA—The cDNA insert of gPCA1 was sequenced on both strands. Analysis of the nucleotide sequence of the gPCA1 insert (GenBank accession number D86050) revealed that it consisted of a 90-bp 5'-untranslated region, a 1,713-bp open

FIG. 1. SDS-polyacrylamide gel (12.5%) electrophoresis of the purified Porphyridium CA. Lanes: A, molecular mass markers (bovine serum albumin, 66 kDa; egg ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle, 36 kDa; carbonic anhydrase from bovine erythrocytes, 29 kDa; trypsinogen from bovine erythrocytes, 24 kDa; trypsin inhibitor from soybean, 20.1 kDa; a-lactalbumin, 14.2 kDa); B, 5 μg of protein from the DEAE-HPLC step. The gel was stained with Coomassie Brilliant Blue R-250.

0.1 mM isopropyl-β-D-thio-galactopyranoside for 5 h at 28 °C. Cells were harvested by centrifugation and resuspended in a lysis buffer containing 1 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, and 50 mM Tris-HCl (pH 9.0). The cells were disrupted by sonication and subjected to centrifugation (12,000 × g, 15 min), and the CA activity of the supernatant fractions was determined as described above.

Western Blotting—Immunoblots were performed on polyvinylidene difluoride membranes (Bio-Rad) using anti-Porphyridium CA antisera (16) and positive signals were identified with goat anti-rabbit IgG ('H+L) horseradish peroxidase conjugate (Bio-Rad).
reading frame encoding 571-amino acid polypeptide with a calculated molecular mass of 62,094 Da, a 141-bp 3'-untranslated region, and a 16-bp poly(A) segment as shown in Fig. 2. The positions of the synthetic PCR primers were indicated by arrows. The first ATG codon at the 91st nucleotide was determined as the initiation codon based on the following observations: 1) no other ATG codon in the frame was found upstream from Leu-78 whose presence was confirmed by peptide sequencing. 2) The nucleotide sequence TCACC just upstream from the first ATG codon resembles the consensus sequence CC(A/G)CC for the eukaryotic initiation site proposed by Kozak (25).

The amino acid sequence of Porphyridium CA deduced from the cDNA nucleotide sequence is also shown in Fig. 2. Amino acid sequences of peptide fragments obtained by the Achromobacter protease I digestion of the purified CA were identical with the amino acid sequences underlined in Fig. 2 except for Met-108 and Asn-389. The differences were explained by assuming the existence of two or more CA isozymes in P. purpureum. In fact, the deduced amino acid sequence from the second clone, gtPCA2, showed complete agreement with the amino acid sequences of these peptide fragments. Briefly, the insert of gtPCA2 (1,905-bp in length) encodes a polypeptide of 571 amino acid residues (62,078 Da) in which 4 residues were substituted when compared with those encoded by gtPCA1: Thr-30, Lys-50, Val-108, and Lys-389. The nucleotide sequence of gtPCA2 has been submitted to GenBank (accession number D86051). The gtPCA1 having the longest insert was used for further analyses.

Although the N-terminal amino acid residue could not be determined by direct protein sequencing, the sequence of highly hydrophobic amino acid residues, such as alanine and proline, in the N-terminal region of the protein encoded in the open reading frame would be regarded as a transit peptide which functions in transport of the nascent enzyme into the chloroplast. The size of the possible mature protein estimated from deduced amino acid sequence showed good agreement with the molecular mass calculated by SDS-PAGE. The presence of a transit peptide is consistent with the chloroplastic location of the enzyme suggested by immunogold electron microscopy (16).

Of particular interest is the finding that in the amino acid sequence of the putative mature CA, extensive sequence similarity was observed between the N- and C-terminal halves (Fig. 3). The alignment was carried out to maximize the similarities between these two halves. When they were compared, 72% of the amino acid residues are identical and 23% are conservative substitutions in the alignment shown in Fig. 3. Furthermore, each half contains a set of amino acid residues conserved in CAs from higher plants and two prokaryotes (Fig. 4). The homologies of the N-terminal half with CAs from Synchococcus (6), E. coli (26), spinach (27), and barley (28) were 29, 27, 28, and 32%, respectively, and those of the C-terminal half were 28, 25, 24, and 28%, respectively. Although no information is available for crystal structure of these CAs, Bracey et al. (7) investigated the role of conserved amino acid residues of spinach CA for the zinc binding by site-directed mutagenesis analysis. Mutation at Cys-150, His-210, or Cys-213 of the spinach CA (numbers used in Ref. 7) caused inactivation of the CA and reduction of zinc binding, suggesting that the active-site zinc of spinach CA is coordinated with these two cysteine and one histidine residues. These three amino acid residues were
found at equivalent positions in the sequence of both N-terminal halves (Cys-149, His-205, and Cys-208) and C-terminal halves (Cys-149, His-205, and Cys-208) and C-terminal halves of Porphyridium CA encoded in gtPCA1 are aligned with Synechococcus IcfA (Ref. 6), E. coli Prokaryotic CA and other CAs show less homology are indicated as dots. Inset, shown by dashes are included to obtain maximum alignment. Equal signs represent the connection of N- and C-terminal halves of Porphyridium CA. Length of the regions in which Porphyridium CA and other CAs show less homology are indicated as numbers of the amino acid residues. The site-directed mutagenesis analysis of spinach CA (7) revealed that the residues below the zinc-liganding.

Expression of N-terminal and C-terminal Halves of CA—In order to demonstrate that the enzyme that can be expressed in E. coli will be functional, the truncated polypeptides in E. coli cells containing pNCA or pCCA by ethoxyzolamide and acetazolamide were 1 and 8, respectively. The I₅₀ values were almost identical to those of the native enzyme: (i) 50% inhibition of activity of the CA purified from E. coli cells was observed by ethoxyzolamide and acetazolamide at a concentration of 2 × 10⁻³ and 6 × 10⁻⁸ M, respectively. (iii) Both the CA purified from E. coli and P. purpureum showed higher activities when it had been kept at an alkaline (pH 9–10) condition (15).
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Table II

Measurements of carbonic anhydrase activity in extracts of E. coli cells transformed with control plasmid, pPCA, pNCA, and pCCA

| Plasmid                | IPTG addition | CA activity (units/mg E. coli protein) |
|------------------------|---------------|---------------------------------------|
| Control plasmid (pUC119)| +             | N.D.A.                                 |
| pPCA                   | +             | 11.65                                  |
| pPCA                   | −             | N.D.A.                                 |
| pNCA                   | +             | 2.28                                   |
| pCCA                   | +             | N.D.A.                                 |

* IPTG, isopropyl-1-thio-β-D-galactopyranoside.

Fig. 5. Western blot analysis of the extracts of E. coli cells expressing the putative mature CA, and the N- and C-terminal halves of the enzyme at equivalent positions, indicating that this CA is a member of prokaryotic-type CAs (Fig. 4). It has been reported that this CA does not cross-react with the spinach CA antibody (16). This might result from relatively broad regions with less similarity, except those containing conserved amino acid residues.

DISCUSSION

CA purified from low-CO₂ cells of P. purpureum with affinity column and DEAE-HPLC column chromatography contained multiple polypeptide species showing similar but distinct electrophoretic mobility on SDS-PAGE, and the separated polypeptides were electrophoretically transferred to a polyvinylidene difluoride membrane and probed as described under “Experimental Procedures.” Ten μg of protein was loaded in each lane. Lane A, extract from cells containing pPCA and expressing the putative mature CA. B, extract from cells containing pNCA and expressing the N-terminal half of the CA. C, extract from cells containing pCCA and expressing the C-terminal half of the CA. On the left are shown the positions of molecular size markers (in kDa).

ever, no detectable CA activity was expressed from E. coli cells containing pCCA (Table II).

Three mammalian hexokinase isozymes (Types I, II, and III) are well characterized examples of the enzymes consisting of highly homologous N-terminal and C-terminal halves (e.g. Ref. 35). It is proposed that these isozymes (Types I, II, and III) of ~100 kDa have evolved by a process of duplication and fusion of a gene encoding an ancestral hexokinase similar to the mammalian glucokinase (Type IV hexokinase) and the yeast enzyme of ~50 kDa. Site-directed mutagenesis studies of the rat Type I isoyme have shown that catalytic activity is associated solely with the C-terminal half while the N-terminal half is catalytically inactive and thought to be involved in a regulatory function (36). On the other hand, more recently, site-directed mutagenesis of rat Type II isoyme showed that both halves of the enzyme retain comparable catalytic activities (37).

In the present study, the N- and C-terminal halves of Porphyridium CA were expressed separately, and it was demonstrated that the N-terminal half was catalytically active, while no catalytic activity was found in the C-terminal half, although both were expressed as soluble proteins. One of the simplest interpretations of this observation is that the N-terminal half expresses the CA activity, while the C-terminal half is not functional due to substitutions in some essential amino acid residues. However, we showed that one molecule of Porphyridium CA binds two zinc atoms, in contrast to other prokaryotic-type CAs which have been shown to bind one zinc atom per
molecule (2). Thus we rather expected that each half of *Porphyridium* CA binds one zinc atom and should exhibit CA activity. We wonder whether the folding of the C-terminal half into a catalytically active form is possible only when the N-terminal half is present. To test this hypothesis, we are in the process of constructing various mutants of *Porphyridium* CA. In any mutant forms thus far obtained, the N-terminal half can take a catalytically active configuration. A functional expression system developed in this study may be useful to further clarify the structure/function relationship and the biochemical and physiological significance of this enzyme structure.

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